THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 12, pp. 10084–10096, March 25, 2011

Signal Transduction of Pregnenolone Sulfate in Insulinoma Cells

ACTIVATION OF EGR-1 EXPRESSION INVOLVING TRPM3, VOLTAGE-GATED CALCIUM CHANNELS, ERK, AND TERNARY COMPLEX FACTORS[*][S]

Received for publication, November 15, 2010, and in revised form, January 20, 2011 Published, JBC Papers in Press, January 21, 2011, DOI 10.1074/jbc.M110.202697

Sabine I. Mayer[‡1], **Isabelle Müller**[‡], **Stefanie Mannebach**[§], **Takeshi Endo**[¶], **and Gerald Thiel**[‡2]

From the Departments of [‡]*Medical Biochemistry and Molecular Biology and* [§]*Experimental and Clinical Pharmacology and Toxicology, University of Saarland Medical Center, D-66421 Homburg, Germany and the* [¶]*Department of Biology, Graduate School of Science, Chiba University, Chiba 263-8522, Japan*

The neurosteroid pregnenolone sulfate acts on the nervous system by modifying neurotransmission and receptor functions, thus influencing synaptic strength, neuronal survival, and neurogenesis. Here we show that pregnenolone sulfate induces a signaling cascade in insulinoma cells leading to enhanced expression of the zinc finger transcription factor Egr-1 and Egr-1-responsive target genes. Pharmacological and genetic experiments revealed that influx of Ca^{2+} ions via transient receptor potential M3 and voltage-gated Ca^{2+} channels, elevation of the cytosolic Ca^{2+} level, and activation of ERK are essential for connecting pregnenolone sulfate stimulation with enhanced Egr-1 biosynthesis. Expression of a dominant-negative mutant of Elk-1, a key regulator of gene transcription driven by a serum response element, attenuated Egr-1 expression following stimulation, indicating that Elk-1 or related ternary complex factors connect the transcription of the *Egr-1* gene with the pregnenolone sulfate-induced intracellular signaling cascade elicited by the initial influx of Ca^{2+}. The newly synthesized Egr-1 was biologically active and bound under physiological conditions to the regulatory regions of the *Pdx-1*, *Synapsin I*, and *Chromogranin B* genes. Pdx-1 is a major regulator of insulin gene transcription. Accordingly, elevated insulin promoter activity and increased mRNA levels of insulin could be detected in pregnenolone sulfate-stimulated insulinoma cells. Likewise, the biosynthesis of synapsin I, a synaptic vesicle protein that is found at secretory granules in insulinoma cells, was stimulated in pregnenolone sulfate-treated INS-1 cells. Together, these data show that pregnenolone sulfate induces a signaling cascade in insulinoma cells that is very similar to the signaling cascade induced by glucose in β-cells.

Steroids synthesized in the central and peripheral nervous system that are, at least in part, independent of steroidogenic gland secretion are termed neurosteroids. They include progesterone, pregnenolone, pregnenolone sulfate, and dehydroepiandrosterone. Pregnenolone sulfate directly acts in the nervous system by modifying neurotransmission, receptor functions, and the strength of synaptic transmission (1). Stimulation with pregnenolone sulfate has been shown to exert modulatory effects on several types of receptors and ion channels including the *N*-methyl-D-aspartate receptor, the γ-amino butyric acid-A receptor (1–6), voltage-gated Ca^{2+} channels, and Kir2.3 K^+ channels (5, 7–9). Intracerebral infusions of pregnenolone sulfate was shown to influence cognitive processes, neuronal survival, and neurogenesis (10, 11).

Interestingly, the molecular cell biology of β-cells shows remarkable similarity to that of neurons. Neuronal genes are not only expressed in neurons, but also in endocrine cells. Pancreatic β-cells express synaptic vesicle proteins such as synapsin I, synaptophysin or synaptotagmin, neurotransmitters, and neurotransmitter-synthesizing enzymes. In line with this, it has recently been reported that the neurosteroid pregnenolone sulfate functions in insulinoma and β-cells by triggering a rapid Ca^{2+} influx into the cells, leading to enhanced insulin secretion (12). Here, we describe the first comprehensive analysis of pregnenolone sulfate-induced signal transduction in insulinoma cells. In addition, this is the first report showing that pregnenolone sulfate changes the genetic pattern of the cells by inducing the biosynthesis of a gene regulatory protein, the zinc finger transcription factor Egr-1. The expression of Egr-1 is regulated in many cell types by environmental signals including hormones, growth factors, and neurotransmitters (13, 14). The newly synthesized Egr-1 in turn couples extracellular signals with long term responses by altering the gene expression pattern of Egr-1 target genes. In INS-1 and MIN6 insulinoma cells the biosynthesis of Egr-1 is strongly stimulated by glucose (15–18). Most interestingly, Egr-1 has recently been shown to induce *Insulin* gene transcription via activation of the transcription factor pancreas duodenum homeobox-1 (Pdx-1)[3] (19), thus providing a link between glucose sensing and transcription of the *Insulin* gene. Here, we show that stimulation of

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 530/C14.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1] Present address: Stanford School of Medicine, Neuroscience Institute, Palo Alto, CA 94304.

[2] To whom correspondence may be addressed: Bldg. 44, University of Saarland Medical Center, D-66421 Homburg, Germany. Tel.: 49-6841-1626506; Fax: 49-6841-1626500; E-mail: gerald.thiel@uks.eu.

[3] The abbreviations used are: Pdx-1, pancreas duodenum homeobox-1; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl) ester; ERK, extracellular signal-regulated protein kinase; MKP, MAP kinase phosphatase; SRE, serum response element; TRP, transient receptor potential; CREB, cAMP-response element-binding protein.

Egr-1 biosynthesis by pregnenolone sulfate requires the influx of Ca^{2+} ions into the cytosol via TRPM3 and voltage-gated Ca^{2+} channels, and activation of ERK and ternary complex factor-mediated transcription. Downstream of Egr-1, we show that newly synthesized Egr-1 is biologically active and activates transcription of its targets, including the genes encoding Pdx-1, synapsin I, and chromogranin B.

MATERIALS AND METHODS

Cell Culture—The rat pancreatic β-cell line INS-1 was derived from cells isolated from an x-ray-induced rat transplantable insulinoma (20). INS-1 cells were kindly provided by Claes B. Wollheim and Susanne Ullrich, Division de Biochimie Clinique, University of Geneva, Switzerland. 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 of penicillin, and 100 μg/ml of streptomycin as described (21). This medium contains 11 mM glucose. All experiments, except the one depicted in Fig. 2*C*, were performed with INS-1 cells cultured in this medium. For the experiment performed with INS-1 cells cultured in low glucose medium (depicted in Fig. 2*C*), we used DMEM without glucose (Sigma number D5030) and added glucose to a final concentration of 2 mM. INS-1 cells were incubated for 24 h in medium without serum. Stimulation with pregnenolone sulfate (50 μM, dissolved in DMSO, Sigma), pregnenolone (50 μM, dissolved in DMSO, Sigma), progesterone (50 μM, dissolved in DMSO, Sigma), or KCl (25 mM) was performed for 1 h if not indicated otherwise. BAPTA-AM, a membrane-permeable form of BAPTA, was purchased from Calbiochem (catalog number 196419). The MAP kinase kinase inhibitor PD98059 was purchased from Axxora (Lauser, Switzerland, catalog number 385-023), dissolved in DMSO and used at a concentration of 50 μM as suggested (22). The voltage-gated Ca^{2+} channel blockers nifedipine and verapamil were purchased from Sigma, dissolved in DMSO, and used at a final concentration of 50 μM. Cells were preincubated with BAPTA-AM, PD98059, EGTA, nifedipine, or verapamil for 1 h.

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

Lentiviral Gene Transfer—All lentiviral transfer vectors used in this study are based on plasmids pFUW or pFUWG (23). The transgenes were expressed under the control of the human ubiquitin-C promoter. The lentiviral transfer vectors pFUW-MKP-1, pFUWΔCnA, pFUW-mycDA-Raf1, pFUWmycPP2C, pFUW-REST/Elk-1ΔC, and pFUW-REST/CREB have been described previously (18, 24–30). To generate an Egr-1_{DBD}/VP16 fusion protein, we cloned the coding region of the transcriptional activation domain of the HSV protein VP16 as an EcoRI/BamHI fragment into plasmid pCMV-FLAG-Egr-1/Zn. This plasmid was cut with NcoI and BamHI and filled in with the Klenow fragment of DNA polymerase I. The fragment was inserted into the HpaI site of plasmid pFUW, thus generating the lentiviral transfer vector pFUW-Egr1Zn/VP16. This plasmid encodes a truncated murine Egr-1 protein encompassing amino acids 322–442, fused to the transcriptional activation domain of VP16. The viral particles were produced as previously described (24) by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein, and the transfer vector.

Lentiviral Expression of Short Hairpin RNAs (shRNAs)—The lentiviral vector pLentiLox3.7 (pLL3.7) was purchased from American Type Culture Collection (Manassas, VA). The sequence used to knock down rat TRPM3 has been described (12). The oligonucleotides for creating RNAi stem loops for pLL3.7 were designed as described (26). The lentiviral transfer vector encoding a ATF2-specific shRNA, used as a negative control, will be described elsewhere.

Reporter Assays—The lentiviral transfer vectors pFWEgr-1.1luc, pFWSRE.luc, pFWEBS2[4]luc, pFWSyIluc, and pFWCg-Bluc have been described elsewhere (18, 26–31). Plasmid Ins-715Luc encoding an insulin promoter/luciferase reporter gene was a kind gift of Michiyo Amemiya-Kudo, Okinawa Memorial Institute for Medical Research, Tokyo, Japan (32). The plasmid was cut with PmeI and BglII and cloned upstream of the luciferase gene, generating the lentiviral transfer vector pFWInsluc. Cell extracts of stimulated cells were prepared using reporter lysis buffer (Promega) and analyzed for luciferase activities as described (33). Luciferase activity was normalized to the protein concentration.

Western Blots—Whole cell extracts, nuclear extracts, and crude membranes were prepared as described (34, 35). Proteins were separated by SDS-PAGE, blotted, and incubated with antibodies directed against Egr-1 (Santa Cruz, Heidelberg, Germany, sc-189), HDAC-1 (Upstate Biotechnology, Lake Placid, NY, 05-100), TRPM3 (12), Calnexin (Stressgen), or Synapsin I (a kind gift of T. C. Südhof, Stanford University). The antibody directed against histone deacetylase-1 (HDAC1) was used as a loading control as previously described (36). To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma, number F3165) at 1:3000 dilution. Antibodies against the myc epitope were prepared from CRL-1729 hybridomas (ATCC). Immunoreactive bands were detected via enhanced chemiluminescence using a 1:1 solution of solution 1 (100 mM Tris-HCl, pH 8.5, 5.4 mM H_2O_2) and solution 2 (2.5 mM Luminol, 400 μM *p*-coumaric acid, 100 mM Tris-HCl, pH 8.5). Densitometric analysis of signal intensities was performed using QuantityOne quantification analysis software (Bio-Rad). Values are expressed as the mean ± S.D. from 3 independent experiments. The statistical difference was analyzed using the Students's *t* test. A p value of <0.05 was considered significant.

