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Thapsigargin-Induced Ca^{2+} Increase Inhibits TGFβ1-mediated Smad2 Transcriptional Responses via Ca^{2+}/Calmodulin-Dependent Protein Kinase II

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ABSTRACT

Transforming growth factor β (TGFβ) signalling plays important roles in a variety of tissues and cell types. Impaired TGFβ signalling contributes to several pathologies, including cancer, fibrosis as well as neurodegenerative diseases. TGFβ receptor type I-mediated phosphorylation of Smad2, the formation of the Smad2–Smad4 complex and translocation to the nucleus are critical steps of the TGFβ signalling pathway. Here, we demonstrate that thapsigargin-mediated increase of intracellular Ca^{2+} concentrations inhibited TGFβ1-induced Smad2 transcriptional activity in the oligodendroglial cell line OLI-neu. We provide evidence that thapsigargin treatment dramatically reduced the nuclear translocation of Smad2 after TGFβ1 treatment but had no effect on its phosphorylation at Ser465/467. Moreover, using Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitors and a constitutively active CaMKII mutant, we provide evidence that the observed inhibition of TGFβ signalling in OLI-neu cells was strongly dependent on Ca^{2+}-mediated CaMKII activation. In summary, this study clearly shows that the TGFβ1-induced Smad2 nuclear translocation is negatively regulated by intracellular Ca^{2+} in OLI-neu cells and that increased intracellular Ca^{2+} concentrations block Smad2-mediated transcription of TGFβ target genes. These results underline the importance of intracellular Ca^{2+} for the regulation of TGFβ signalling. J. Cell. Biochem. 111: 1222–1230, 2010.

KEY WORDS: THAPSIGARGIN; CaMKII; TGFβ1; Smad2; CALCIUM

Transforming growth factor β (TGFβ) signalling plays important roles in a variety of different cell types and tissues during development and adulthood [Wu and Hill, 2009]. Moreover, impaired TGFβ signalling contributes to several pathologies, including cancer, fibrosis as well as neurodegenerative diseases such as Alzheimer's disease [Blobe et al., 2000; Wyss-Coray, 2006]. The different TGFβ isoforms (TGFβ1, 2, 3) bind to and stimulate the formation of heteromeric complexes of type I (TGFβRI) and type II (TGFβRII) serine/threonine kinase receptors [Yamashita et al., 1994]. The constitutively active TGFβRII phosphorylates the TGFβRI, which interacts with and phosphorylates the receptor-associated Smads (R-Smads), Smad2 and Smad3 [Wrana et al., 1994; Abdollah et al., 1997]. Activated R-Smads form a heterotrimeric complex with Smad4 and subsequently translocate to the nucleus to initiate the transcription of TGFβ target genes [Massagué and Wotton, 2000]. The duration and intensity of TGFβ signalling determines the transcriptional responses and cellular effects, respectively. Thus, a complex network of proteins is involved in fine-tuning TGFβ responses in a cell. For instance, TGFβ signalling is blocked by the inhibitory Smad7 [Nakao et al., 1997], which stably interacts with

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the TGFβRI and, thereby, abrogates the association and phosphorylation of R-Smads [Hayashi et al., 1997]. In contrast, the TGFβ-induced early genes Klf10 and Klf11 amplify TGFβ signalling responses by inhibiting the transcription of Smad7 [Johnsen et al., 2002; Gohla et al., 2008]. Recently, it has been shown that Smads are direct substrates of the Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) in Cos1 and HEK-293 cells. Activation of CaMKII by thapsigargin treatment resulted in inhibition of nuclear translocation of Smad2 and Smad4 [Wicks et al., 2000].

In this study, we used the oligodendroglial cell line OLI-neu to analyse the effect of changing intracellular calcium concentrations ($[Ca^{2+}]_i$) on TGFβ1 signalling. We demonstrate that thapsigargin-mediated increase of $[Ca^{2+}]_i$ concentrations inhibited Smad2 nuclear translocation and abrogated Smad2 transcriptional responses. Moreover, using CaMKII inhibitors and constitutively active CaMKII (caCaMKII), we show that the observed inhibition of TGFβ signalling was strongly dependent on CaMKII. Together, we clearly demonstrate that increased intracellular Ca^{2+} concentrations downregulated TGFβ-induced Smad2-mediated gene transcription in the oligodendroglial cell line OLI-neu by inhibiting Smad2 nuclear translocation. This study underlines the role of intracellular Ca^{2+} in modulating TGFβ signalling in cells of glial origin.

