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Pregnenolone sulfate activates basic region leucine zipper transcription factors in insulinoma cells - Role of voltage gated Ca^{2+} channels and transient receptor potential TRPM3 channels

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Running title: Pregnenolone sulfate activates bZIP transcription factors

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Abbreviations: AP-1, activator protein-1; bZIP, basic region leucine zipper; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; SRE, serum response element; SRF, serum response factor; TRE, phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element; TRP, transient receptor potential

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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 upregulated in insulinoma cells and pancreatic β-cells in primary culture following pregnenolone sulfate stimulation. Upregulation 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 motives function as pregnenolone sulfate response elements. In addition, phosphorylation of CREB is induced and transcription of a cAMP response element (CRE)-controlled reporter gene is stimulated following 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 M3 (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.

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Introduction

Steroids, including progesterone, pregnenolone, pregnenolone sulfate, and dehydroepiandrosterone, that are synthesized in the central and peripheral nervous system, at least in part independent 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.

Elevated levels of glucose have a profound effect on gene transcription. In particular, the activity of the transcription factor AP-1 is upregulated in glucose-stimulated in insulinoma cells (Glauser et al., 2006; Müller et al., 2010). We asked whether pregnenolone sulfate stimulation also increases AP-1 activity and 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 upregulates 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(TPA)-responsive element (TRE) and the cyclic AMP response element (CRE) as pregnenolone sulfate response elements. We further addressed the role of L-type Ca^{2+} channels and TRPM3 channels within the signaling cascade that connects pregnenolone stimulation with enhanced bZIP activity and bZIP-regulated transcription.

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Materials and Methods

Cell culture

The rat pancreatic β-cell line INS-1 were kindly provided by Claes B. Wollheim, University of Geneva, Switzerland, and Susanne Ullrich, University of Tübingen, Germany. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin as described (Groot et al., 2000). This medium contains 11 mM glucose. The experiments were performed with INS-1 cells cultured in low glucose medium using DME medium without glucose (Sigma # D5030) supplemented with glucose to a final concentration of 2 mM. AtT20 corticotrophs were cultured as described (Hohl and Thiel, 2005). The cells were incubated for 24 hours in medium without serum. Stimulation with pregnenolone sulfate (50 μM, dissolved in DMSO, Sigma-Aldrich), pregnenolone (50 μM, dissolved in DMSO, Sigma-Aldrich), progesterone (50 μM, dissolved in DMSO. Sigma-Aldrich), or KCl (25 mM)/FPL64176 (2.5 or 5 μM) was performed as indicated. The L-type voltage-gated Ca^{2+} channel blocker verapamil were purchased from Sigma-Aldrich, dissolved in DMSO and used at a final concentration of 50 μM. Cells were preincubated with verapamil for 1 hour.

Primary culture of pancreatic islets

Pancreatic islets were prepared following digestion of mouse pancreata with collagenase (Roche, 1 mg/ml). The islets were broken up into individual cells by shaking in divalent-free cation solution supplemented with trypsin. Dispersed cells were plated on plastic Petri dishes for stimulation. Dispersed islet cells were maintained for up to 48 hours under the same conditions as INS-1 cells. Cells were cultured in DME containing 2 mM glucose for 24 hours in medium without fetal bovine serum. Stimulation with pregnenolone sulfate (50 μM) was performed for 1 hour.

Lentiviral gene transfer

The lentiviral transfer vectors pFUW-REST/Elk-1ΔC and pFUW-REST/CREB have been described previously (Mayer et al., 2008; Mayer and Thiel, 2009; Mayer et al., 2009; Rössler and Thiel, 2009; Müller et al., 2010). The viral particles were produced as previously described (Stefano et al., 2006) by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein, and the transfer vector.

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Lentiviral expression of short hairpin RNAs (shRNAs)

The lentiviral vector pLentiLox3.7 (pLL3.7) was purchased from American Type Culture Collection (Manassas, VA). The lentiviral transfer vector pLLTRPM3, used to knock-down TRPM3 expression, has been described (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/+170JUNCAT5 and -1600/+170Δ2TREΔAP1CAT-5 were kind gifts of P. Angel, DKFZ, 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-Junluc 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 pc-FosCRE^{4}luc, that 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 lentivial transfer vector, generating plasmid pFWc-Fos(CRE)^{4}luc. The lentiviral transfer vector pFUWluc, encoding luciferase under the control of the human ubiquitin C promoter, was construcetd by inserting the coding region for luciferase, derived from plasmid pGL3-Promoter, into the BamHI and EcoRI sites of pFUWG. INS-1 cells, and AtT20 cells were infected with recombinant lentiviruses expressing promoter/luciferase reporter genes. Cells were stimulated with pregnenolone sulfate or KCl/FPL64176 for 24 hours and with glucose for 48 hours. Cell extracts of stimulated cells were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities as described (Thiel et al., 2000). Luciferase activity was normalized to the protein concentration.

Western blots

Whole cell extracts, nuclear extracts and crude membranes were prepared as described (Kaufmann and Thiel, 2002). Proteins were separated by SDS-PAGE, blotted and incubated with antibodies directed against either c-Jun (Santa Cruz, Heidelberg, Germany, # sc-1694), the phosphorylated form of c-Jun (Upstate Biotechnology, Lake Placid, NY, # 06-659), c-Fos (Santa Cruz, Heidelberg, Germany, # sc52), CREB (Upstate Biotechnology, Lake Placid, NY,

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06-863), or the phosphorylated form of CREB (Upstate Biotechnology, Lake Placid, NY, # 06-519). The antibody directed against histone deacetylase-1 (HDAC1) was used as a loading control as previously described (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, Steinheim, Germany, # F3165) at 1:3000 dilution. Immunoreactive bands were detected via enhanced chemiluminescence as described (Spohn et al., 2010; Mayer et al., 2011). Values are expressed as the mean +/- SD from 3 independent experiments.