RT-PCR—RT-PCR was performed as previously described (37). The primers are listed in Table 1. Quantitative real time PCR was performed using SYBR Green and gene-specific primers on a Stratagene Mx3000P. The primers are listed in Table 1. Total RNA isolated from islet cells was purified with the Qiagen RNeasy Plus Micro Kit (catalog number 74034). RNA samples

TABLE 1
Sequence of the primers used for ChIP and RT-PCR experiments

Genes	Forward primer	Reverse primer	Size of product
			bp
Gene-specific primers for RT-PCR			
GAPDH	ccctgcatccactggtgctgc	cattgagagcaatgccagccc	292
Insulin	gtacctggtgtgtggggaac	ccagttggtagagggagcag	200
β-Actin	ggctgtattcccccttccatcg	ccagttggtaacaatgccatgt	153
Egr-1	agcgaacaaccctatgagcac	tcgtttggctgggataactcg	99
Gene-specific primers for ChIP-PCR			
CgB	cctgagatccacagcacctg	ggcctggctcttatgaagg	204
Insulin	gtccccaacaactgcaactt	aggaggggtaggtaggcag	261
PDX-1	cttagctggtcagtgacaga	ggttaacaacatcaggctga	183
Synapsin I	gagccttactacgggtccag	ggtgaggtaggggagtttgg	245

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 s, primer annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. Gene expression values were calculated based on the comparative threshold cycle (C_t) method as described in Applied Biosystems User Bulletin No. 2, normalized to the expression of β-actin, and displayed as fold-induction relative to unstimulated cells.

Chromatin Immunoprecipitation (ChIP)—ChIP experiments were performed as described elsewhere (38) with modifications. All washing procedures were performed at 4 °C. Immunocomplexes were eluted from the beads with 2 × 250 μl of elution buffer at room temperature. The PCR fragments were purified using the Qiaquick PCR purification kit (Qiagen, 28106). The primers are listed in Table 1. The anti-Egr-1 antibody used for immunoprecipitation was purchased from Santa Cruz (sc-189).

Flow Cytometry—Analysis of at least 10,000 EGFP expressing cells was performed on a MoFlo cell sorter (Cytomation) 48 h after infection with pLentiLox3.7 constructs. The fluorescence of EGFP was excited using the 488 nm line of a argon laser and emitted light passing 530/40 (FL1) and 580/30 (FL2) bandpass filters was detected and analyzed in an uncompensated manner.

RESULTS

Stimulation of INS-1 Pancreatic β-Cells with Pregnenolone Sulfate Induces the Biosynthesis of Biologically Active Egr-1—Recently it has been shown that insulin-secreting rat INS-1 insulinoma cells are responsive to pregnenolone sulfate stimulation, leading to a rapid influx of Ca^{2+} ions into the cells (12). Therefore, we used INS-1 cells as a cellular model system to analyze the signal transduction pathway induced by pregnenolone sulfate. INS-1 cells were serum-starved for 24 h in medium containing 11 mM glucose. Cells were stimulated with pregnenolone sulfate (5–100 μM) for 1 h (Fig. 1*A*). The cells were harvested, nuclear extracts were prepared, and Egr-1 expression was analyzed via immunoblotting. Egr-1 immunoreactivity was almost undetectable in the absence of stimulation. Pregnenolone sulfate at concentrations of 50 or 100 μM strikingly increased the biosynthesis of Egr-1 (Fig. 1*A*). In contrast, incubation of the cells with pregnenolone or progesterone did not induce Egr-1 expression (Fig. 1*B*).

The ability of Egr-1 to activate transcription depends upon concentrations of Egr-1 negative cofactors NAB1 and NAB2. These proteins bind to Egr-1 and block transcriptional activation via Egr-1 (33, 39, 40). Thus, elevated Egr-1 protein levels do not automatically indicate an increased transcription of Egr-1 target genes. We therefore determined the transcription of an Egr-1-responsive target gene in pregnenolone sulfate-stimulated INS-1 cells using a chromosomally embedded Egr-1-responsive luciferase reporter gene. The reporter gene has been integrated into the genome of the cells via lentiviral gene transfer. A schematic depiction of the integrated provirus is shown in Fig. 1*C*. The implanted transcription unit encodes the luciferase reporter gene, controlled by a minimal promoter consisting of four binding sites for Egr-1 termed EBS, a TATA box, and an initiator element. The infected cells were stimulated with pregnenolone sulfate and gene transcription of the integrated reporter was measured. The results show that treatment of INS-1 cells with pregnenolone sulfate significantly increased the transcription of the Egr-1-responsive reporter gene (Fig. 1*C*), indicating that biologically active Egr-1 had been synthesized.

Stimulation with Pregnenolone Sulfate Increases the Egr-1 mRNA Concentration in Cultured Cells from Isolated Mouse Pancreatic Islets—To show that pregnenolone sulfate activates Egr-1 expression also in primary cultured cells, we isolated pancreatic islets and kept the cells in a short term culture. Cells were stimulated with pregnenolone sulfate (50 μM) for 1 h. RNA was isolated, reversed transcribed, and gene expression was monitored using quantitative real time PCR. Fig. 1*D* shows that stimulation with pregnenolone sulfate significantly increased the Egr-1 mRNA concentration.

TRPM3 Is Required for Pregnenolone Sulfate-stimulated Egr-1 Expression in INS-1 Cells Cultured in Low Glucose Containing Medium—It has been reported that stimulation of TRPM3 channels is involved for pregnenolone sulfate-induced Ca^{2+} signals in INS-1 cells (12). To assess the involvement of TRPM3 channel activation in the signal transduction leading to enhanced Egr-1 expression in INS-1 cells, we expressed a TRPM3-specific shRNA in INS-1 cells using lentiviral gene transfer. As a control, ATF2-specific shRNAs were expressed. The provirus contains, in addition to the transcription unit expressing shRNAs, a second transcription unit that encodes EGFP under control of the cytomegalovirus promoter/enhancer. Expression of EGFP was used to measure the infection

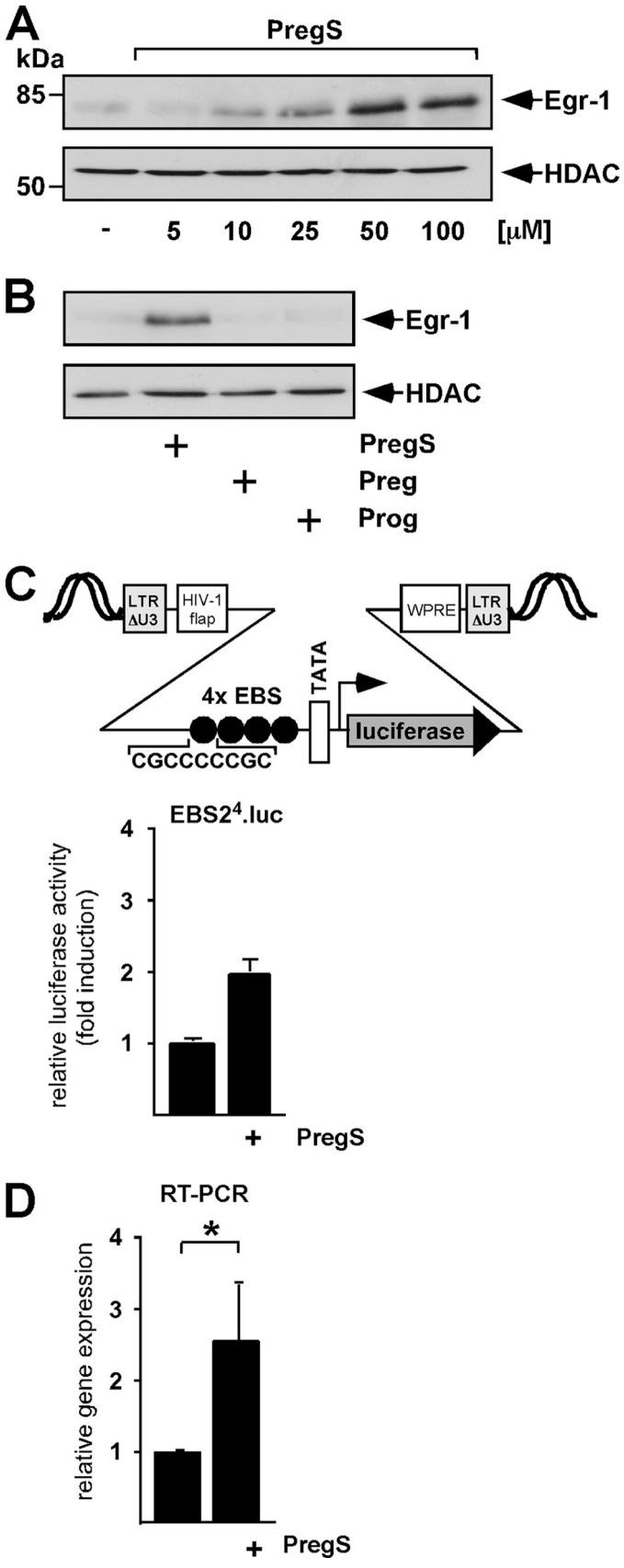

FIGURE 1. **Pregnenolone sulfate triggers the biosynthesis of biologically active Egr-1 in INS-1 insulinoma cells.** *A*, induction of Egr-1 biosynthesis in pregnenolone sulfate-stimulated INS-1 insulinoma cells. Cells were serum-starved for 24 h and then stimulated with pregnenolone sulfate (*PregS*, 50–100 μM) for 1 h as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control. *B*, INS-1 insulinoma cells were stimulated with pregnenolone sulfate (50 μM), pregnenolone (*Preg*, 50 μM), or progesterone (*Prog*, 50 μM). Egr-1 expression was assessed by Western blotting. *C*, the newly synthesized Egr-1 protein is biologically active. INS-1 cells were infected with a recombinant lentivirus encoding an Egr-1 responsive reporter gene. The infected cells were stimulated with pregnenolone sulfate for 24 h. Cell extracts were prepared and analyzed for luciferase activities, which were normalized to the protein concentrations. Each experiment illustrated and in all subsequent figures was repeated a minimum of three times with consistent results (*, $p < 0.05$). *D*, quantitative real-time PCR of RNA isolated from stimulated (*PregS*, 50 μM) or not stimulated pancreatic islet cells in primary short term culture. The Egr-1 signal was normalized to the β-actin signal and the fold-stimulation was calculated using $2^{-\Delta\Delta C_t}$ (*, $p < 0.05$).

rate following lentiviral gene transfer. Fig. 2*A* shows that EGFP was expressed in almost all cells indicating a high rate of infection. Corresponding analysis of the infection rate by flow cytometry demonstrated >95% green fluorescent cells (supplemental Fig. S1). The high expression rate of a TRPM3-specific shRNA in insulinoma cells following lentiviral infection allowed us to use shRNA-mediated knockdown of TRPM3 for biochemical analysis.