MATERIALS AND METHODS

MATERIALS

Bapta, caffeine, thapsigargin and the CaMKII inhibitor KN-93 were purchased from Sigma (Deisenhofen, Germany). KN-62 and veratridine were from Calbiochem (Darmstadt, Germany). Dantrolene was purchased from TOCRIS (Bristol, UK).

PLASMIDS AND REPORTER CONSTRUCTS

The pCMV-βGal reporter driving expression of β-galactosidase under the control of the human cytomegalovirus promoter (CMV) was purchased from Clontech (Heidelberg, Germany). pSBE-Luc was kindly provided by Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala University, Sweden). The construct pJPA7-CaMKIIca coding for a constitutively active His-282-Arg mutant was a kind gift from Tom Soderling (Oregon Health & Sciences University, Portland, OR). The generation of the construct has been described previously [Chang et al., 1998].

CELL CULTURE

OLI-neu, an immortalized oligodendrocyte precursor cell line [Jung et al., 1995], was cultured in DMEM (Gibco) supplemented with 10 μg/ml apo-Transferrin (Sigma), 5.2 ng/ml sodium selenite (Sigma), 10 μg/ml insulin (Sigma), 16 μg/ml putrescine (Sigma), 62 ng/ml progesterone (Sigma), 340 ng/ml Tri-iodo-L-Thyronine (Sigma), 1% horse serum (Gibco), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). All flasks and plates were coated with poly-D-lysine (Sigma) prior to plating OLI-neu cells. For TGFβ1 treatment, cells were switched to medium containing 0.2% horse serum 2 h prior to treatment.

Mink lung epithelial cells (MLEC), which are stably transfected with an expression construct containing the truncated plasminogen-activator inhibitor 1 (PAI-1) promoter fused to the luciferase reporter gene, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 250 μg/ml geneticin (Invitrogen) [Abe et al., 1994]. TGFβ1 treatment was performed under serum-free conditions.

Ca^{2+} IMAGING AND DATA EVALUATION

OLI-neu cells were transferred to a recording chamber, and 200 μl of bath solution/medium containing 10 μM Fluo-4/AM (Molecular Probes, Leiden, The Netherlands) was added. Fluo-4/AM was dissolved in DMSO (Sigma) and Pluronic F-127 (Molecular Probes). After incubation on a shaker at room temperature (RT) for 15 min, the cells were washed several times with bath solution/medium and placed on the microscope stage of an Axiovert 100M (Zeiss, Jena, Germany) to which a laser scanning unit (LSM 510, Zeiss) was attached. Before starting the Ca^{2+} imaging experiments, the cells were rinsed with bath solution/medium for at least 5 min. Fluorescence images (excitation at 488 nm; emission >505 nm) of the cells were acquired at 0.5 Hz and 786 ms exposure time per image with about 10 images taken as control images before the onset of thapsigargin wash-in. Thapsigargin was dissolved in bath solution/medium (1.5 mM stock) and used at a final concentration of 1.5 μM. The bath solution/medium was applied by gravity feed from a storage syringe through a funnel drug applicator to the recording chamber. Outflow was through a syringe needle. The fluorescence changes ΔF/F were calculated for individual cells as $\Delta F/F = (F_1 - F_2)/F_2$, where F_1 was the fluorescence averaged over the pixels of a cell, while F_2 was the average fluorescence of that cell prior to thapsigargin application, averaged over five images. Image analysis was performed using custom programs written in MATLAB (MathWorks, Natick).

WESTERN BLOT

Total proteins were isolated from OLI-neu cells after lysis in NP40 buffer supplemented with Complete Protease Inhibitor cocktail (Roche). The nuclear protein fractions were obtained using a subcellular protein extraction kit (Calbiochem) according to the manufacturer's instructions. Twenty-five micrograms of protein was loaded per lane of 9–12% SDS–PAGE and separated by electrophoresis. Proteins were transferred onto PVDF membranes (Immobilon, Milipore, Schwalbach, Germany). After transfer, the membranes were washed with TBST and blocked with 10% dry milk (Roth, Karlsruhe, Germany) in TTBS for 2 h at RT. Membranes were incubated with primary antibodies (anti-phospho-Smad2-Ser465/467, Cell Signaling 1:1,000; anti-Smad2/3, Cell Signaling 1:1,000), anti-phosphoCaMKII-Thr286 1:1,000, Cell Signaling; anti-CaMKII 1:1,000, Cell Signaling) at 4°C overnight with gently shaking. After incubation with horseradish peroxidase-conjugated secondary antibodies, labeled proteins were detected by using the Super-Signal™ detection Kit (Pierce). To confirm equal protein loadings, membranes were reprobed with a Gapdh antibody (1:10,000, Abcam) for cytosolic fractions and total protein lysates and Histone 2A antibody (1:1,000, Cell Signaling) for nuclear fractions. All blots were captured with Amersham Hyperfilm™ ECL (GE Healthcare).