RT-PCR

RT-PCR was performed as previously described (Bauer et al., 2007). Primers specific for detection of TRPM3 mRNA, termed 9-12, have been published recently (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 reaction. Cav1.3 expression was detected in RT-PCR experiments using the primers 5´-GAC TCA GAT TAC AAC CCA GG-3´ and 5´-GTG 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. The primers 5´-AGA GCG 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 TTC 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 Qiagen RNeasy Plus Micro Kit (Cat.No: 74034). RNA samples were reverse transcribed into cDNA with RevertAid M-MuLV RT (Fermentas) in the presence of RNase Inhibitor (Fermentas). The PCR conditions were: one cycle at 95°C for 10 min, 40 amplification cycles, each cycle consisted of denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Gene expression values were calculated based on the comparative threshold cycle (Cq) method as described in Applied Biosystems User Bulletin No. 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 +/- SD from three independent experiments. Statistical probability is expressed as $\star P < 0.05$, $\star\star P < 0.01$, and $\star\star\star P < 0.001$. Values were considered significant when $P < 0.05$.

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Results

Upregulation 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., 2006). Recently, we 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 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 hours in medium containing 2 mM glucose. Cells were stimulated with either pregnenolone sulfate, pregnenolone, or progesterone for twenty-four hours. Fig. 1B shows that pregnenolone sulfate stimulation enhanced transcription mediated by AP-1, while 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´. Fig.1C shows that stimulation of reporter gene transcription by pregnenolone sulfate was strikingly reduced when the TRE was mutated. This experiments reveals 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 (supplement Fig. 1). Thus, cells had to be cultured in serum-free medium to measure the effect of pregnenolone sulfate stimulation upon gene transcription.

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Upregulation of c-Jun and c-Fos expression in pregnenolone sulfate-stimulated insulinoma cells and pancreatic β-cells

The activator protein-1 (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. Fig. 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 (JNK) and phosphorylation of c-Jun is required for activation of the transcriptional activation potential of c-Jun. Fig. 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 hour. RNA was isolated, reversed transcribed and gene expression was monitored using quantitative real-time PCR. Fig. 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 insulioma 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 motives termed jun1TRE and jun2TRE. Fig. 3B shows that stimulation of insulinoma cells with pregnenolone sulfate significantly increased c-Jun promoter controlled reporter gene transcription. However, upregulation of an integrated c-Jun promoter/luciferase reporter, where both TRE-like motives 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

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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 cyclic AMP response element (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-1ΔC (Fig. 3E). This mutant retains the DNA-binding and SRF interaction domains, but lacks the C-terminal activation domain of Elk-1. REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST, a FLAG epitope for immunological detection and a nuclear localization signal (NLS). 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 basic region leucine zipper (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 NLS. 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. Figs. 3G and 3H show that expression of both REST/Elk-1ΔC and REST/CREB abolished the upregulation 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 the signaling cascade induced by glucose in β-cells (Mayer et al., 2011). Recently, we showed 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. Fig. 4A shows that treatment of the

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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, activation of transcription is only regulated by the CREs. 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 upregulation of c-Jun and c-Fos expression in insulinoma

Recently, we 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 following lentiviral infection was demonstrated recently (Mayer et al., 2011), allowing biochemical analysis of the cells that expressed the TRPM3-specific shRNA. Fig. 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 a Western blot analysis showing that the upregulation 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 (Figs. 5C, D). In contrast, the knock-down of TRPM3 did not influence the upregulation 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 (Figs. 5E, F).

TRPM3 is required for pregnenolone sulfate-induced transcripion of a CRE-controlled reporter gene in insulinoma cells

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Fig. 5G shows that the upregulation 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 upregulation of c-Jun and c-Fos promoter activity, the enhanced transcription of a CRE-regulated 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 Ca^{2+} channels in the transcriptional response to pregnenolone sulfate stimulation

Activation of L-type voltage-dependent Ca^{2+} channels is necessary for glucose signaling in pancreatic β-cells. In neurons, pregnenolone sulfate activates L-type voltage-gated Ca^{2+} channels (Chen and Sokabe, 2005; Hige et al., 2006; Chen et al., 2010). Recently, we showed that L-type voltage-gated Ca^{2+} channels are required for the pregnenolone sulfate-triggered upregulation 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 Ca^{2+} channels in pregnenolone sulfate-stimulated transcription in insulinoma cells cultured in low glucose medium. Incubation of the cells with the L-type voltage-gated Ca^{2+} channel blocker verapamil completely blocked the transcriptional upregulation of reporter gene transcription controlled by the collagenase, c-Jun, or c-Fos promoters, respectively (Fig. 6A-C). In addition, pregnenolone sulfate-induced upregulation 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 in INS-1 insulinoma cells cultured in medium containing 2 mM glucose L-type voltage-gated Ca^{2+} channels are required for the pregnenolone sulfate-triggered upregulation of reporter gene transcription controlled by either by the collagenase promoter, the c-Jun promoter, the c-Fos promoter, or multiple CRE motives. Thus, both TRPM3 and L-type voltage-gated Ca^{2+} channels are necessary to connect pregnenolone sulfate stimulation with enhanced transcription.

Expression of TRPM3 and L-type voltage-gated Ca^{2+} channels in islets, insulinoma cells and corticotrophs

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To define the role of either TRPM3 and L-type voltage-gated Ca^{2+} channels, we searched for a cell line that expresses L-type voltage-gated Ca^{2+} channels, but lacks expression of TRPM3 Ca^{2+} channels. RT-PCR experiments using 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. 7A, 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 reaction involving 35 amplification cycles, indicating that AtT20 cells express trace amounts of TRPM3. We used AtT20 cells to answer the question whether expression of Cav1.2 and Cav1.3 is sufficient to activate transcription following pregnenolone sulfate-stimulation. Fig. 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 upregulation 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 following pregnenolone sulfate stimulation. These results were corroborated by a Western blot analysis. Fig. 8B shows that pregnenolone sulfate stimulation fails to induce c-Fos and c-Jun expression in AtT20 corticotrophs, while activation of L-type voltage-gated Ca^{2+} channels by KCl/FPL64176-stimulation induced the biosynthesis of c-Fos and c-Jun.