We analyzed the specificity of the TRPM3-specific shRNA. INS-1 cells were either mock infected or infected with lentivirus encoding shRNAs directed against either TRPM3 or ATF2. Cell extracts were prepared and analyzed for TRPM3 immunoreactivity. In INS-1 cells expressing a TRPM3-specific shRNA, expression of TRPM3 was significantly reduced (Fig. 2*B*), indicating that expression of a shRNA specific for TRPM3 induced down-regulation of TRPM3 expression.

We used INS-1 cells expressing shRNAs specific for either TRPM3 or ATF2 to assess the role of TRPM3 in the signaling cascade leading to enhanced Egr-1 expression following pregnenolone sulfate stimulation. Cells were cultured in medium containing either 2 or 11 mM glucose. Fig. 2*C* shows that the up-regulation of Egr-1 expression was significantly impaired in INS-1 cells that expressed the TRPM3-specific shRNA and were cultured in medium containing 2 mM glucose. In contrast, when the cells were cultured in medium containing 11 mM glucose, Egr-1 biosynthesis was up-regulated to a similar degree in pregnenolone sulfate-stimulated INS-1 cells that expressed either ATF2 or TRPM3-specific shRNAs (Fig. 2*D*). These data indicate that TRPM3 activation is not or only marginally involved under these conditions for the signaling cascade that leads to the biosynthesis of Egr-1 as a result of pregnenolone sulfate stimulation, suggesting that another component of the plasma membrane transduces the pregnenolone sulfate signal into the cells.

Pharmacological Inhibition of Voltage-gated Ca^{2+} Channels Blocks the Pregnenolone Sulfate-induced Expression of Egr-1 in INS-1 Insulinoma Cells—Activation of voltage-dependent Ca^{2+} channels is necessary for glucose signaling in pancreatic β-cells. In neurons, it has been shown that pregnenolone sulfate activates voltage-gated Ca^{2+} channels (6–8). Given the fact that TRPM3 channels play no or only a marginal role as signal transducer for pregnenolone sulfate in INS-1 cells cultured in medium containing 11 mM glucose, we assessed the involvement of voltage-gated Ca^{2+} channels. Stimulation of β-cells

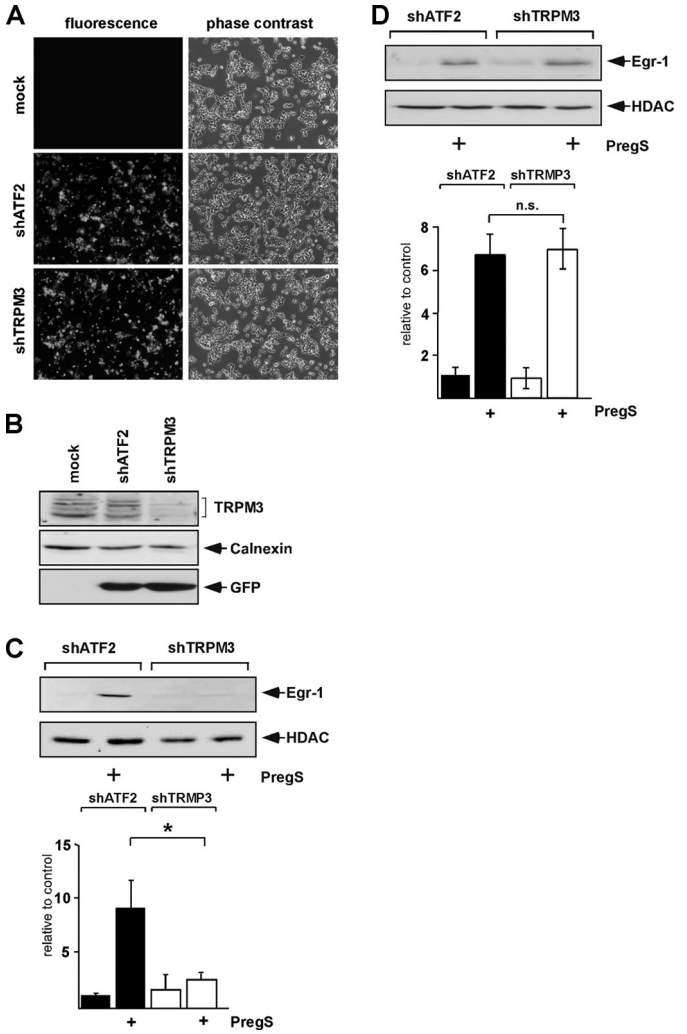

FIGURE 2. **Activation of transient receptor potential M3 (*TRPM3*) is required for pregnenolone sulfate-induced Egr-1 expression in INS-1 cells cultured in low glucose medium.** *A*, INS-1 cells were either mock-infected or infected with lentiviruses that encoded for TRPM3 or ATF2-specific shRNAs. In addition, expression of EGFP was induced from a second transcription unit. Green fluorescence and phase-contrast images are depicted. *B*, down-regulation of TRPM3 expression in INS-1 cells that express TRPM3-specific shRNAs. INS-1 cells were either mock-infected or infected with lentiviruses expressing TRPM3 or ATF2-specific shRNAs. Membrane proteins and whole cell extracts were prepared and analyzed for the expression of TRPM3, calnexin (as loading marker) and GFP. *C* and *D*, INS-1 cells cultured in medium containing either 2 (*C*) or 11 mM glucose (*D*) were infected with lentiviruses to express either TRPM3-specific or ATF2-specific shRNAs. Cells were serum-starved for 24 h and then stimulated with pregnenolone sulfate (*PregS*, 50 μM) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control (*, $p < 0.05$; *N.S.*, not significant ($p > 0.05$).

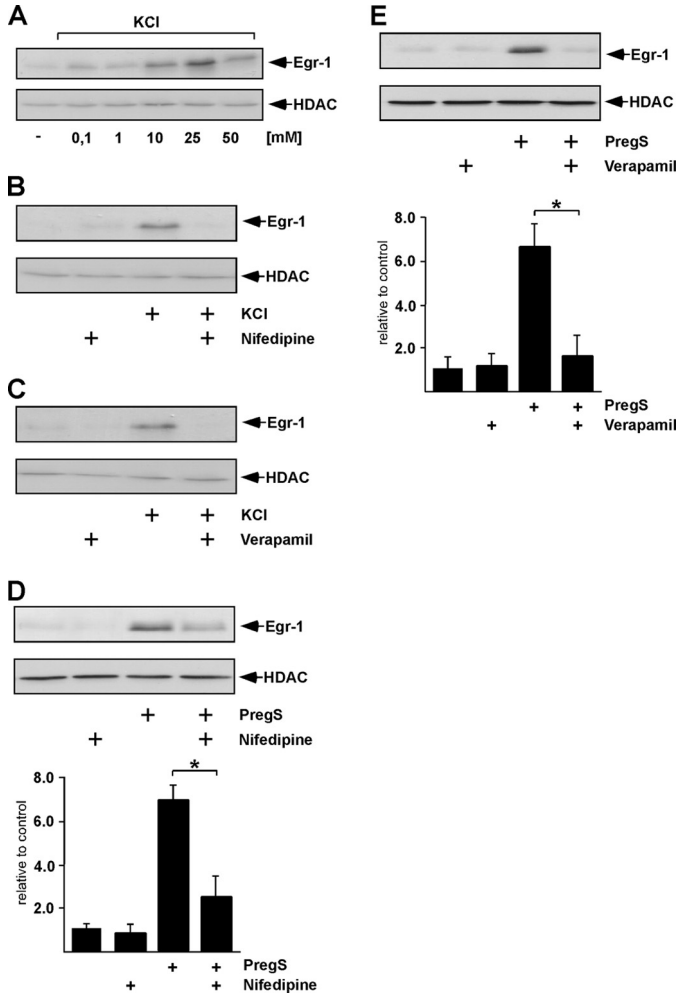

FIGURE 3. **Role of L-type Ca^{2+} channels for pregnenolone sulfate-induced Egr-1 expression in INS-1 cells.** *A*, induction of Egr-1 biosynthesis in KCl-stimulated INS-1 insulinoma cells. Cells were serum-starved for 24 h and then stimulated with KCl (25 mM) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control. *B* and *C*, expression of Egr-1 was impaired in KCl-treated INS cells preincubated with the voltage-gated Ca^{2+} channel blockers nifedipine (50 μM) or verapamil (50 μM). *D* and *E*, INS-1 cells were serum-starved for 24 h, preincubated for 1 h with either nifedipine (50 μM) or verapamil, and then stimulated with pregnenolone sulfate (*PregS*, 50 μM) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1 (*, $p < 0.05$).

with KCl leads elevation of the intracellular Ca^{2+} concentration via activation of these channels (41). Thus, we used the stimulation with KCl as a control. Fig. 3*A* shows that Egr-1 is synthesized in INS-1 cells that had been treated with KCl. Incubation of the cells with the voltage-gated Ca^{2+} channel blockers nifedipine or verapamil completely blocked Egr-1 expression following stimulation of the cells with KCl (Fig. 3, *B* and *C*). Next, we assessed the importance of voltage-gated Ca^{2+} channels for pregnenolone sulfate-induced up-regulation of Egr-1 in INS-1 cells. Fig. 3, *D* and *E*, shows that pregnenolone sulfate-induced Egr-1 expression in INS-1 was completely blocked by preincubation of the cells with either nifedipine or verapamil. Together, these data indicate that voltage-gated Ca^{2+} channels are involved in pregnenolone sulfate-triggered up-regulation of Egr-1 biosynthesis in INS-1 insulinoma cells cultured in medium containing 11 mM glucose.

Essential Role of Extracellular and Intracellular Ca^{2+} Ions in Pregnenolone Sulfate-induced Up-regulation of Egr-1 Expression in INS-1 Insulinoma Cells—The previous experiments have shown that either TRPM3 or voltage-gated Ca^{2+} channels are involved in the signal transduction of pregnenolone sulfate across the plasma membrane, suggesting an influx of Ca^{2+} ions from outside into the cells. Thus, we next assessed the role of extracellular and intracellular Ca^{2+} ions in pregnenolone sulfate-induced up-regulation of Egr-1 expression in INS-1 insulinoma cells.

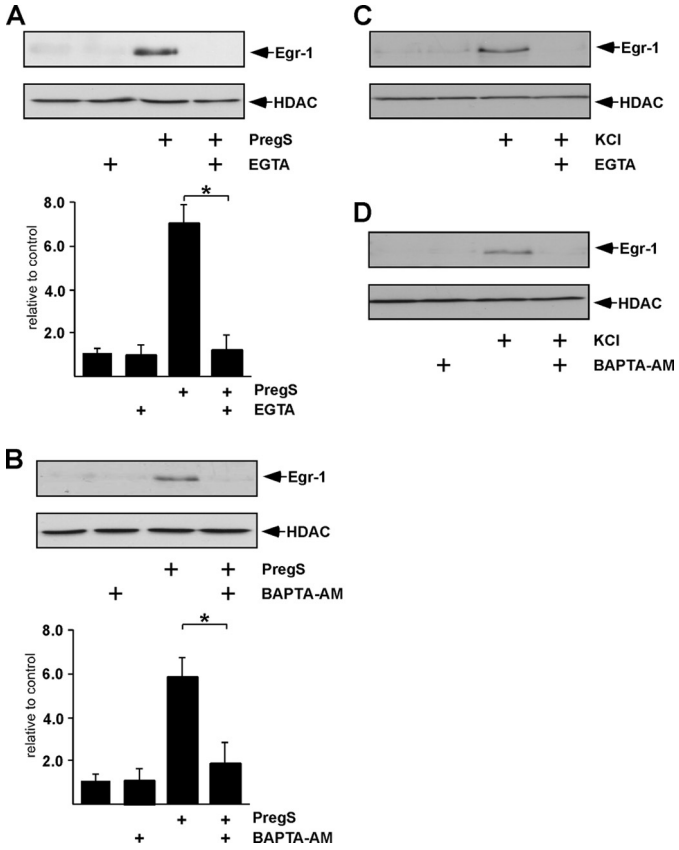

FIGURE 4. **Chelation of extracellular or intracellular Ca^{2+} attenuates expression of Egr-1 in pregnenolone sulfate or KCl-stimulated INS-1 cells.** INS-1 cells were preincubated for 1 h with EGTA (500 μM) (*A* and *C*) or BAPTA-AM (25 μM) (*B* and *D*). Cells were stimulated with either pregnenolone sulfate (*PregS*, 50 μM) or KCl (25 mM) (*C* and *D*) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control (*, $p < 0.05$).