LUCIFERASE REPORTER ASSAY

OLI-neu cells were placed with a density of 1×10^5 cells/well of a 48-well plate. Twenty-four hours after plating, the cells were transfected with 250 ng TGF-β responsive Smad-binding element luciferase construct (pSBE-Luc) and 100 ng of pCMV-βGal internal control vector using the Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After additional 24 h, cells were treated with 5 ng/ml TGFβ1 (Peprotech, Hamburg, Germany) and different Ca^{2+} regulators and CamKII inhibitors KN-93 (Sigma) and KN-62 (Calbiochem). Finally, cells were collected in lysis buffer (Tropix, Applied Biosystems) and analysed for luciferase activity in a luminometer (Lumat LB5076, Berthold, Bad Wildbad, Germany). Every assay was performed in duplicates and mean luciferase values were normalized to the mean β-galactosidase activity.

IMMUNOCYTOCHEMISTRY

Cells were fixed with 4% para formaldehyde for 30 min and were incubated with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 10% Goat serum to block the nonspecific protein binding. Cells were incubated with primary antibodies (anti-CamKII, Cell Signaling, 1:1,000; anti-phosphoCamKII-Thr286, Cell Signaling, 1:1,000; anti-Smad1/2/3, Santa Cruz, 1:100) overnight at 4°C, and incubated with the secondary antibody (FITC-conjuncted anti-mouse IgG 1:200, Sigma; Cy3-conjugated anti-mouse IgG 1:200, Abcam) at RT for 2 h. Nuclear DNA was stained by incubation with 100 ng/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) in PBS at RT for 3 min. Phase contrast and fluorescence images were captured using the Leica AF6000 imaging system (LEICA, Wetzlar, Germany).

STATISTICS

All data were expressed as means ± SEM. Statistical analysis was performed using the paired Student's *t*-test to compare the treated group with the control. For multiple comparisons between different treated groups, statistical differences were evaluated using one-way ANOVA and Bonferoni's post hoc test for multiple comparisons using the program GraphPad Prism5 (GraphPad Software Inc.). Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

RESULTS

THAPSIGARGIN INHIBITED TGFβ1-INDUCED Smad TRANSCRIPTIONAL RESPONSES

In order to analyse the effects of changing $[Ca^{2+}]_i$ concentrations on TGFβ1 signalling, OLI-neu cells were pretreated with different Ca^{2+} regulating substances. As shown in Figure 1A, treatment of OLI-neu cells with TGFβ1 (5 ng/ml) significantly increased Smad-mediated transcription as monitored using the pSBE-Luc reporter construct. Pretreatment with thapsigargin (1.5 μM), caffeine (3 mM) and veratridine (10 μM), which increase $[Ca^{2+}]_i$, dramatically decreased TGFβ1-induced Smad activity with the strongest effect being observed in the presence of thapsigargin. Dantrolene and bapta (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), both of which decreasing $[Ca^{2+}]_i$, had no significant effects on TGFβ1-induced Smad activity. Since we observed the highest reduction of Smad transcriptional responses after thapsigargin treatment, we focused on the thapsigargin-mediated effects on TGFβ1 signalling during this study. To confirm our results we used the TGFβ-responsive cell line MLEC [Abe et al., 1994] which is stably