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Discussion

The neurosteroid pregnenolone sulfate acts on the nervous system by regulating neurotransmission and receptor functions, leading to changes in synaptic strength, neuronal survival and neurogenesis. Recently, we showed 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 β-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 β-cells, including Egr-1 (Frödin et al., 1995; Josefsen et al., 1999; Bernal-Mizrachi et al., 2000; Mayer and Thiel, 2009), CREB (Wang et al., 2008; Mayer and Thiel, 2009), Elk-1 (Bernal-Mizrachi et al., 2001; Mayer and Thiel 2009), 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., 2006). Recently, we showed 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. Frequently, reporter genes are introduced into cultured cells via transient transfection of plasmids. This approach has the disadvantage that the structure of these plasmids may be incompletely organized in comparison to cellular chromatin (Smith and Hager, 1997), and may thus resemble a prokaryotic gene organisation 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 analyse AP-1-mediated gene

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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 upregulation 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 upregulated in insulinoma cells and pancreatic islets following 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 upregulation of reporter gene transcription solely controlled by CRE motives.

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 transcription (Mayer et al., 2011). In the nervous system, pregnenolone sulfate-induced Ca^{2+} influx into neurons may occur by activating neurotransmitter receptors (i.e NMDA receptors) or by stimulating L-type voltage-gated Ca^{2+} channels. The analysis of perforant path-granule cell synaptic transmission revealed that the presynaptic effect of pregnenolone sulfate was – at least in part - attenuated 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 upregulation of Egr-1 required L-type voltage-gated Ca^{2+} channels, when the cells were cultured in a medium

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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 upregulation (Mayer et al., 2011). The role of L-type voltage-gated Ca^{2+} channels was not assessed under these conditions. Here, we performed a series of experiments to clarify the roles of TRPM3 and L-type voltage-gated Ca^{2+} channels in transcription of pregnenolone sulfate treated insulinoma cells. The results based on genetic and pharmacological experiments showed that both TRPM3 and L-type voltage-gated Ca^{2+} channels are needed to stimulate gene expression by pregnenolone sulfate in INS-1 cells that were cultured in low glucose medium. We propose that activation of TRPM3 by pregnenolone sulfate triggers an influx of Ca^{2+} ions into the cells as described (Wagner et al., 2008), leading to a subsequent activation of L-type Ca^{2+} channels. The initial Ca^{2+} influx into the cells, mediated by TRPM3 activation, seems not to be sufficient to induce a signaling cascade toward the nucleus, as pregnenolone sulfate induced gene transcription was impaired in verapamil-treated 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 carboxyl-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 following 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 shows that expression of L-type voltage-gated Ca^{2+} channels is not sufficient to obtain a transcriptional response following 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, i.e. NMDA receptors in neurons or TRPM3 in insulinoma cells, to allow pregnenolone sulfate regulated gene transcription. We speculate that the interaction of pregnenolone sulfate to L-

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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 the 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
Conduced experiments: Müller, Rössler
Contributed new reagents: Thiel
Wrote the manuscript: Thiel

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FOOTNOTE

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LEGENDS FOR FIGURES

FIGURE 1

Upregulation 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 phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) within the collagenase promoter is depicted. (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 either pregnenolone sulfate (PregS, 50 μM), pregnenolone (Preg, 50 μM), or progesterone (Prog, 50 μM) for twenty four hours. (C) INS-1 cells were infected with recombinant lentiviruses encoding collagenase promoter/luciferase reporter genes with an intact or a mutated TRE. The cells were serum-starved for twenty-four hours and then stimulated with either pregnenolone sulfate (PregS, 50 μM) for twenty four hours. (D) INS-1 cells were infected with recombinant lentiviruses encoding an ubiquitin C promoter/luciferase reporter gene. The cells were serum-starved for twenty-four hours and then stimulated with either pregnenolone sulfate (PregS, 50 μM) for twenty four hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration (✶✶✶, $P < 0.001$).

FIGURE 2

Pregnenolone sulfate triggers the biosynthesis of c-Jun and c-Fos in INS-1 insulinoma cells and in pancreatic islets

(A) Induction of c-Jun and c-Fos biosynthesis in pregnenolone sulfate-stimulated INS-1 insulinoma cells. Cells were serum-starved for twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibodies directed against either c-Jun, c-Fos or the phosphorylated form of c-Jun. The antibody directed against HDAC1 was used as a loading control. (B) Quantitative real-time PCR of RNA isolated from stimulated (PregS, 50 μM) or not stimulated pancreatic islet cells in primary short-term culture. The c-Jun and c-Fos signal was normalized to the 18S rRNA signal and the fold-stimulation was calculated using the $2^{-\Delta\Delta Ct}$ equation.

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FIGURE 3

Stimulation with pregnenolone sulfate upregulates c-Jun and c-Fos promoter activity in insulinoma cells

(A, B) Transcriptional upregulation 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 motives is depicted. Both TREs are mutated in the c-Jun promoter ΔTRE/luciferase reporter gene. (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 twenty-four hours as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. (C, D) Transcriptional upregulation 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 cyclic AMP response element (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 pregnenolone sulfate (PregS, 50 μM) for twenty-four hours 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-1ΔC 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 S383 and S389. 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-1ΔC lacks the phosphorylation-regulated activation domain, but retains the DNA and SRF binding domains. The phosphorylation-dependent transcriptional activation domain of CREB (kinase-inducible domain; KID) 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. (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 kDa are shown on the left. (G, H)

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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 a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The infected cells were treated with pregnenolone sulfate (PregS, 50 μM) for twenty-four hours as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

FIGURE 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 twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) 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. (C) INS-1 insulinoma cells were infected with a recombinant lentivirus encoding the c-Fos CRE/luciferase reporter gene. The cells were incubated with medium containing 0.05% serum and 2 mM glucose for twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM). Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

FIGURE 5

Expression of TRPM3 is required for pregnenolone sulfate-induced c-Jun and c-Fos expression and CRE-controlled transcription in insulinoma cells

(A, C) INS-1 insulionoma 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 twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) for twenty-four hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. (B, D) INS-1 insulinoma

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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 twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) for 1 hour. 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, 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 twenty-four hours and then stimulated with either glucose (11 mM) (E) for 48 hours, or with KCl (25 mM)/FPL64176 (2.5 μM) (F) for twenty-four hours. 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 twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) for twenty-four hours. Cell extracts were prepared and analyzed for luciferase activities.