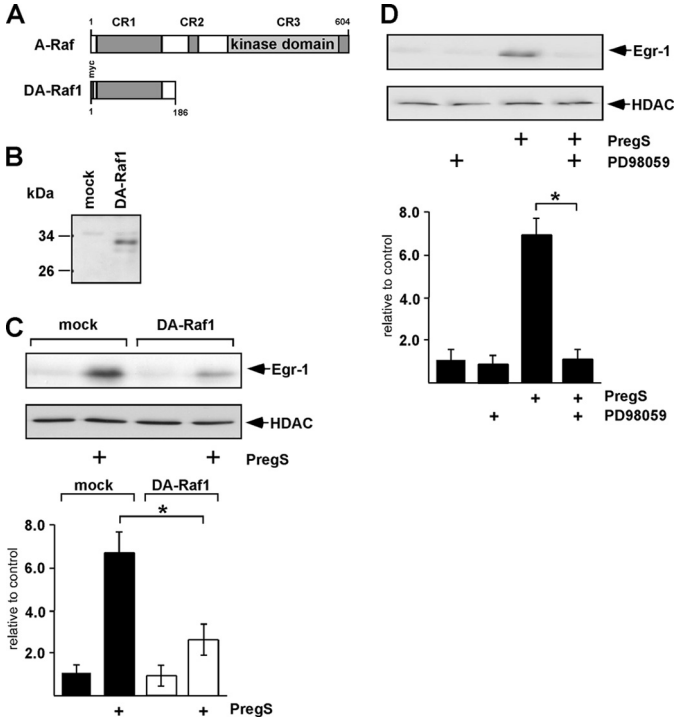

FIGURE 5. **Essential role of ERK activation for the pregnenolone sulfate-triggered up-regulation of Egr-1 expression in INS-1 cells.** *A*, modular structure of A-Raf and the DA-Raf1. *B*, expression of DA-Raf1 in lentiviral-infected INS-1 is detected using an antibody against the N-terminal myc tag. Molecular mass markers in kDa are shown on the *left*. *C*, INS-1 insulinoma cells were infected with a lentivirus encoding DA-Raf1. As a control INS-1 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (*mock*). The cells were serum starved for 24 h. Stimulation with pregnenolone sulfate (PregS, 50 μM) was performed for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1. *D*, INS-1 insulinoma cells were preincubated with PD98059 (50 μM) for 1 h and then stimulated with pregnenolone sulfate (*PregS*, 50 μM) for 1 h. Nuclear extracts were prepared and analyzed for Egr-1 expression (*, $p < 0.05$).

First, we tested whether extracellular Ca^{2+} is required to induce Egr-1 expression in pregnenolone sulfate-stimulated INS-1 cells. We used EGTA to buffer the Ca^{2+} ions in the medium. Fig. 4*A* shows that under these conditions the up-regulation of Egr-1 following pregnenolone sulfate stimulation was completely blocked, indicating that an influx of Ca^{2+} ions into INS-1 cells is essential to connect pregnenolone sulfate stimulation with enhanced Egr-1 expression. Second, we tested whether elevated intracellular Ca^{2+} levels are required to induce Egr-1 expression in pregnenolone sulfate-stimulated INS-1 cells. The pregnenolone sulfate-induced elevation of $[Ca^{2+}]_i$ was precluded by preincubation with BAPTA-AM. As a result, the stimulus-induced biosynthesis of Egr-1 was completely blocked (Fig. 4*B*). Hence, an influx of Ca^{2+} ions into the cells via TRPM3 and voltage-gated Ca^{2+} channels and the subsequent elevation of $[Ca^{2+}]_i$ is essential for induction of Egr-1 biosynthesis following stimulation of the cells with pregnenolone sulfate. As a control, we tested the effects of EGTA and BAPTA-AM preincubation on the biosynthesis of Egr-1 following KCl treatment. Fig. 4, *C* and *D*, shows that Egr-1 is not synthesized in KCl-treated INS-1 cells when either the extracellular Ca^{2+} concentration was reduced or elevation of the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was prevented.

Activation of ERK Is Essential for the Induction of Egr-1 Biosynthesis in INS-1 Cells Stimulated with Pregnenolone Sulfate—Elevation of the intracellular Ca^{2+} concentrations often triggers an activation of ERK, a crucial factor for induction of Egr-1 biosynthesis in many cell types, including insulinoma cells (18, 42). The connection between an elevated Ca^{2+} concentration and activation of the ERK signaling pathway is accomplished by PKC, most likely PKCα and PKCβII (18, 43). We assessed the role of ERK in pregnenolone sulfate-treated INS-1 cells using genetic and pharmacological tools. First, we inhibited Raf, a MAP kinase kinase kinase, via expression of a dominant-negative antagonist of the Ras/ERK pathway. Second, we treated INS-1 cells with PD98059, a compound that inhibits phosphorylation of the MAP kinase kinase by Raf. Fig. 5*A* shows the modular structure of DA-Raf1, a splicing isoform of A-Raf that functions as an antagonist of the Ras/Raf-ERK1/2 pathway (44). Cellular proteins of mock-infected INS-1 cells or cells infected with a myc-tagged DA-Raf1 encoding lentivirus were fractionated by SDS-PAGE. The fusion protein was identified by Western blot analysis using an antibody targeting the myc epitope (Fig. 5*B*). Next, the functional implication of DA-Raf1 expression was assessed (Fig. 5*C*). The results show that expression of DA-Raf1 significantly reduced the up-regulation of Egr-1

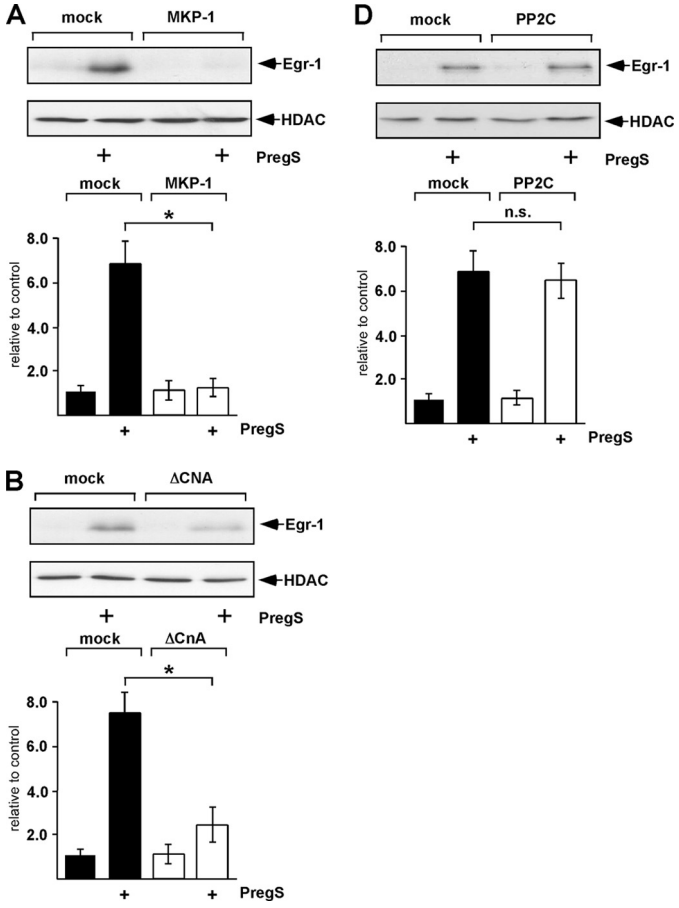

FIGURE 6. **Expression of MKP-1 or ΔCnA, a constitutively active mutant of calcineurin A, attenuates expression of Egr-1 in pregnenolone sulfate-stimulated INS-1 cells.** INS-1 cells were infected with recombinant lentiviruses encoding either MKP-1 (*A*), ΔCnA, a constitutively active mutant of calcineurin A (*B*), or PP2C (*C*). The transgenes were expressed under control of the human ubiquitin C promoter. As a control mock-infected cells were analyzed. The cells were serum-starved for 24 h and then treated with pregnenolone sulfate (*PregS*, 50 μM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control (*, $p < 0.05$; *n.s.*, $p > 0.05$).

expression in pregnenolone sulfate-stimulated INS-1 cells. Likewise, preincubation of the cells with PD98059 efficiently blocked up-regulation of Egr-1 in pregnenolone sulfate-stimulated insulinoma cells. Together, these data indicate that ERK1/2 activation is a key event in controlling Egr-1 expression as a result of pregnenolone sulfate treatment.

Expression of MKP-1 or ΔCnA, a Constitutively Active Form of Calcineurin A, Impairs Pregnenolone Sulfate-induced Up-regulation of Egr-1—MKP-1, the phosphatase that dephosphorylates and inactivates ERK in the nucleus, is synthesized in different cell types following ERK activation (28, 30). Hence, MKP-1 is part of a negative feedback loop inducing dephosphorylation and inactivation of nuclear ERK. Having shown that ERK activation is a key step in the signaling cascade leading to *Egr-1* gene transcription in INS-1 cells, we tested whether overexpression of MKP-1 counteracts the stimulus-induced biosynthesis of Egr-1. Fig. 6*A* shows that the biosynthesis of Egr-1 was impaired in pregnenolone sulfate-stimulated INS-1 cells that had been infected with a MKP-1-encoding lentivirus. These data indicate that active ERK in the nucleus was required within the signaling cascade.

The Ca^{2+}-regulated phosphatase calcineurin negatively regulates the transcriptional activity of the ternary complex factor Elk-1 (45, 46), a major regulator of *Egr-1* gene transcription (26, 27, 30). Hence, calcineurin may be part of a negative feedback loop inducing dephosphorylation and inactivation of ternary complex factors. Calcineurin is composed of two polypeptides, calcineurin A and B. We expressed a constitutively active calcineurin A mutant termed ΔCnA that lacks the calmodulin binding site and the C-terminal autoinhibitory domain and that does not require Ca^{2+} ions for activation. Fig. 6*B* shows that expression of ΔCnA significantly reduced expression of Egr-1. In contrast, expression of protein phosphatase 2C (PP2C), which has not been correlated with the Ca^{2+}/ERK/Elk-1/Egr-1 signaling pathway, did not impair pregnenolone sulfate-induced up-regulation of Egr-1 (Fig. 6*C*).