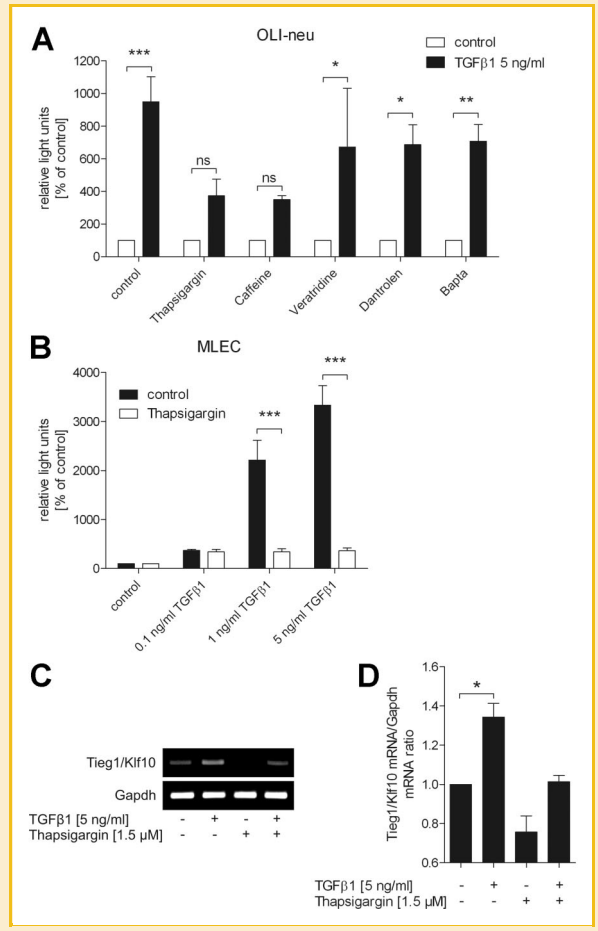

Fig. 1. TGFβ1-induced transcription is inhibited in the presence of elevated intracellular Ca^{2+} concentrations. A: OLI-neu cells were transfected with the reporter plasmid pSBE-Luc to monitor Smad-mediated transcriptional activation and pCMV-βGal as internal control plasmid. After 24 h the cells were treated with thapsigargin (1.5 μM), caffeine (3 mM), veratridine (10 μM), which increase intracellular Ca^{2+} concentrations and dantrolene (50 μM) and BAPTA (1 μM), which decrease intracellular Ca^{2+} concentrations. Subsequently, cells were treated with TGFβ1 (5 ng/ml) or left untreated. After 8 h of incubation the cells were lysed and protein lysates were used for the analysis of luciferase activity. Data are given as means from six independent experiments ± SEM. B: The TGFβ-responsive cell line MLEC coding for the firefly luciferase gene under the control of the plasminogen activator inhibitor (PAI) promoter was treated with increasing doses of TGFβ1 in the presence of thapsigargin (1.5 μM). After 24 h the cells were harvested and protein lysates were analysed for luciferase activity. Data are given as means from four independent experiments ± SEM. *P*-values derived from two-way ANOVA are $^{**}<0.01$ and $^{***}<0.001$. Transcriptional activation of the TGFβ-inducible gene Tieg1/Klf10 was analysed using RT-PCR. Cells were treated for 16 h as indicated. One representative RT-PCR result is shown C: Treatment of OLI-neu cells with TGFβ1 significantly increases Tieg1/Klf10 mRNA levels, whereas treatment with thapsigargin in combination with TGFβ1 failed to activate Tieg1/Klf10 transcription (D). Data are given as means from three independent experiments ± SEM. *P*-values derived from Student's *t*-test are $^{*}<0.05$.

transfected with the truncated PAI-1 promoter fused to the firefly luciferase gene. Figure 1B shows that thapsigargin significantly decreased the Smad-mediated transcription of the firefly luciferase gene after TGFβ1 treatment with different concentrations. We further used Tieg1/Klf10 as a well-described TGFβ-target gene in OLI-neu cells [Bender et al., 2004] to show that thapsigargin has a functional impact in TGFβ1-mediated transcription. As shown in Figure 1C,D, Tieg1/Klf10 was significantly upregulated after TGFβ1 treatment. Combination of TGFβ1 and thapsigargin failed to upregulate Tieg1/Klf10 in OLI-neu cells. Interestingly, treatment with thapsigargin alone reduced the levels of Tieg1/Klf10 mRNA compared to the control, suggesting endogenous TGFβ signalling under control conditions.

THAPSIGARGIN INCREASED $[Ca^{2+}]_i$ IN OLI-NEU CELLS

To detect thapsigargin-induced $[Ca^{2+}]_i$ increase in OLI-neu cells, we used the calcium-sensitive dye Fluo-4/AM. After incubation of the cells with Fluo-4/AM, thapsigargin (1.5 μM) was added and changes in fluorescence intensity were monitored as described in the Materials and Methods Section. Figure 2 shows representative images of OLI-neu cells before (A), at the peak (B) and after (C) addition of thapsigargin. After thapsigargin washout, the cells recovered and $[Ca^{2+}]_i$ reached basal levels. The response time curves of three representative cells are displayed in Figure 2D. Note that some cells were not capable to restore $[Ca^{2+}]_i$ and consequently die (e.g., Cell #1, Fig. 2D). This results clearly demonstrate that thapsigargin effectively increases $[Ca^{2+}]_i$ in OLI-neu cells.

