Signal Transduction of Pregnenolone Sulfate in Insulinoma Cells

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

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

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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 Ca2+ ions via transient receptor potential M3 and voltage-gated Ca2+ channels, elevation of the cytosolic Ca2+ 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 Ca2+. The newly synthesized Egr-1 was biochemically 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-aminobutyric acid-A receptor (1–6), voltage-gated Ca2+ channels, and Kir2.3 K+ channels (5, 7–9). Intracerebral infusions of pregnenolone sulfate were 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 Ca2+ 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.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 51 and 52.

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3 The abbreviations used are: Pdx-1, pancreas duodenum homeobox-1; BAPTA-AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N’,N’-tetraacetic acid tetracetate (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 \(\beta\)-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 \(\mu\)M \(\beta\)-mercaptoethanol, 100 units/ml of penicillin, and 100 \(\mu\)g/ml of streptomycin as described (21). This medium contains 11 mM glucose. All experiments, except the one depicted in Fig. 2C, were performed with INS-1 cells cultured in this medium. Stimulation with pregnenolone sulfate (50 \(\mu\)M, dissolved in DMSO, Sigma), pregnenolone (50 \(\mu\)M, dissolved in DMSO, Sigma), progesterone (50 \(\mu\)M, 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 196419). The MAP kinase kinase inhibitor PD98059 was purchased from Axxora (Lauser, Switzerland, catalog number 196419). For the experiment performed with INS-1 cells cultured in low glucose medium (depicted in Fig. 2), we used DMEM without glucose (Sigma without 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 \(\mu\)M, dissolved in DMSO, Sigma), pregnenolone (50 \(\mu\)M, dissolved in DMSO, Sigma), progesterone (50 \(\mu\)M, 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 \(\mu\)M 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 \(\mu\)M. 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 \(\mu\)M) 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, pFUW\(\Delta\)CnA, pFUW-myCDA-Raf1, pFUWmycPP2C, pFUW-REST/Elk-1\(\Delta\)C, and pFUW-REST/CREB have been described previously (18, 24–30). To generate an Egr-1DBD/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 Hpal 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 enve 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, pFWSuperLuc, pFWEs2\(\alpha\)Luc, pFWSyLuc, and pFWCgBluc 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 Pmel and BglIII 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\(_2\)O\(_2\)) and solution 2 (2.5 mM Luminol, 400 \(\mu\)M 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’ 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
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**TABLE 1**

| Genes          | Forward primer | Reverse primer | Size of product |
|----------------|----------------|----------------|-----------------|
| GAPDH          | cctgctacetcgagctgcgc | cattgagacacatgacctgc | 292             |
| Insulin        | gtacctgtttagggagagac | cccatttgttagaggagagac | 200             |
| β-Actin        | ggtctatgaccctctcatcg | cccattgttagaacatgactag | 153             |
| Egr-1          | aggcaacacccctgtagacac | tgcgatgctgagcatacctg | 99              |

Gene-specific primers for ChIP-PCR

| Genes | Forward primer | Reverse primer | Size of product |
|-------|----------------|----------------|-----------------|
| CgB   | cctgagatcaccagcagtg | gccctggctcttataggaag | 204             |
| Insulin | gttcccaaactgcaagtctt | aggagggtagttagagaaag | 261             |
| PDX-1  | ttagcttgccctgagcaga | gttcacaactgacgctga | 183             |
| Synapsin 1 | gagctctactacggtcctg | ggtgaggtaggggagttgg | 245             |

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. 1C. 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. 1C), 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. 1D 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<sup>2+</sup> 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...
rate following lentiviral gene transfer. Fig. 2A 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 (supplementary 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. 2B), 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. 2C 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. 2D). 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 \(\beta\)-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 \(\beta\)-cells