The Proximal Serum Response Elements of the Egr-1 Promoter Are Essential for the Up-regulation of Egr-1 Expression in Pregnenolone Sulfate-treated Insulinoma Cells—The 5′-flanking region of the *Egr-1* gene contains five serum response elements, and these motifs are responsible for the induction of *Egr-1* gene transcription by various extracellular signaling molecules (13, 14). To identify genetic elements that mediate pregnenolone sulfate responsiveness of the *Egr-1* gene we inserted Egr-1 promoter/luciferase reporter genes into the chromatin of INS-1 cells using lentiviral gene transfer. The transfer vector pFWEgr-1.1luc encodes an Egr-1 promoter/luciferase reporter gene that contains 239 nucleotides of the human *Egr-1* gene 5′ upstream region, including a cyclic AMP response element and the proximal serum response elements (SREs), together with 235 nucleotides of the 5′-nontranslated region. The transfer vector pFWSREluc encodes the luciferase gene under control of the two proximal SREs of the Egr-1 promoter upstream of a minimal promoter. Fig. 7*A* shows a schematic depiction of the integrated proviruses encoding the Egr-1 promoter/luciferase reporter genes. INS-1 cells were infected with recombinant lentiviruses and stimulated with pregnenolone sulfate. The addition of pregnenolone sulfate induced reporter gene transcription to similar levels for both transcription units (Fig. 7*A*), indicating that the proximal cluster of SREs is sufficient for the up-regulation of Egr-1 transcription in pregnenolone sulfate-stimulated INS-1 cells.

Suppression of Ternary Complex Factor Activity Blocks the Up-regulation of Egr-1 Expression in Pregnenolone-stimulated INS-1 Pancreatic β-Cells—Given the importance of the proximal SREs within the Egr-1 promoter, we directly assessed the impact of ternary complex factor activation on the regulation of *Egr-1* gene transcription. To overcome the problem associated with redundancy of functions between the ternary complex factors, we expressed a dominant-negative mutant of the ternary complex factor Elk-1, termed REST/Elk-1ΔC (Fig. 7*B*). This mutant retains the DNA binding and serum response factor 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 (47), a FLAG epitope for immunological detection and a nuclear localization signal. Nuclear proteins of mock-infected

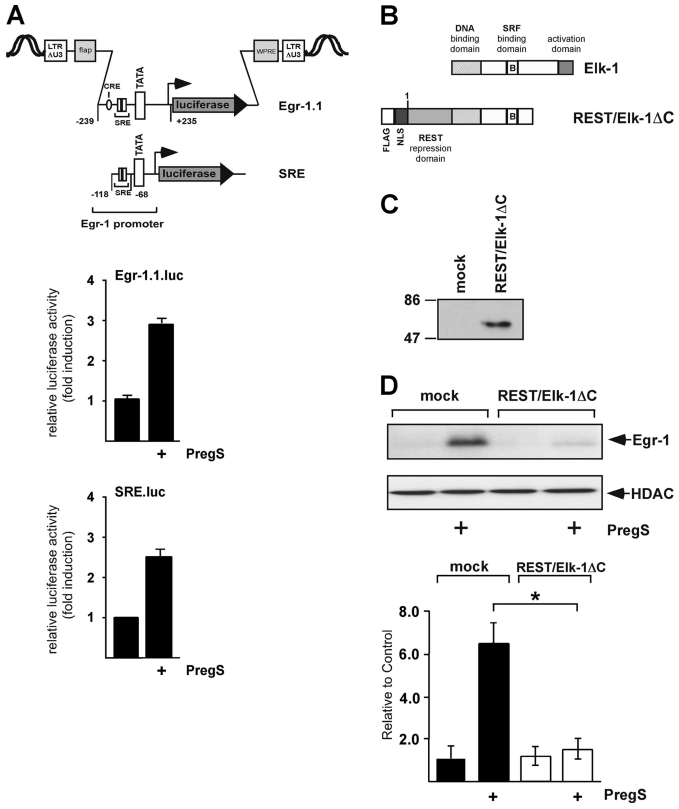

FIGURE 7. **Essential role of ternary complex factor activation for the pregnenolone sulfate-induced up-regulation of Egr-1 expression in INS-1 cells.** *A*, transcriptional up-regulation of Egr-1 promoter/luciferase reporter genes in pregnenolone sulfate-stimulated INS-1 cells. Schematic representation of the integrated proviruses encoding Egr-1 promoter/luciferase reporter genes. The cyclic AMP response element (*CRE*) and the proximal SREs are depicted. The U3 region of the 5′ long terminal repeat of the transfer vector is deleted. The woodchuck hepatitis virus post-transcriptional regulatory element (*WPRE*) and the HIV flap element are indicated. INS-1 cells were infected with recombinant lentiviruses prepared with transfer vectors pFWEgr-1.1luc or pFWEgr-1SREluc. The infected cells were treated with vehicle or pregnenolone sulfate (denoted "+") for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. *B*, schematic representation of wild-type Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. *C*, Western blot analysis of mock-infected INS-1 cells or cells infected with a recombinant lentivirus encoding REST/Elk-1ΔC. The Western blot was probed with an antibody against the FLAG tag. Molecular mass markers in kDa are shown on the *left*. *D*, expression of REST/Elk-1ΔC blocked pregnenolone sulfate-induced up-regulation of Egr-1 in INS-1 cells. Cells were either mock infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC and stimulated with pregnenolone sulfate as indicated. Nuclear extracts were prepared and subjected to Western blot analysis. The blot was incubated with an antibody directed against Egr-1 (*, $p < 0.05$).

INS-1 cells or cells infected with a REST/Elk-1ΔC encoding lentivirus were fractionated by SDS-PAGE. The fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 7*C*). Fig. 7*D* shows that expression of REST/Elk-1ΔC completely prevented the induction of Egr-1 biosynthesis in INS-1 cells that had been stimulated with pregnenolone sulfate. We conclude that ternary complex factor activation is essential for inducing Egr-1 transcription in pregnenolone sulfate-stimulated INS-1 cells.

Recently, we showed that suppression of CREB activity impaired the up-regulation of Egr-1 biosynthesis in glucose, tolbutamide, and KCl-stimulated MIN6 insulinoma cells (18). Thus, we assessed the involvement of CREB in the signaling cascade connecting pregnenolone sulfate stimulation with enhanced Egr-1 biosynthesis using a dominant-negative mutant of CREB termed REST/CREB (supplemental Fig. S2*A*). The 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 tag, and an nuclear localization signal. The fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (supplemental Fig. S2*B*). Expression of REST/CREB had no effect upon the biosynthesis of Egr-1 in pregnenolone sulfate-stimulated INS-1 cells (supplemental Fig. S2*C*).

Chromatin Immunoprecipitation Reveals Binding of Egr-1 to the Pdx-1 Gene in Pregnenolone Sulfate-stimulated INS-1 Insulinoma Cells—In Fig. 1, we have shown that pregnenolone sulfate stimulation leads to the biosynthesis of biologically active Egr-1. We therefore tested whether Egr-1 binds under physiological conditions in a stimulus-dependent manner to *Egr-1* target genes. Egr-1 has been shown to bind to the *Pdx-1* gene, a major regulator of insulin expression (19). The binding site in the *Pdx-1* regulatory region is depicted in Fig. 8*A*. Cross-linked and sheared chromatin prepared from unstimulated INS-1 cells and INS-1 cells stimulated with pregnenolone sulfate was immunoprecipitated with an antibody directed against Egr-1. Fig. 8*A* shows that Egr-1 bound under physiological conditions to the regulatory region of the *Pdx-1* gene when the cells had been stimulated with pregnenolone sulfate. No binding of Egr-1 to the *Insulin* gene was observed (Fig. 8*B*).

Pregnenolone Sulfate Stimulation Up-regulates Insulin Promoter Activity and Insulin Expression in INS-1 Insulinoma Cells—Given the fact that Pdx-1 regulates insulin expression, we measured insulin promoter activity. We implanted an insulin promoter/luciferase reporter gene into the chromatin of INS-1 cells. Fig. 8*C* shows a schematic depiction of the integrated provirus. The infected cells were stimulated with pregnenolone sulfate. As a control, mock-infected cells were analyzed. Fig. 8*C* shows that pregnenolone sulfate stimulation enhanced transcription of the integrated insulin promoter/luciferase reporter gene. Likewise, forced expression of an Egr-1/VP16 fusion protein that lacked the NAB1/2 binding site activated transcription of the insulin promoter/reporter gene in INS-1 cells (Fig. 8*D*), indicating that Egr-1 expression triggers an up-regulation of insulin biosynthesis. Fig. 8*E* shows that elevated insulin mRNA levels could be detected in INS-1 cells that had been treated with pregnenolone sulfate.

Pregnenolone Sulfate Stimulation Up-regulates Synapsin I Promoter Activity and Synapsin I Expression in INS-1 Insulinoma Cells—Synapsin I is a synaptic vesicle-associated protein that is also expressed in β-cells (48). The regulatory region of the *Synapsin I* gene contains a binding site for Egr-1 (Fig. 9*A*) and binding of Egr-1 to this site has been shown *in vitro* (49). ChIP experiments showed that Egr-1 bound under physiological conditions to the regulatory region of the *Synapsin I* gene when the cells had been stimulated with pregnenolone sulfate (Fig. 9*A*).