NORMAL Smad2 PHOSPHORYLATION AT Ser465/467 AFTER THAPSIGARGIN TREATMENT

TGFβ1 downstream signalling is mediated by the receptor-associated Smads (R-Smads), Smad2 and Smad3, which are activated by phosphorylation and translocate to the nucleus to initiate transcription of target genes [Shi and Massagué, 2003]. Previous studies showed that Smad2 is the predominantly expressed R-Smad in OLI-neu cells [Gohla et al., 2008]. Therefore, we analysed the time course of Smad2 phosphorylation in OLI-neu cells. Figure 3A shows that Smad2 was rapidly phosphorylated after TGFβ1 treatment and levels of phospho-Smad2 were stable up to 8 h, the time point used for luciferase experiments. In a next step, we

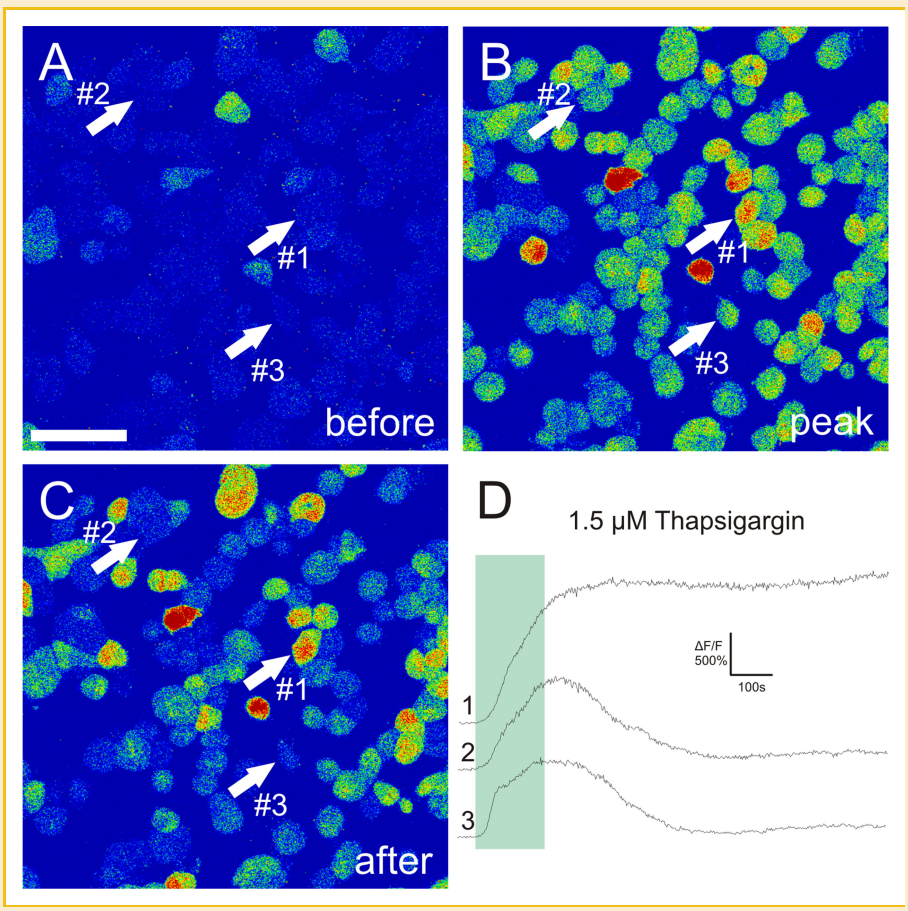

Fig. 2. Thapsigargin induced $[Ca^{2+}]_i$ increases in OLI-neu cells. Increases in intracellular Ca2+ concentrations were monitored using the Ca^{2+} sensitive indicator dye Fluo-4/AM. Sequence of three pseudo-coloured images of the same OLI-neu cells showing that thapsigargin (1.5 μM) increased the fluorescence in individual cells. A: Image taken before the application of thapsigargin. B: Image taken at the peak of the response. C: Image taken after washout of thapsigargin and return to baseline fluorescence. Arrows mark three representative cells whose time response curves are depicted (D). The grey-shaded box indicates the time of thapsigargin application. Note that some cells fail to return to baseline fluorescence (e.g., cell #1) indicating cell death. Scale bar indicates 50 μm.