![FIGURE 1. Pregnenolone sulfate triggers the biosynthesis of biologically active Egr-1 in INS-1 insulinoma cells.](image)
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. 3A 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
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. 4A 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+}], was precluded by preincubation with BAPTA-AM. As a result, the stimulus-induced biosynthesis of Egr-1 was completely blocked (Fig. 4B). 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+}], 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+}], 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/Raf-ERK1/2 pathway. Second, we treated INS-1 cells with PD98059, a compound that inhibits phosphorylation of the MAP kinase kinase by Raf. Fig. 5A 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. 5B). Next, the functional implication of DA-Raf1 expression was assessed (Fig. 5C). The results show that expression of DA-Raf1 significantly reduced the up-regulation of Egr-1.
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 ∆CnA, 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. 6A 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 Ca²⁺-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 ∆CnA that lacks the calmodulin binding site and the C-terminal autoinhibitory domain and that does not require Ca²⁺ ions for activation. Fig. 6B shows that expression of ∆CnA significantly reduced expression of Egr-1. In contrast, expression of protein phosphatase 2C (PP2C), which has not been correlated with the Ca²⁺/ERK/Elk-1/Egr-1 signaling pathway, did not impair pregnenolone sulfate-induced up-regulation of Egr-1 (Fig. 6C).

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 pFWSRluc encodes the luciferase gene under control of the two proximal SREs of the Egr-1 promoter upstream of a minimal promoter. Fig. 7A 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. 7A), 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-1∆C (Fig. 7B). This mutant retains the DNA binding and serum response factor interaction domains, but lacks the C-terminal activation domain of Elk-1. REST/Elk-1∆C 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
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 (supplemental Fig. S2A). 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. S2B).

Expression of REST/CREB had no effect upon the biosynthesis of Egr-1 in pregnenolone sulfate-stimulated INS-1 cells (supplemental Fig. S2C).

Pregnenolone Sulfate Stimulation Up-regulates 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. 8A. 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. 8A 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. 8B).

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. 8C 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. 8C 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. 8D), indicating that Egr-1 expression triggers an up-regulation of insulin biosynthesis. Fig. 8E 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. 9A) 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. 9A).

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/
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**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 (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. 9B). Accordingly, Western blot analysis revealed that pregnenolone sulfate-treated INS-1 cells expressed higher levels of synapsin I in comparison to untreated cells (Fig. 9C).

_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. 9D). 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. 9E). 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\(_{\text{ATP}}\_\) 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 reveal 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).
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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-1C). 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)\(_6\)GG, 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 \textit{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 \textit{Egr-1} expression (18). The analysis of \textit{Egr-1} promoter/luciferase reporter genes revealed that the most proximal SREs of the \textit{Egr-1} promoter are involved for transduction of pregnenolone sulfate signaling to the \textit{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 \(\text{Ca}^{2+}\)-dependent protein phosphatase that dephosphorylates and inactivates Elk-1 (45, 46), blocked pregnenolone sulfate-induced up-regulation of \textit{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 \textit{Egr-1}.

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

\textit{Synapsin I} and \textit{Chromogranin B} are proteins of synaptic and secretory granules, respectively. \textit{Synapsin I} was originally discovered in neurons, but expression of \textit{synapsin I}, like other neuronal proteins, has been detected in pancreatic \textit{\(\beta\)-cells}. \textit{Synapsin I} was found on insulin secretory granules in insulinoma cells (48). \textit{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 \textit{synapsin I} and \textit{Chromogranin B} encoding genes contain binding sites for \textit{Egr-1} in their regulatory regions (26, 49). The results presented here reveal that \textit{Egr-1} binds in insulinoma cells to the \textit{Synapsin I} and \textit{Chromogranin B} genes under physiological conditions when the cells had been stimulated with pregnenolone sulfate. Moreover, elevated \textit{synapsin I} and \textit{Chromogranin B} promoter activities have been detected in pregnenolone sulfate-stimulated INS-1 cells. Thus, \textit{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 \textit{INS-1} insulinoma cells promotes expression of \textit{Egr-1} using a signaling cascade involving activation of TRPM3 and voltage-gated \(\text{Ca}^{2+}\) channels, an influx of \(\text{Ca}^{2+}\) ions into the cells, and activation of ERK1/2. In the nucleus, the ternary complex factor Elk-1 connects the signaling cascade with the \textit{Egr-1} gene. The newly synthesized \textit{Egr-1} is biologically active and binds to the \textit{Ins-1}, \textit{Synapsin I}, and \textit{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.

\textbf{Acknowledgments—}We thank Thomas S{"u}dhof for anti-synapsin I antibodies, Michiyo Amemiya-Kudo for plasmid \textit{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 R{"o}ssler, and Stephan Philipp for critical reading of the manuscript.

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