Stimulation of INS-1 cells that had an integrated synapsin I promoter/luciferase reporter gene with pregnenolone sulfate revealed that reporter gene transcription was significantly enhanced. Likewise, forced expression of an Egr-1/

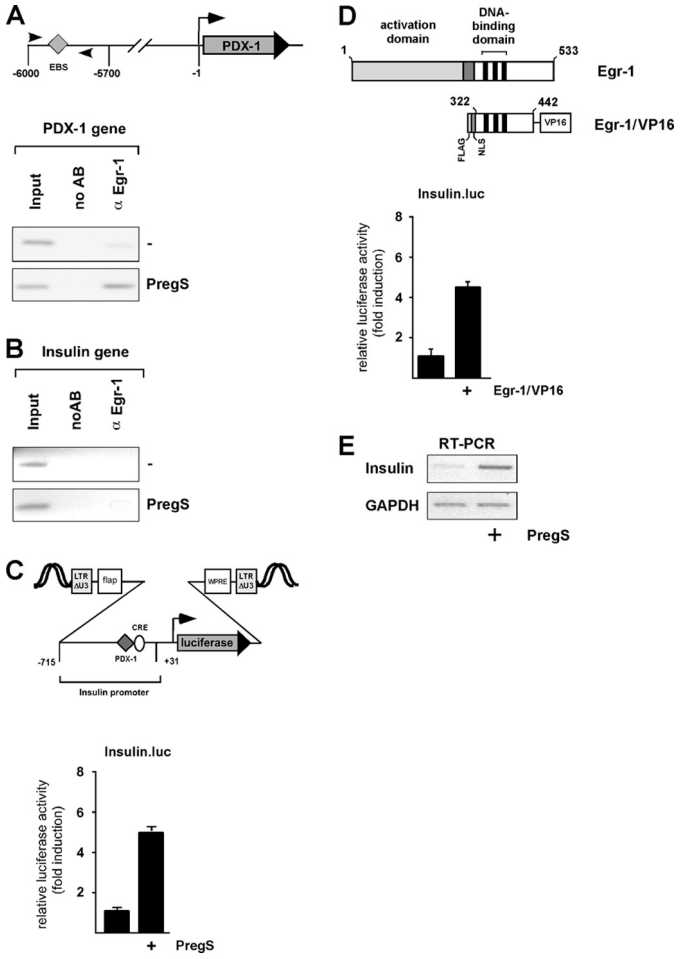

FIGURE 8. **Egr-1 binds to the *Pdx-1* gene in pregnenolone sulfate-stimulated INS-1 cells and regulates insulin expression.** *A*, schematic representation of the *Pdx-1* gene. The locations of the Egr-1 binding site (*EBS*) and the PCR primers used for the ChIP experiments are depicted. ChIP was performed with chromatin isolated from INS-1 cells that had been stimulated with pregnenolone sulfate (50 μM, 1 h). As a control, chromatin of unstimulated cells was analyzed. Cross-linked and sheared chromatin was immunoprecipitated with an antibody directed against Egr-1. As a negative control, ChIP was performed with preimmune serum (*no AB*). As a positive control, an aliquot of the total chromatin was analyzed by PCR (*Input*). PCR primers were used to amplify the proximal region of the Pdx-1 (*A*) or insulin (*B*) promoters. *C*, schematic representation of an integrated provirus encoding the insulin promoter/luciferase reporter gene. The cyclic AMP response element (*CRE*) and the binding site for Pdx-1 are depicted. INS-1 insulinoma cells were infected with a recombinant lentivirus encoding an insulin promoter/luciferase reporter gene. The infected cells were stimulated with pregnenolone sulfate for 24 h. Cell extracts were prepared and analyzed for luciferase activities normalized to the protein concentrations. *D*, modular structure of Egr-1 and Egr-1 mutant Egr-1/VP16. INS-1 insulinoma cells were double-infected with recombinant lentiviruses encoding an insulin promoter/luciferase reporter gene and the Egr-1 mutant Egr-1/VP16. 24 h later, cell extracts were prepared and analyzed for luciferase activities. *E*, pregnenolone sulfate stimulation leads to increased insulin mRNA levels. INS-1 cells were serum starved for 24 h and then stimulated with pregnenolone sulfate (*PregS*, 50 μM) for 1 h. Total RNA was isolated, the mRNA reverse transcribed, and the cDNA was analyzed by PCR.

VP16 fusion protein activated synapsin I promoter/luciferase reporter gene transcription (Fig. 9*B*). Accordingly, Western blot analysis revealed that pregnenolone sulfate-treated INS-1 cells expressed higher levels of synapsin I in comparison to untreated cells (Fig. 9*C*).

Egr-1 Regulates Chromogranin B Gene Transcription in Pregnenolone Sulfate Stimulated in INS-1 Insulinoma Cells—Recently, we have shown that Egr-1 binds to the *Chromogranin B* gene in buserelin-stimulated gonadotrophs (26). The granins are a group of acidic proteins of secretory granules that are highly expressed in neuroendocrine and pancreatic β-cells (38, 50). ChIP experiments showed that Egr-1 bound to the regulatory region of the *Chromogranin B* gene when the cells had been stimulated with pregnenolone sulfate (Fig. 9*D*). In contrast, we did not detect binding of Egr-1 in unstimulated cells. Likewise, transcription of a nucleosomal-embedded chromogranin B promoter/reporter gene was enhanced in INS-1 cells that had either been stimulated with pregnenolone sulfate or expressed an Egr-1/VP16 fusion protein (Fig. 9*E*). Together, these data reveal that the genes encoding Pdx-1, synapsin I, and chromogranin B are *bona fide* target genes of Egr-1 in INS-1 insulinoma cells.

DISCUSSION

We are interested in understanding the mechanisms of selective gene transcription following cellular stimulation. The neurosteroid pregnenolone sulfate functions on insulinoma and triggers a rapid influx of Ca^{2+} ions into these cells (12). The objective of this study was to investigate the signaling cascade induced by pregnenolone sulfate in insulinoma cells. In this study, we have shown that influx of Ca^{2+} ions via TRPM3 and voltage-gated Ca^{2+} channels, and activation of ERK and ternary complex factors are integral parts of the signaling cascade connecting pregnenolone sulfate stimulation with enhanced *Egr-1* gene transcription. This is the first report showing that stimulation with pregnenolone sulfate leads to transcriptional changes in the cells. Interestingly, the signaling molecules required to induce Egr-1 in pregnenolone sulfate-stimulated cells are also necessary in glucose-stimulated insulinoma cells (15–18), indicating that glucose and pregnenolone sulfate induce a similar, if not identical signaling pathway in insulinoma and β-cells (Fig. 10). The effect of pregnenolone sulfate on Egr-1 expression has also been shown to occur in pancreatic islet cells in primary culture.

There is no dispute about the essential role of increased Ca^{2+} concentration in glucose-induced signaling in β-cells. The metabolism of glucose increases the concentration of ATP that, in turn, induces closure of the nucleotide-regulated potassium channel K_{ATP} in the plasma membrane, leading to depolarization of the membrane and subsequent activation of voltage-gated Ca^{2+} channels. Ca^{2+} influx via these voltage-dependent Ca^{2+} channels is necessary for the glucose-induced insulin secretion. Accordingly, inhibition of voltage-gated Ca^{2+} channels by dihydropyridine nifedipine blocks glucose-induced insulin secretion as well as glucose-induced up-regulation of Egr-1 expression (18, 51, 52). Likewise, pharmacological activation of voltage-gated Ca^{2+} channels increases insulin secretion in the absence of glucose (52). Pregnenolone sulfate stimulation has been shown to induce an influx of Ca^{2+} ions into neurons or pancreatic β-cells (5, 7, 12, 53). Using pharmacological tools, we have shown in this study that the influx of Ca^{2+} ions and the subsequent rise of the intracellular Ca^{2+} concentration is absolutely essential to continue the pregnenolone sulfate-induced signaling cascade in INS-1 cells.

Pregnenolone sulfate-induced Ca^{2+} influx into neurons may occur by activating neurotransmitter receptors (*i.e. N*-methyl-

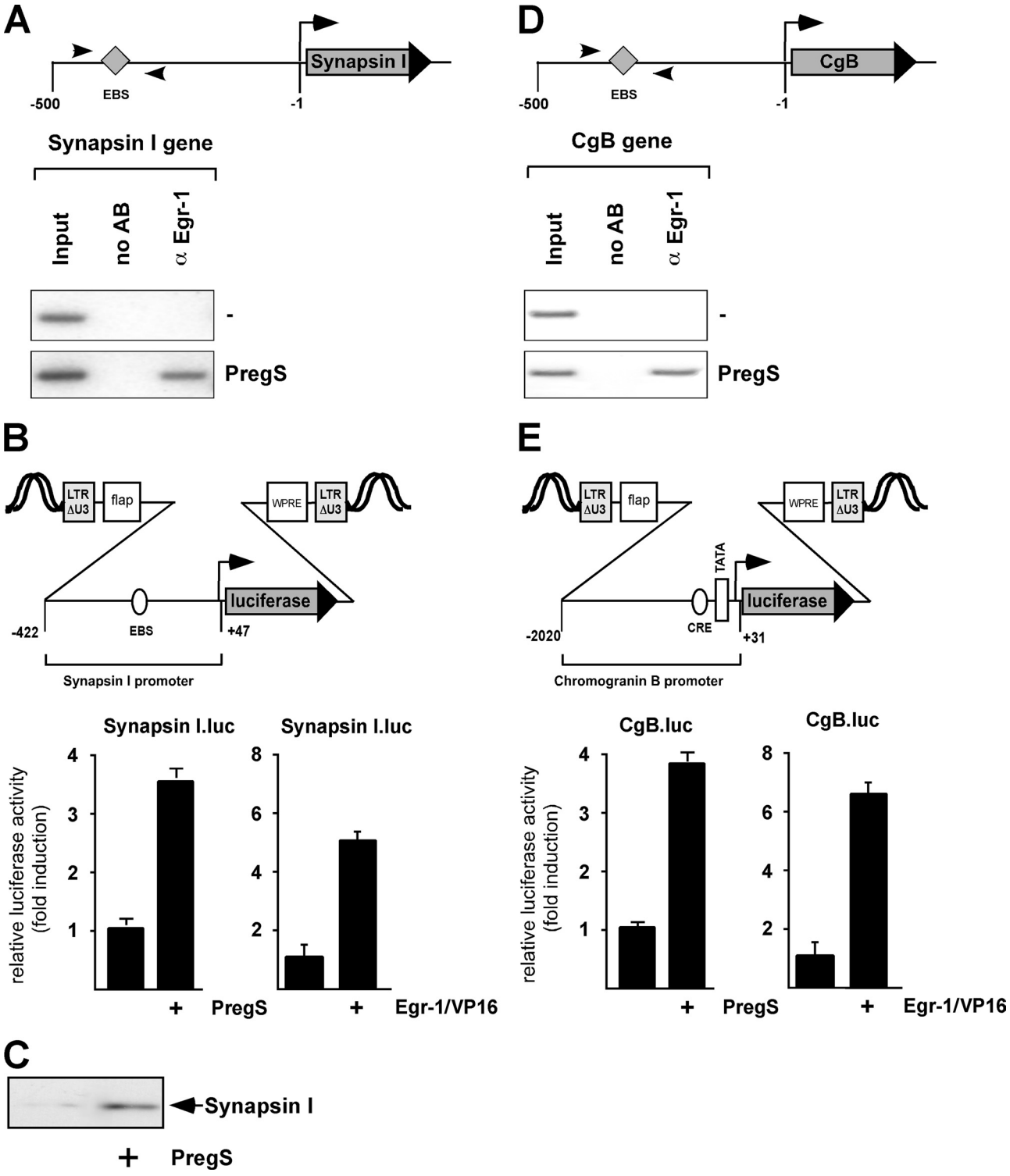

FIGURE 9. **Egr-1 binds to the *Synapsin I* and *Chromogranin B* genes in pregnenolone sulfate-stimulated INS-1 cells and up-regulates synapsin I and chromogranin B promoter activity.** *A*, schematic representation of the *Synapsin I* gene. The location of the Egr-1 binding site (*EBS*) and the PCR primers used for ChIP are depicted. ChIP experiments reveal binding of Egr-1 to the synapsin I promoter in pregnenolone sulfate-stimulated INS-1 cells. *B*, INS-1 insulinoma cells were infected with a recombinant lentivirus encoding a synapsin I promoter/luciferase reporter gene. The infected cells were stimulated with pregnenolone sulfate for 24 h. Cell extracts were prepared and analyzed for luciferase activities (*left panel*). INS-1 insulinoma cells were double infected with recombinant lentiviruses encoding a synapsin I promoter/luciferase reporter gene and the Egr-1 mutant Egr-1/VP16. 24 h later, cell extracts were prepared and analyzed for luciferase activities (*right panel*). *C*, increased levels of synapsin I in pregnenolone sulfate-stimulated INS-1 cells. Cells were stimulated with pregnenolone sulfate (*PregS*, 50 μM). Whole cell extracts were prepared and subjected to Western blot analysis using an antibody directed against synapsin I. *D*, schematic representation of the *Chromogranin B* gene. The locations of the Egr-1 binding site (*EBS*) and PCR primers used for ChIP are depicted. ChIP experiments reveals binding of Egr-1 to the chromogranin B promoter in pregnenolone sulfate-stimulated INS-1 cells. *E*, INS-1 insulinoma cells were infected with a recombinant lentivirus encoding a chromogranin B promoter/luciferase reporter gene. The infected cells were stimulated with pregnenolone sulfate for 24 h (*left panel*). INS-1 insulinoma cells were double infected with recombinant lentiviruses encoding a chromogranin B promoter/luciferase reporter gene and the Egr-1 mutant Egr-1/VP16. 24 h later, cell extracts were prepared and analyzed for luciferase activities (*right panel*).