FIGURE 6

Activation of L-type Ca^{2+} channels is essential for pregnenolone sulfate-induced c-Jun and c-Fos expression and CRE-controlled transcription in insulinoma cells

INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a lentivirus encoding luciferase under the control of the collagenase promoter (A), the c-Jun promoter (B), the c-Fos promoter (C) or a CRE-controlled promoter (D). Cells were serum-starved for twenty-four hours and then stimulated with pregnenolone sulfate (PregS, 50 μM) in the presence or absence of verapamil (50 μM) for twenty-four hours. (E) INS-1 cells were infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene. Cells were serum-starved for twenty-four hours and then stimulated with KCl/FPL64176 (25mM; 2.5 μM) in the presence or absence of verapamil for twenty-four hours.

FIGURE 7

Expression of TRPM3, Cav1.2 und Cav1.3 in islets, insulinoma cells and corticotrophs

RNA was prepared from islets, INS insulinoma cells and AtT20 corticotrophs. Expression of TRPM3, Cav1.2 and Cav.1.3 was monitored via RT-PCR using gene specific primers. As a control, expression of GAPDH was assessed.

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FIGURE 8

Stimulation with KCl/FPL64176, but not with pregnenolone sulfate, increases AP-1 activity and upregulates c-Fos and c-Jun expression in pituitary corticotrophs

(A) AtT20 corticotrophs 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 either pregnenolone sulfate (PregS, 50 μM), or KCl/FPL64176 (25 mM, 5 μM) for twenty four hours (✶, $p < 0.05$). (B) Induction of c-Jun and c-Fos expression in AtT20 corticotrophs. Cells were serum-starved for twenty-four hours and then stimulated with either pregnenolone sulfate (PregS, 50 μM), or KCl/FPL64176 (25 mM, 5 μM) for 1 hour as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibodies directed against either c-Jun, or c-Fos The antibody directed against HDAC1 was used as a loading control.

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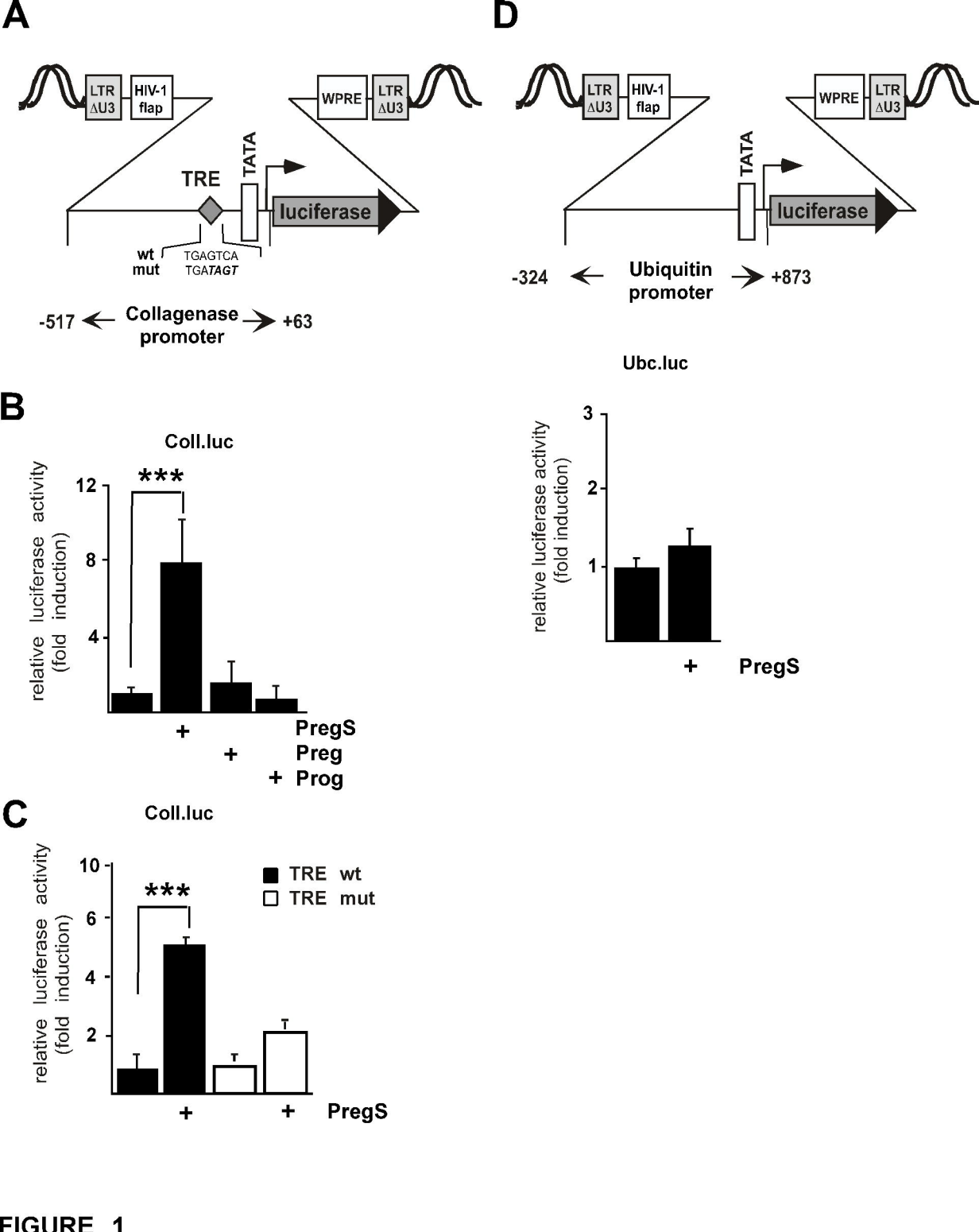
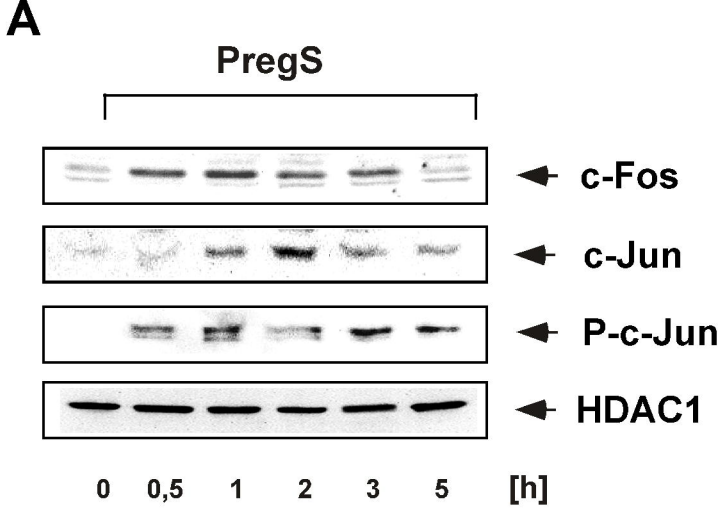