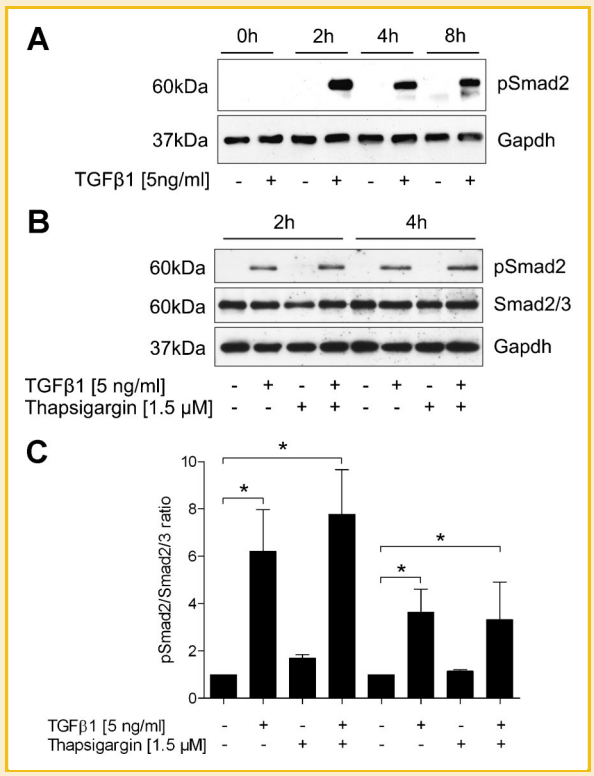

Fig. 3. Normal Smad2 phosphorylation at Ser465/467 after thapsigargin treatment in OLI-neu cells. A: OLI-neu cells were treated with TGFβ1 (5 ng/ml) for the indicated time points, harvested and total protein lysates were used for analysis of Smad2 phosphorylation. Smad2 phosphorylation was detectable from 2 to 8 h after TGFβ1 treatment. Gapdh was used as control for equal protein loading. B: Treatment of OLI-neu cells with thapsigargin has no effect on TGFβ1-mediated Smad2 phosphorylation at Ser465/467. Cells were treated with TGFβ1, thapsigargin and TGFβ1/thapsigargin for 2 and 4 h. No differences in phospho-Smad2 levels were detectable between TGFβ1-treated and TGFβ1/thapsigargin-treated cells. One representative Western blot result from three independent experiments is shown. C: Densitometric evaluation of phospho-Smad2 band intensities confirmed this result. Data are given as means from three independent experiments ± SEM. *P*-values derived from one-way ANOVA is $^{*}<0.05$.

analysed the phosphorylation of Smad2 at Ser465/467 after treatment with TGFβ1, thapsigargin and TGFβ1/thapsigargin. Figure 3B shows that treatment of OLI-neu cells with TGFβ1/thapsigargin did not reduce the levels of phosphorylated Smad2 in total protein lysates after 2 and 4 h. Total levels of Smad2/3 were not changed in differently treated groups. Gapdh was used to determine equal protein loading. These results clearly demonstrate that thapsigargin-mediated increase of $[Ca^{2+}]_i$ did not impair the phosphorylation of Smad2 at Ser465/467.

IMPAIRED NUCLEAR TRANSLOCATION OF Smad2 IN THE PRESENCE OF THAPSIGARGIN

As a consequent step we next analysed the nuclear translocation of Smad2 after treatment of OLI-neu cells with TGFβ1 and thapsigargin using immunocytochemistry and isolation of nuclear protein fractions. Figure 4B,C show a predominant cytosolic staining for Smad1/2/3 under control conditions. Treatment with