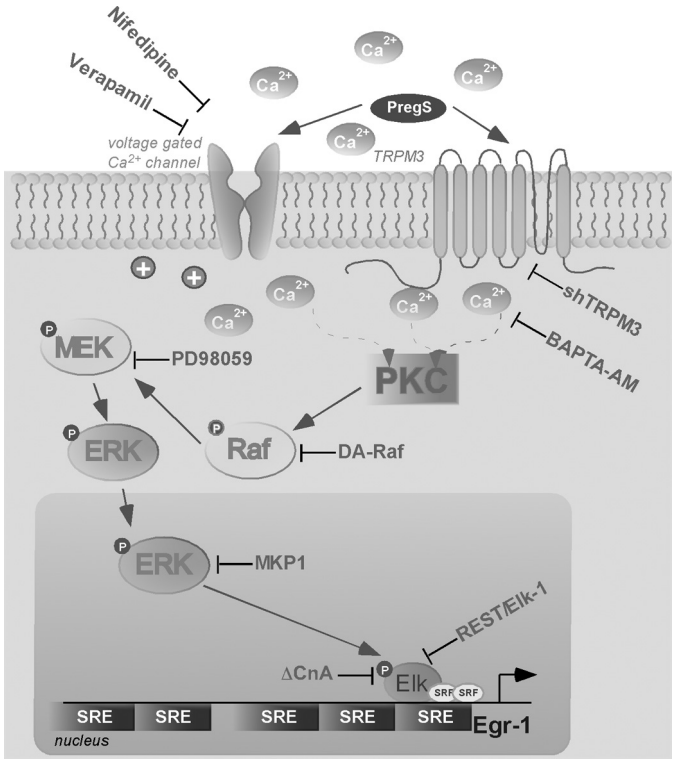

FIGURE 10. **Pregnenolone sulfate induced intracellular signaling pathways leading to Egr-1 expression in INS-1 cells.** Stimulation of insulinoma cells with pregnenolone sulfate leads to the activation of TRPM3 and voltage-gated Ca^{2+} channels in insulinoma cells. Up-regulation of Egr-1 expression was blocked by either expression of a TRPM3-specific shRNA or by pharmacological inhibitors of voltage-gated Ca^{2+} channels (nifedipine, verapamil). As a result, the cytosolic Ca^{2+} concentration is increased via an influx of Ca^{2+} ions from the outside. The increase of the intracellular Ca^{2+} concentration could be prevented by pretreating the cells with BAPTA-AM. Elevation of the intracellular Ca^{2+} concentration leads to activation of the ERK signaling cascade, mediated by PKC that directly or indirectly regulates Raf activity. The compound PD98059 was used to inhibit phosphorylation of the MAP kinase kinase by Raf, thus blocking the stimulus-induced phosphorylation and activation of ERK. Expression of a splice form of A-Raf, *DA-Raf*, was used as a tool to show the importance of Raf in the signaling cascade leading to enhanced Egr-1 expression in pregnenolone sulfate-stimulated INS-1 cells. A major nuclear substrate for ERK is the ternary complex factor Elk-1, an essential component of the SRE ternary complex. The Egr-1 promoter contains five SREs that mediate signal-induced activation of *Egr-1* gene transcription. Stimulus-induced Egr-1 biosynthesis could be blocked by expressing a dominant-negative mutant of Elk-1 (termed *REST/Elk-1ΔC*). The phosphatases MKP-1 and calcineurin functions as negative regulators of this signaling cascade by dephosphorylating ERK and Elk-1, respectively.

D-aspartate receptors) or by stimulating voltage-gated Ca^{2+} channels (6–8). 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 voltage-gated Ca^{2+} channel blocker nifedipine (6). Likewise, pregnenolone sulfate facilitates glutamate release from calyx synapses via the direct modulation of presynaptic voltage-dependent Ca^{2+} channels (7). A recent study showed that long-term potentiation induced by conditioning electric stimuli at 20 Hz was dependent on L-type voltage-gated Ca^{2+} channels (8). In this study, we have shown for the first time that pregnenolone sulfate also targets voltage-gated Ca^{2+} channels in insulinoma cells. Thus, voltage-gated Ca^{2+} channels are intermediate steps in the signaling cascade triggered by pregnenolone sulfate in neurons and pancreatic β-cells. In addition, the requirement of TRPM3 channels has been clearly demonstrated in the analysis of INS-1 cells expressing a TRPM3-specific shRNA, supporting previously published data (12). Thus, pregnenolone sulfate targets both voltage-gated Ca^{2+} channels and TRPM3 channels in insulinoma cells. Likewise, both *N*-methyl-D-aspartate receptors and voltage-gated Ca^{2+} channels have been identified as targets for pregnenolone sulfate in neurons. For the regulation of *N*-methyl-D-aspartate receptor activity by pregnenolone sulfate, it has been shown that the extracellular loop between the 3rd and 4th transmembrane domain (M3-M4 loop) of the NR2 subunit is essential to confer sensitivity of the receptor to pregnenolone sulfate (5). Accordingly, the simplest hypothesis would be that pregnenolone sulfate binds to extracellular domains of both TRPM3 and voltage-gated Ca^{2+} channels and thereby modulates their activity. The sites attributed to pregnenolone sulfate binding to the TRPM3 channel and the voltage-gated Ca^{2+} channel have yet to be identified as well as the molecular mechanism of channel activation by this neurosteroid.

Stimulation of pancreatic β-cells with glucose has been reported to activate ERK with Ca^{2+} ions as the essential mediator between glucose stimulation and ERK1/2 activation (15, 43, 53). Additionally, ERK activation is the major stimulus for induction of *Egr-1* gene transcription (13, 14). Accordingly, the signaling cascade leading to Egr-1 expression in glucose-stimulated insulinoma cells relies on ERK1/2 activation (18). Using genetic and pharmacological tools, we have shown in this study that ERK1/2 activation is also required in pregnenolone sulfate-stimulated INS-1 cells for up-regulation of Egr-1 expression. The experiments showed clearly that inhibition of ERK activation by PD98059 interfered with up-regulation of Egr-1 expression in pregnenolone sulfate-treated cells. In addition, overexpression of MKP-1, an enzyme that dephosphorylates and inactivates ERK and other MAP kinases in the nucleus, completely blocked stimulus-induced Egr-1 biosynthesis, indicating that MKP-1 functions as a nuclear shut-off device that interrupts the signaling cascades induced by pregnenolone sulfate stimulation. Furthermore, these experiments indicate that nuclear translocation of the phosphorylated ERK1/2 is required to stimulate Egr-1 expression. Interestingly, pregnenolone sulfate stimulation also induces a sustained activation of ERK2 in the hippocampus (8), indicating that pregnenolone sulfate signaling is similar in neurons and insulin-secreting cells.

In the nucleus, phosphorylated ERK1/2 is able to change the transcriptional program by phosphorylating transcriptional regulatory proteins. One of the major substrates of ERK1/2 is Elk-1, a ternary complex factor that connects the ERK signaling cascade with serum response element-mediated transcription. Elk-1 is phosphorylated by several protein kinases including ERK, leading to enhanced DNA binding activity, ternary complex formation, and SRE-mediated transcription. The Egr-1 promoter contains five SREs encompassing the consensus sequence $CC(A/T)_6GG$, also termed the CArG box. In addition, multiple binding sites for ternary complex factors (Ets) are adjacent to the CArG boxes having the Ets consensus core sequence GGA(A/T). The SREs within the Egr-1 promoter mediate signal-induced activation of *Egr-1* gene transcription (25). Thus, transcriptional activation of Egr-1 is often preceded by the activation of Elk-1. Stimulation of insulinoma cells with glucose has

been shown to induce the phosphorylation of Elk-1 (54). Recently, we confirmed these data and showed additionally that phosphorylated Elk-1 binds under physiological conditions to the regulatory region of the *Egr-1* gene in insulinoma cells that had been stimulated with glucose (18). In addition, loss-of-function experiments unequivocally showed that ternary complex factor activation is a key event for the glucose-induced up-regulation of Egr-1 expression (18). The analysis of Egr-1 promoter/luciferase reporter genes revealed that the most proximal SREs of the Egr-1 promoter are involved for transduction of pregnenolone sulfate signaling to the *Egr-1* gene. The necessity of ternary complex factor activation was shown by using a dominant-negative version of Elk-1 in loss-of-function experiments. These data were corroborated by experiments showing that calcineurin, a Ca^{2+}-dependent protein phosphatase that dephosphorylates and inactivates Elk-1 (45, 46), blocked pregnenolone sulfate-induced up-regulation of Egr-1 expression. Activation of calcineurin may function as a negative feedback loop leading to the dephosphorylation of Elk-1. Together with previously published data, we conclude that ternary complex factor activation is essential for connecting either glucose or pregnenolone sulfate stimulation with enhanced expression of Egr-1.

Egr-1 has been proposed to regulate insulin biosynthesis (18, 19). Recently, it has been shown that Egr-1 enhances insulin expression via up-regulation of the transcription factor Pdx-1 (19), providing a link between glucose sensing and transcription of the *Insulin* gene. This study shows for the first time that Egr-1 binds under physiological conditions to the regulatory region of the Pdx-1 gene in pregnenolone sulfate-stimulated insulinoma cells. Pdx-1 is a major regulator of the *Insulin* gene. Accordingly, elevated insulin mRNA could be detected in pregnenolone sulfate-stimulated insulinoma cells. β-Cells are very sensitive to changes in Pdx-1 expression. Transgenic mice with one Pdx-1 allele inactivated had impaired glucose tolerance and secreted less insulin in a glucose tolerance test (55), indicating an inability of the Pdx-1 heterozygous mice to respond to glucose stimulation. Furthermore, Pdx-1 directly influences the exocytotic machinery by regulating expression of synaptotagmin 1 (56), a Ca^{2+} sensor involved in Ca^{2+}-dependent insulin secretion.