A
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TRE
TATA
luciferase
wt TGAGTCA
mut TGATAGT
-517
Collagenase promoter
+63
D
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TATA
luciferase
-324
Ubiquitin promoter
+873
Ubc.luc
relative luciferase activity (fold induction)
3
2
1
+
PregS
B
Coll.luc
relative luciferase activity (fold induction)
***
12
8
4
+
PregS
+
Preg
+
Prog
C
Coll.luc
relative luciferase activity (fold induction)
***
10
6
4
2
TRE wt
TRE mut
+
+
PregS

FIGURE 1


A
PregS
c-Fos
c-Jun
P-c-Jun
HDAC1
0
0,5
1
2
3
5
[h]

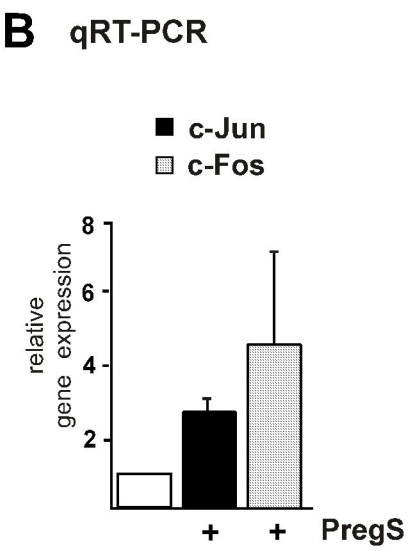

B
qRT-PCR
c-Jun
c-Fos
relative gene expression
8
6
4
2
+
+
PregS

FIGURE 2

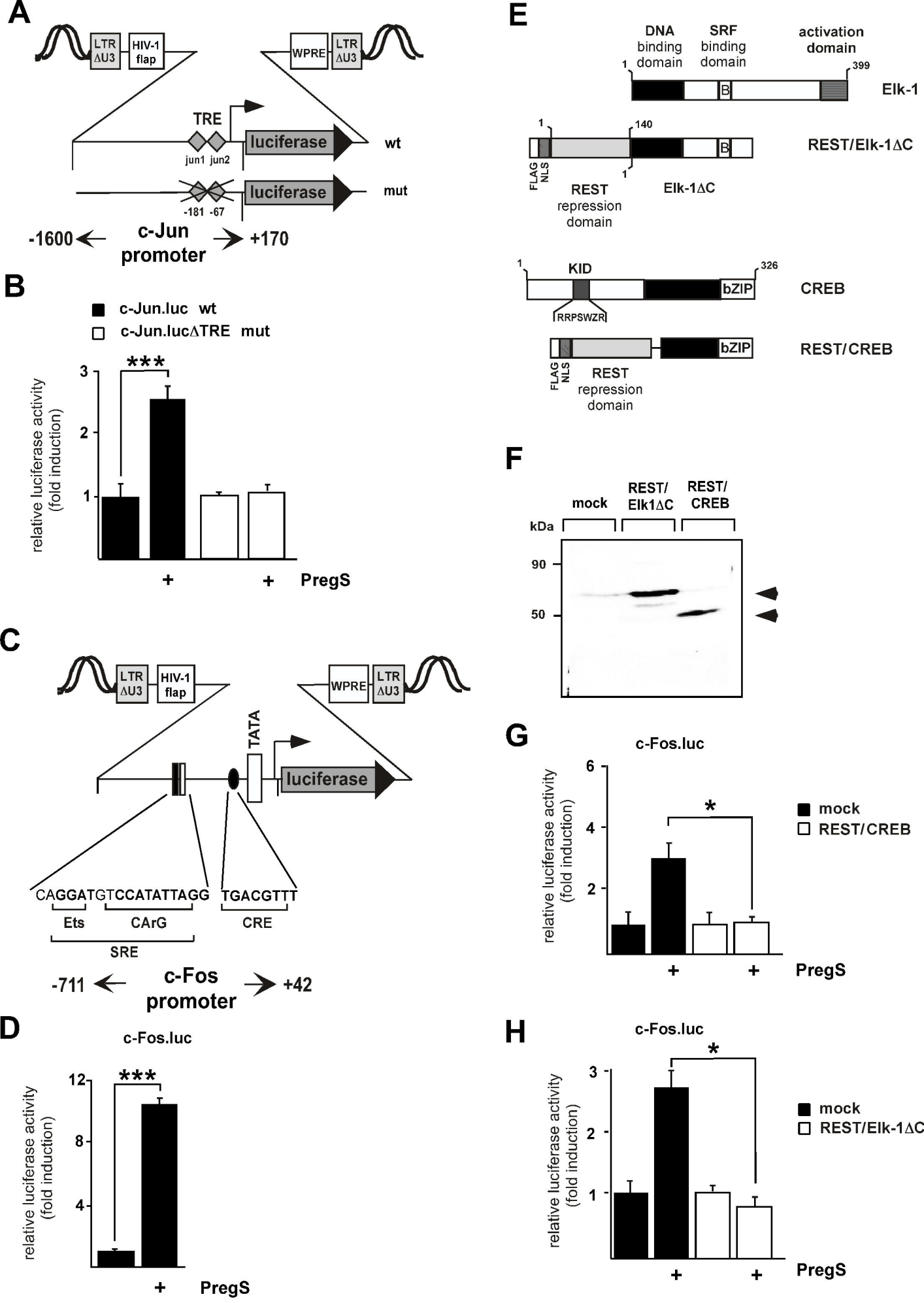

A
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TRE
jun1 jun2
luciferase
wt
luciferase
mut
-181 -67
-1600
c-Jun promoter
+170
B
c-Jun.luc wt
c-Jun.lucΔTRE mut
***
relative luciferase activity (fold induction)
3
2
1
+
+
PregS
C
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TATA
luciferase
CAGGATGTCCATATTAGG
TGACGTTT
Ets
CArG
CRE
SRE
-711
c-Fos promoter
+42
D
c-Fos.luc
***
relative luciferase activity (fold induction)
12
8
4
+
PregS
E
DNA binding domain
SRF binding domain
activation domain
1
399
B
Elk-1
1
140
1
B
REST/Elk-1ΔC
FLAG NLS
REST repression domain
Elk-1ΔC
1
KID
326
bZIP
CREB
RRPSWZR
bZIP
REST/CREB
FLAG NLS
REST repression domain
F
mock
REST/ Elk1ΔC
REST/ CREB
kDa
90
50
G
c-Fos.luc
*
relative luciferase activity (fold induction)
6
4
2
mock
REST/CREB
+
+
PregS
H
c-Fos.luc
*
relative luciferase activity (fold induction)
3
2
1
mock
REST/Elk-1ΔC
+
+
PregS

FIGURE 3

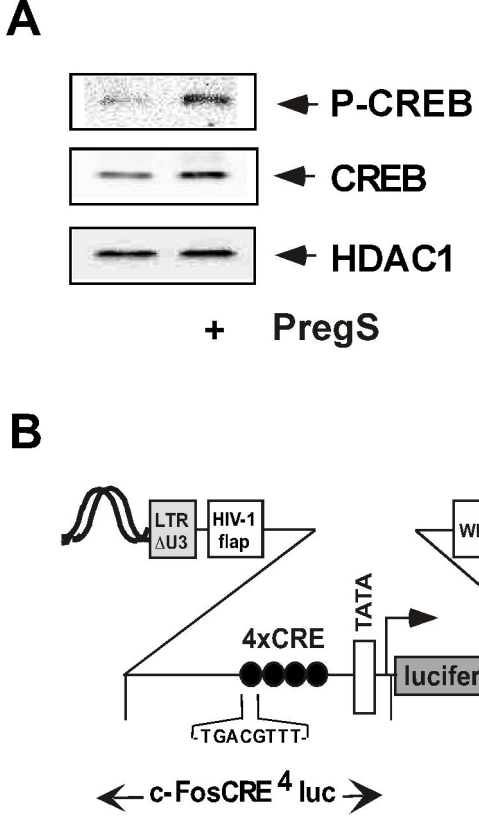

A
P-CREB
CREB
HDAC1
+ PregS
B
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TATA
4xCRE
luciferase
TGACGTTT
c-FosCRE 4 luc
C
c-FosCRE4luc
***
relative luciferase activity (fold induction)
6
4
2
+ PregS