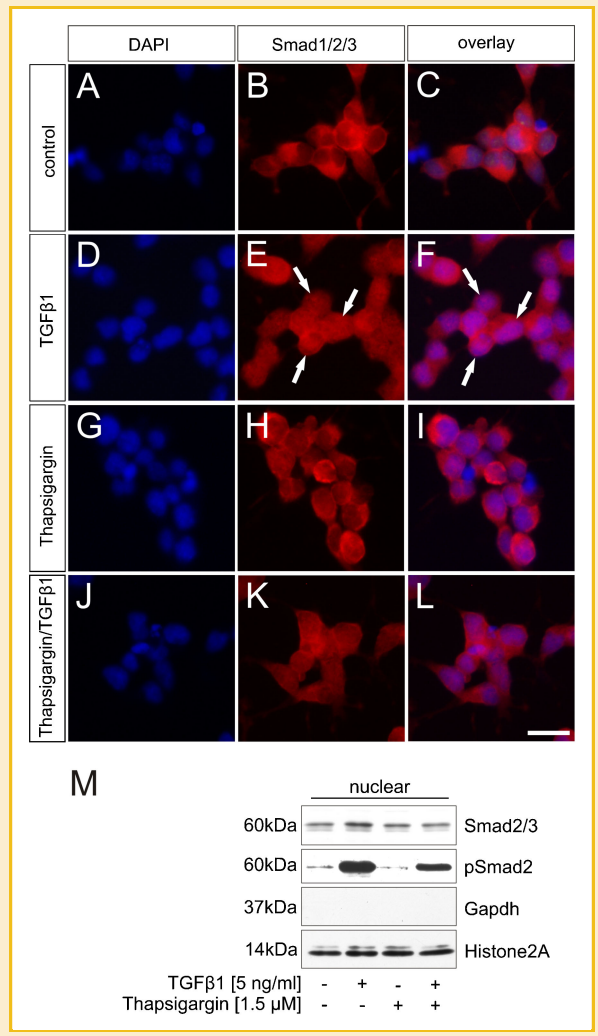

Fig. 4. Smad nuclear translocation is impaired in the presence of thapsigargin. OLI-neu cells were treated for 2 h, subsequently fixed and stained with a Smad1/2/3 antibody. Nuclei were counterstained with DAPI. Under control conditions and after thapsigargin treatment the Smad1/2/3 signal is predominantly located in the cytoplasm (B, C, H, I). TGFβ1 treatment resulted in a nuclear translocation (E, F). After treatment of OLI-neu cells with TGFβ1 in combination with thapsigargin, reduced nuclear translocation of Smad2 was detectable (K, L). Arrows mark nuclear Smad1/2/3 signal after TGFβ1 treatment. DAPI was used as a nuclear counterstain (A, D, G, J). Scale bar indicates 20 μm. M: Nuclear protein fractions were isolated after 8 h using a sub-cellular protein extraction kit (Calbiochem). Phosphorylated Smad2 was detectable in nuclear fractions of TGFβ1 treated OLI-neu cells. Thapsigargin dramatically reduced the levels of pSmad2 in nuclear fractions of TGFβ1 treated cells indicating impaired nuclear translocation of pSmad2. Histone2A was used as loading controls for nuclear fractions. Gapdh was used to exclude cytosolic proteins in the samples. The blots are representative of three independent experiments with similar results.

TGFβ1 for 2 h resulted in a strong nuclear staining pattern (Fig. 4E,F), whereas thapsigargin treatment showed a similar signal as the control cells (Fig. 4H,I). Figure 4K,L show that co-treatment of OLI-neu cells with TGFβ1 and thapsigargin reduced the nuclear accumulation of Smad2 after 2 h. To strengthen these results we extracted nuclear proteins from OLI-neu cells after 8 h treatment and used immunoblotting to analyse the levels of phosphorylated Smad2

proteins. After treatment with TGFβ1 the levels of nuclear Smad2/3 and the levels of phospho-Smad2 strongly increased (Fig. 4M). Co-treatment with TGFβ1 and thapsigargin also resulted in increased nuclear levels of phospho-Smad2. However, compared to TGFβ1 treatment the levels of phospho-Smad2 in the TGFβ1/thapsigargin group were dramatically lower. The total nuclear levels of Smad2/3 in the TGFβ1/thapsigargin group were similar to the control group. These results indicate that the nuclear translocation of Smad2 is impaired in the presence of thapsigargin.

INCREASED PHOSPHORYLATION OF CaMKII AFTER THAPSIGARGIN TREATMENT

The increase of $[Ca^{2+}]_i$ leads to activation of several Ca^{2+}-dependent proteins, among them CaMKII. Recently, it has been shown that CaMKII is able to interact with Smad2 in COS1 and HEK-293 cells in a Ca^{2+}-dependent manner [Wicks et al., 2000]. Figure 5A,B show that CaMKII was expressed in OLI-neu cells and that treatment with thapsigargin rapidly induced phosphorylation of CaMKII at Thr286. Immunocytochemistry after 2 h treatment with 1.5 μM thapsigargin revealed increased immunoreactivity for phosphorylated CaMKII (Fig. 5D,F) compared to untreated control cells (Fig. 5C,E).