Synapsin I and chromogranin B are proteins of synaptic and secretory granules, respectively. Synapsin I was originally discovered in neurons, but expression of synapsin I, like other neuronal proteins, has been detected in pancreatic β-cells. Synapsin I was found on insulin secretory granules in insulinoma cells (48). Chromogranin B belongs to the granins, a group of acidic proteins of secretory granules that are highly expressed in endocrine cells (38, 50), and thought to play important roles in the sorting, packaging, and processing of secreted peptides (57). Both the synapsin I and chromogranin B encoding genes contain binding sites for Egr-1 in their regulatory regions (26, 49). The results presented here reveal that Egr-1 binds in insulinoma cells to the *Synapsin I* and *Chromogranin B* genes under physiological conditions when the cells had been stimulated with pregnenolone sulfate. Moreover, elevated synapsin I and chromogranin B promoter activities have been detected in pregnenolone sulfate-stimulated INS-1 cells. Thus, Egr-1 may directly influence exocytosis in insulin-secreting cells by regulating the expression of components of the secretory machinery.

In summary, we present here the first comprehensive analysis of pregnenolone sulfate signaling in insulinoma cells. The results show that pregnenolone sulfate stimulation of INS-1 insulinoma cells promotes expression of Egr-1 using a signaling cascade involving activation of TRPM3 and voltage-gated Ca^{2+} channels, an influx of Ca^{2+} ions into the cells, and activation of ERK1/2. In the nucleus, the ternary complex factor Elk-1 connects the signaling cascade with the *Egr-1* gene. The newly synthesized Egr-1 is biologically active and binds to the *Pdx-1*, *Synapsin I*, and *Chromogranin B* genes, suggesting that insulin biosynthesis and secretion may be regulated by pregnenolone sulfate stimulation. Accordingly, higher levels of insulin mRNA were detected in pregnenolone sulfate-stimulated INS-1 cells. The signaling cascades induced by long term stimulation of insulinoma cells with either glucose or pregnenolone sulfate is very similar, if not identical.

Acknowledgments—We thank Thomas Südhof for anti-synapsin I antibodies, Michiyo Amemiya-Kudo for plasmid Ins-715Luc, Stephan Philipp for help with flow cytometry, J. Oberwinkler for the suggestion to test pregnenolone sulfate signaling in medium containing 2 mM glucose; Karl Bach for excellent technical help, and Libby Guethlein, Oliver Rössler, and Stephan Philipp for critical reading of the manuscript.

REFERENCES

1. Baulieu, E. E. (1998) *Psychoneuroendocrinology* **23,** 963–987
2. Compagnone, N. A., and Mellon, S. H. (2000) *Front. Neuroendocrinol.* **21,** 1–56
3. Akk, G., Bracamontes, J., and Steinbach, J. H. (2001) *J. Physiol.* **532,** 673–684
4. Gibbs, T. T., Russek, S. J., and Farb, D. H. (2006) *Pharmacol. Biochem. Behav.* **84,** 555–567
5. Horak, M., Vlcek, K., Chodounska, H., and Vyklicky, L., Jr. (2006) *Neuroscience* **137,** 93–102
6. Chen, L., and Sokabe, M. (2005) *J. Neurophysiol.* **94,** 4131–4144
7. Hige, T., Fujiyoshi, Y., and Takahashi, T. (2006) *Eur. J. Neurosci.* **24,** 1955–1966
8. Chen, L., Cai, W., Chen, L., Zhou, R., Furuya, K., and Sokabe, M. (2010) *Hippocampus* **20,** 499–512
9. Kobayashi, T., Washiyama, K., and Ikeda, K. (2009) *PLoS ONE* **4,** e63118
10. Mayo, W., Le Moal, M., and Abrous, D. N. (2001) *Horm. Behav.* **40,** 215–217
11. Charalampopoulos, I., Remboutsika, E., Margioris, A. N., and Gravanis, A. (2008) *Trends Endocrinol. Metab.* **19,** 300–307
12. Wagner, T. F., Loch, S., Lambert, S., Straub, I., Mannebach, S., Mathar, I., Düfer, M., Lis, A., Flockerzi, V., Philipp, S. E., and Oberwinkler, J. (2008) *Nat. Cell. Biol.* **10,** 1421–1430
13. Thiel, G., and Cibelli, G. (2002) *J. Cell. Physiol.* **193,** 287–292
14. Rössler, O. G., Stefano, L., Bauer, I., and Thiel, G. (2006) in *Transcription Factors in the Nervous System, Development, Brain Function, and Disease* (Thiel, G., ed) pp. 379–395, Wiley-VCH, Weinheim, Germany
15. Frödin, M., Sekine, N., Roche, E., Filloux, C., Prentki, M., Wollheim, C. B., and Van Obberghen, E. (1995) *J. Biol. Chem.* **270,** 7882–7889
16. Josefsen, K., Sørensen, L. R., Buschard, K., and Birkenbach, M. (1999) *Diabetologia* **42,** 195–203
17. Bernal-Mizrachi, E., Wice, B., Inoue, H., and Permutt, M. A. (2000) *J. Biol. Chem.* **275,** 25681–25689
18. Mayer, S. I., and Thiel, G. (2009) *Eur. J. Cell Biol.* **88,** 19–33

19. Eto, K., Kaur, V., and Thomas, M. K. (2007) *J. Biol. Chem.* **282,** 5973–5983
20. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A., and Wollheim, C. B. (1992) *Endocrinology* **130,** 167–178
21. Groot, M., Boxer, L. M., and Thiel, G. (2000) *Eur. J. Cell Biol.* **79,** 924–935
22. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95–105
23. Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002) *Science* **295,** 868–872
24. Stefano, L., Al Sarraj, J., Rössler, O. G., Vinson, C., and Thiel, G. (2006) *J. Neurochem.* **97,** 92–104
25. Bauer, I., Hohl, M., Al-Sarraj, A., Vinson, C., and Thiel, G. (2005) *Arch. Biochem. Biophys.* **438,** 36–52
26. Mayer, S. I., Dexheimer, V., Nishida, E., Kitajima, S., and Thiel, G. (2008) *Endocrinology* **149,** 6311–6325
27. Mayer, S. I., Willars, G. B., Nishida, E., and Thiel, G. (2008) *J. Cell. Biochem.* **105,** 1267–1278
28. Rössler, O. G., Henss, I., and Thiel, G. (2008) *Arch. Biochem. Biophys.* **470,** 93–102
29. Mayer, S. I., Rössler, O. G., Endo, T., Charnay, P., and Thiel, G. (2009) *J. Cell Sci.* **122,** 3340–3350
30. Rössler, O. G., and Thiel, G. (2009) *BMC Mol. Biol.* **10,** 40
31. Ekici, M., Schmitz, F., Hohl, M., Seigel, G. M., and Thiel, G. (2008) *Neurochem. Int.* **53,** 165–172
32. Amemiya-Kudo, M., Oka, J., Ide, T., Matsuzaka, T., Sone, H., Yoshikawa, T., Yahagi, N., Ishibashi, S., Osuga, J., Yamada, N., Murase, T., and Shimano, H. (2005) *J. Biol. Chem.* **280,** 34577–34589
33. Thiel, G., Kaufmann, K., Magin, A., Lietz, M., Bach, K., and Cramer, M. (2000) *Biochim. Biophys. Acta* **1493,** 289–301
34. Kaufmann, K., and Thiel, G. (2001) *J. Neurol. Sci.* **189,** 83–91
35. Kaufmann, K., and Thiel, G. (2002) *J. Cell. Biochem.* **85,** 381–391
36. Spohn, D., Rössler, O. G., Philipp, S. E., Raubuch, M., Kitajima, S., Griesemer, D., Hoth, M., and Thiel, G. (2010) *Mol. Pharmacol.* **78,** 865–876
37. Bauer, I., Al Sarraj, J., Vinson, C., Larsen, R., and Thiel, G. (2007) *J. Cell. Biochem.* **100,** 242–255
38. Hohl, M., and Thiel, G. (2005) *Eur. J. Neurosci.* **22,** 2216–2230
39. Russo, M. W., Sevetson, B. R., and Milbrandt, J. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6873–6877
40. Svaren, J., Sevetson, B. R., Apel, E. D., Zimonjic, D. B., Popescu, N. C., and Milbrandt, J. (1996) *Mol. Cell. Biol.* **16,** 3545–3553
41. Satin, L. S. (2000) *Endocrine* **13,** 251–262
42. Kaufmann, K., Bach, K., and Thiel, G. (2001) *Biol. Chem.* **382,** 1077–1081
43. Benes, C., Poitout, V., Marie, J. C., Martin-Perez, J., Roisin, M. P., and Fagard, R. (1999) *Biochem. J.* **340,** 219–225
44. Yokoyama, T., Takano, K., Yoshida, A., Katada, F., Sun, P., Takenawa, T., Andoh, T., and Endo, T. (2007) *J. Cell Biol.* **177,** 781–793
45. Sugimoto, T., Stewart, S., and Guan, K. L. (1997) *J. Biol. Chem.* **272,** 29415–29418
46. Tian, J., and Karin, M. (1999) *J. Biol. Chem.* **274,** 15173–15180
47. Thiel, G., Lietz, M., and Cramer, M. (1998) *J. Biol. Chem.* **273,** 26891–26899
48. Matsumoto, K., Ebihara, K., Yamamoto, H., Tabuchi, H., Fukunaga, K., Yasunami, M., Ohkubo, H., Shichiri, M., and Miyamoto, E. (1999) *J. Biol. Chem.* **274,** 2053–2059
49. Thiel, G., Schoch, S., and Petersohn, D. (1994) *J. Biol. Chem.* **269,** 15294–15301
50. Giordano, T., Brigatti, C., Podini, P., Bonifacio, E., Meldolesi, J., and Malosio, M. L. (2008) *Diabetologia* **51,** 997–1007
51. Trus, M., Corkey, R. F., Nesher, R., Richard, A. M., Deeney, J. T., Corkey, B. E., and Atlas, D. (2007) *Biochemistry* **46,** 14461–14467
52. Yang, M., Chisholm, J. W., Soelaiman, S., and Shryock, J. C. (2010) *Mol. Cell. Endocrinol.* **315,** 308–313
53. Arnette, D., Gibson, T. B., Lawrence, M. C., January, B., Khoo, S., McGlynn, K., Vanderbilt, C. A., and Cobb, M. H. (2003) *J. Biol. Chem.* **278,** 32517–32525
54. Bernal-Mizrachi, E., Wen, W., Srinivasan, S., Klenk, A., Cohen, D., and Permutt, M. A. (2001) *Am. J. Physiol. Endocrinol. Metab.* **281,** E1286–E1299
55. Brissova, M., Shiota, M., Nicholson, W. E., Gannon, M., Knobel, S. M., Piston, D. W., Wright, C. V., and Powers, A. C. (2002) *J. Biol. Chem.* **277,** 11225–11232
56. Nakajima-Nagata, N., Sugai, M., Sakurai, T., Miyazaki, J., Tabata, Y., and Shimizu, A. (2004) *Biochem. Biophys. Res. Commun.* **318,** 631–635
57. Glombik, M. M., Krömer, A., Salm, T., Huttner, W. B., and Gerdes, H. H. (1999) *EMBO J.* **18,** 1059–1070