FIGURE 4

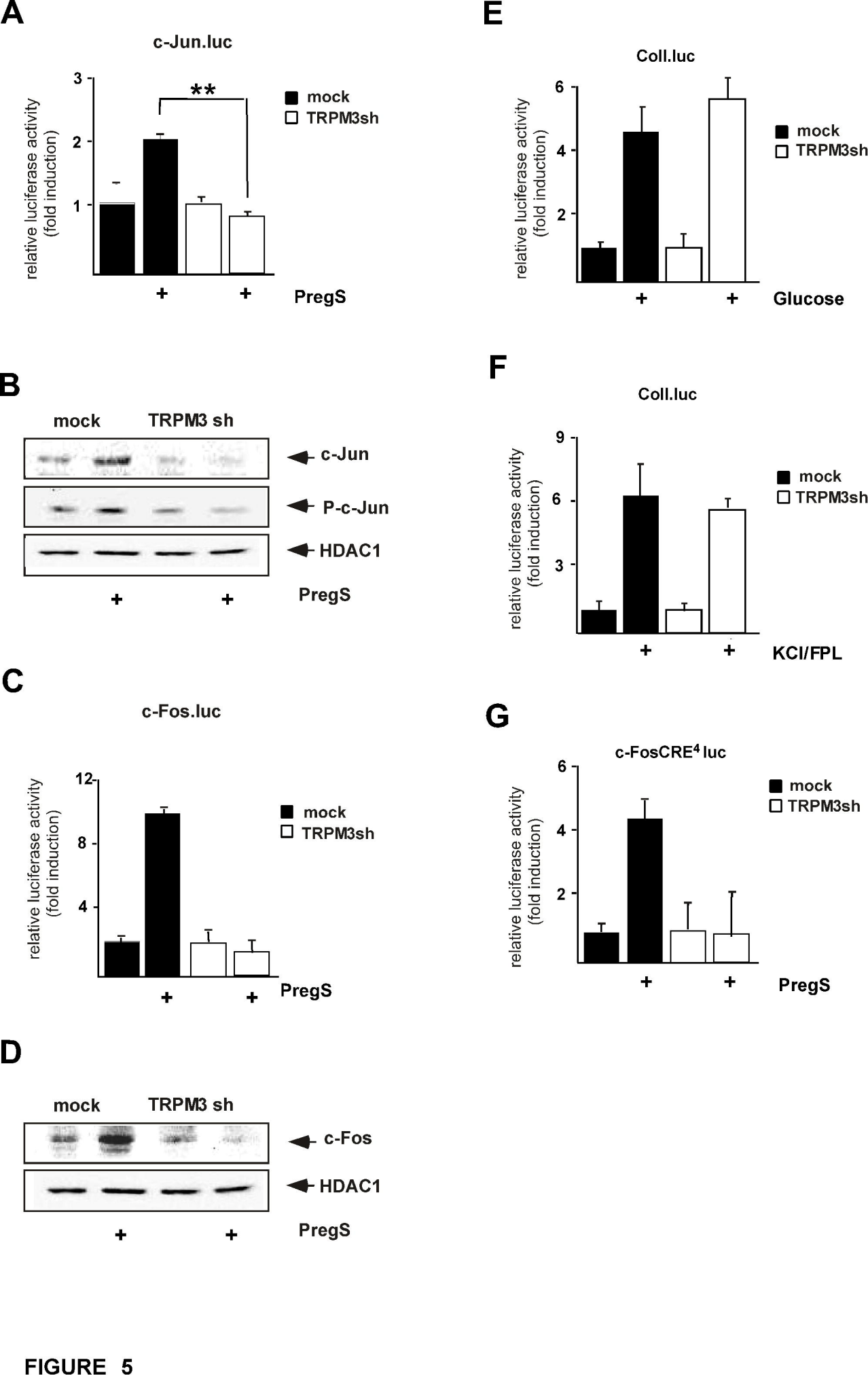

A
c-Jun.luc
relative luciferase activity (fold induction)
3
2
1
**
mock
TRPM3sh
+
+
PregS
B
mock
TRPM3 sh
c-Jun
P-c-Jun
HDAC1
+
+
PregS
C
c-Fos.luc
relative luciferase activity (fold induction)
12
8
4
mock
TRPM3sh
+
+
PregS
D
mock
TRPM3 sh
c-Fos
HDAC1
+
+
PregS
E
Coll.luc
relative luciferase activity (fold induction)
6
4
2
mock
TRPM3sh
+
+
Glucose
F
Coll.luc
relative luciferase activity (fold induction)
9
6
3
mock
TRPM3sh
+
+
KCl/FPL
G
c-FosCRE4 luc
relative luciferase activity (fold induction)
6
4
2
mock
TRPM3sh
+
+
PregS

FIGURE 5

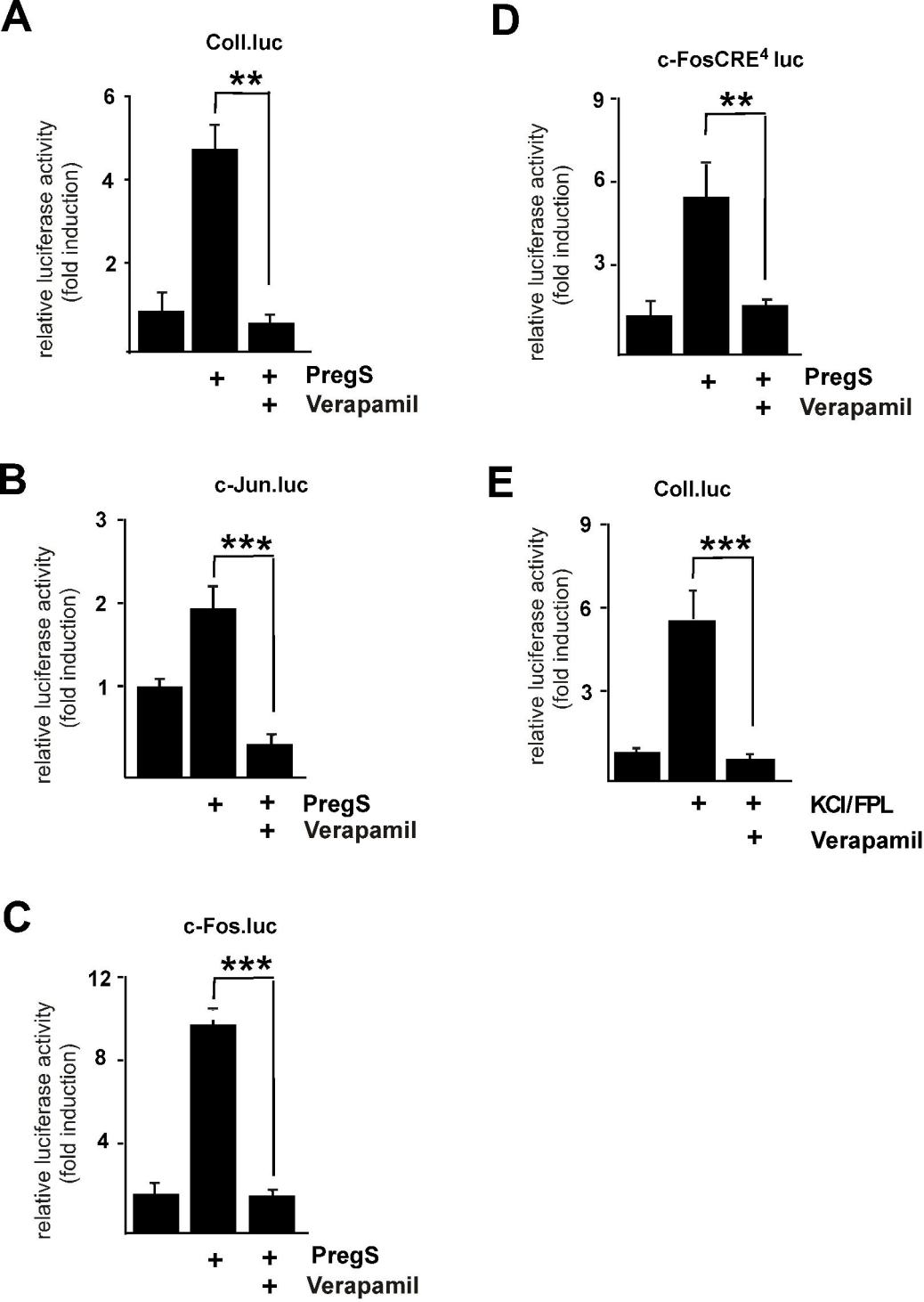

A
Coll.luc
**
relative luciferase activity (fold induction)
6
4
2
+ + PregS
+ Verapamil
B
c-Jun.luc
***
relative luciferase activity (fold induction)
3
2
1
+ + PregS
+ Verapamil
C
c-Fos.luc
***
relative luciferase activity (fold induction)
12
8
4
+ + PregS
+ Verapamil
D
c-FosCRE4 luc
**
relative luciferase activity (fold induction)
9
6
3
+ + PregS
+ Verapamil
E
Coll.luc
***
relative luciferase activity (fold induction)
9
6
3
+ + KCl/FPL
+ Verapamil

FIGURE 6

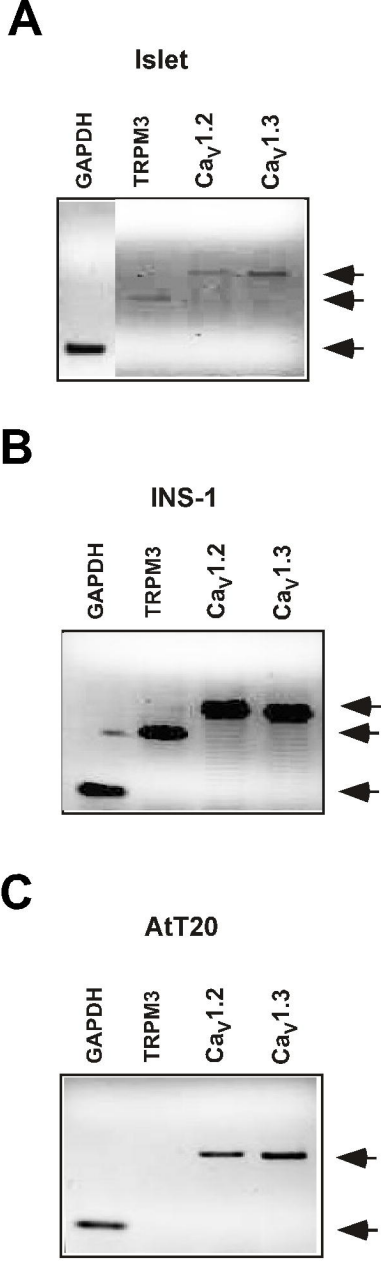

A
Islet
GAPDH
TRPM3
$Ca_V1.2$
$Ca_V1.3$
B
INS-1
GAPDH
TRPM3
$Ca_V1.2$
$Ca_V1.3$
C
AtT20
GAPDH
TRPM3
$Ca_V1.2$
$Ca_V1.3$

FIGURE 7

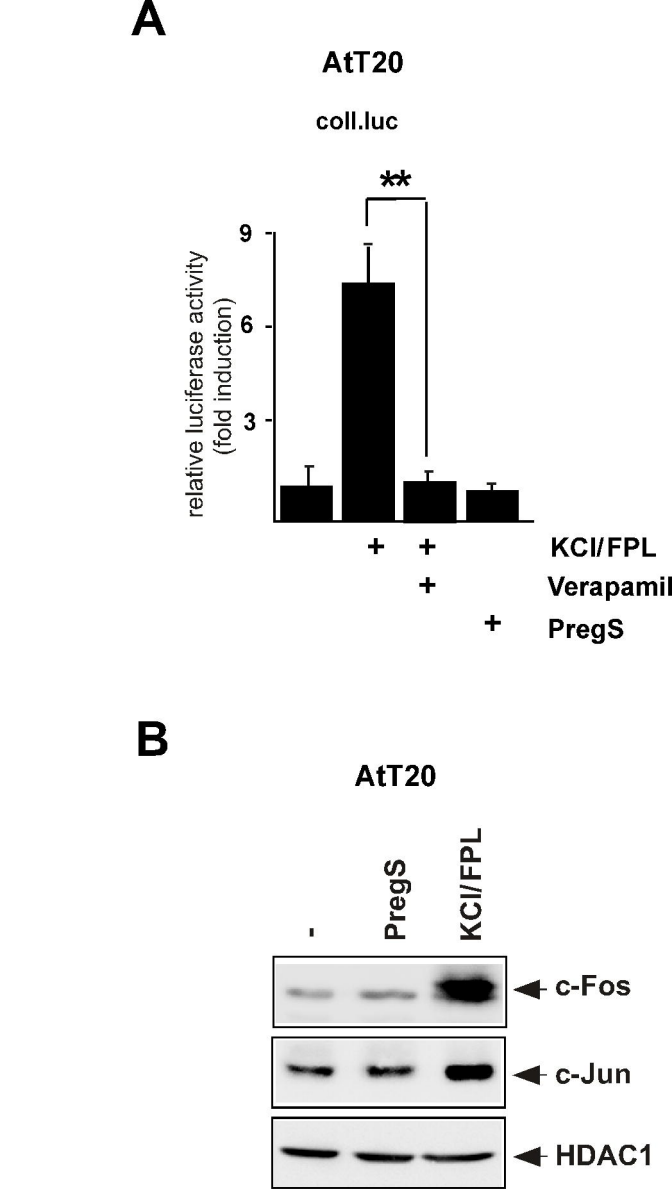

A
AtT20
coll.luc
**
relative luciferase activity
(fold induction)
9
6
3
+ +
KCl/FPL
+
Verapamil
+
PregS
B
AtT20
-
PregS
KCl/FPL
c-Fos
c-Jun
HDAC1

FIGURE 8

Journal: Molecular Pharmacology
Title: Pregnenolone sulfate activates basic region leucine zipper transcription factors in insulinoma cells - Role of voltage gated Ca^{2+} channels and transient receptor potential TRPM3 channels
Authors: Isabelle Müller, Oliver G. Rössler, and Gerald Thiel

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 (✶✶✶, $P < 0.001$).

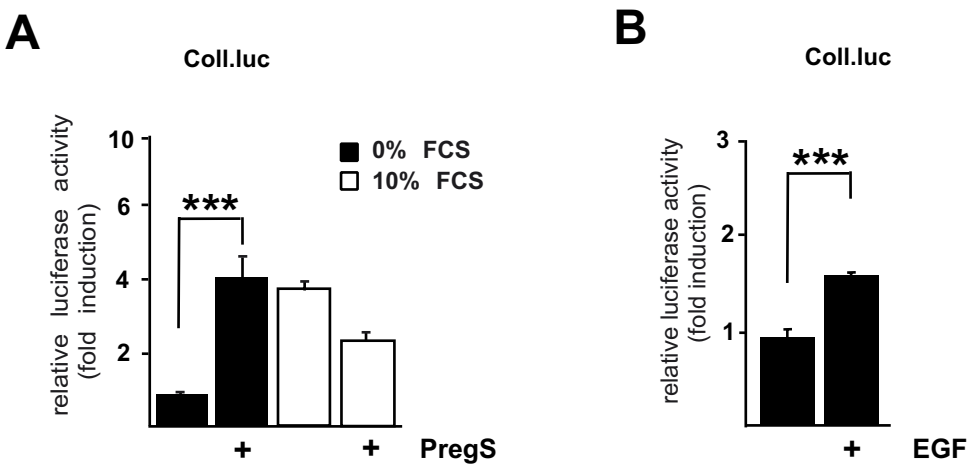


Fig.S1