THAPSIGARGIN-MEDIATED DECREASE IN Smad TRANSCRIPTIONAL RESPONSES WAS CaMKII-DEPENDENT

In order to analyse whether the observed reduction in Smad-mediated transcriptional responses after thapsigargin treatment were CaMKII dependent, we used the CaMKII inhibitors KN-62 and KN-93. Figure 6A shows that incubation of MLEC with the CaMKII inhibitor KN-93 was sufficient to abrogate the thapsigargin-induced reduction of Smad transcriptional responses after TGFβ1 treatment in a dose-dependent manner. To exclude KN-93-induced Smad activation, we included a single treatment with KN-93 (10 μM) which did not influence Smad-mediated transcription. Further, we treated OLI-neu cells with the CaMKII inhibitor KN-62 at

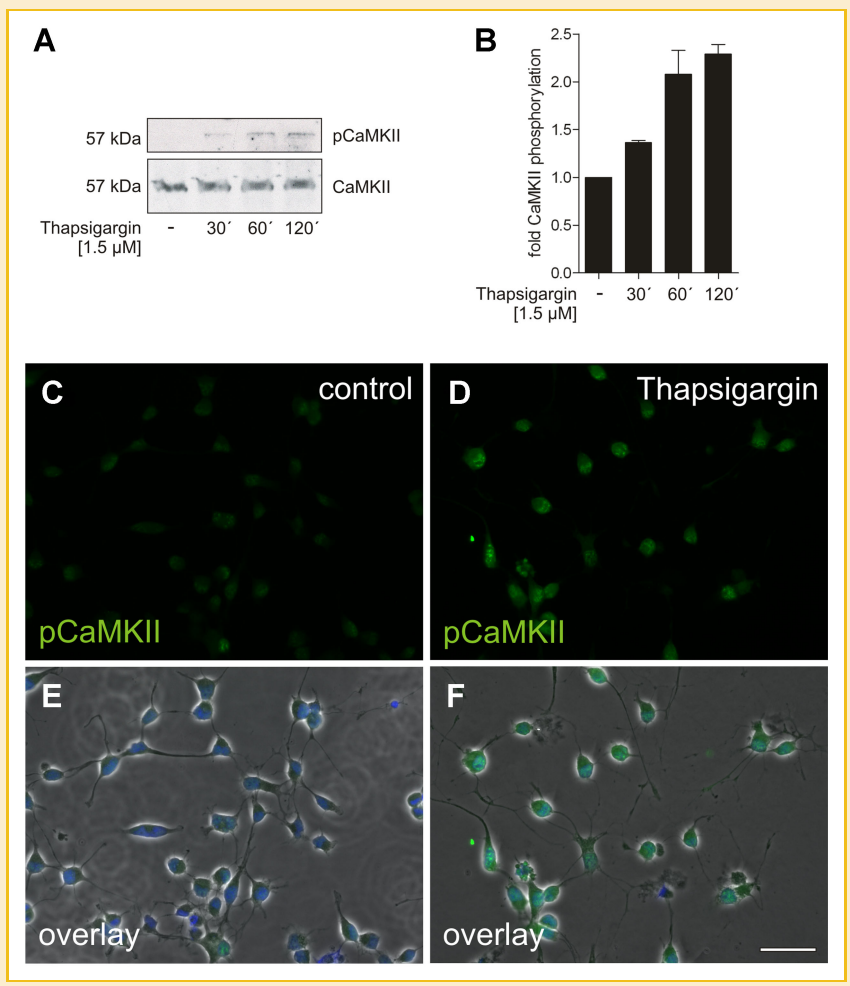

Fig. 5. Thapsigargin-induced CaMKII phosphorylation in OLI-neu cells. A: CaMKII is expressed in OLI-neu cells and is rapidly phosphorylated after thapsigargin treatment. One representative blot from three independent experiments is shown. B: Densitometric analysis revealed a twofold increase in pCaMKII levels after 60 min of treatment. Data are given as means from three different experiments ± SEM. Immunocytochemistry confirmed that treatment of OLI-neu cells with thapsigargin (1.5 μM) for 2 h induced phosphorylation of CaMKII at Thr286 (C–F). Again, strong fluorescence signals were observed in the nucleus and the cytosol. 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. Scale bars indicate 25 μm.

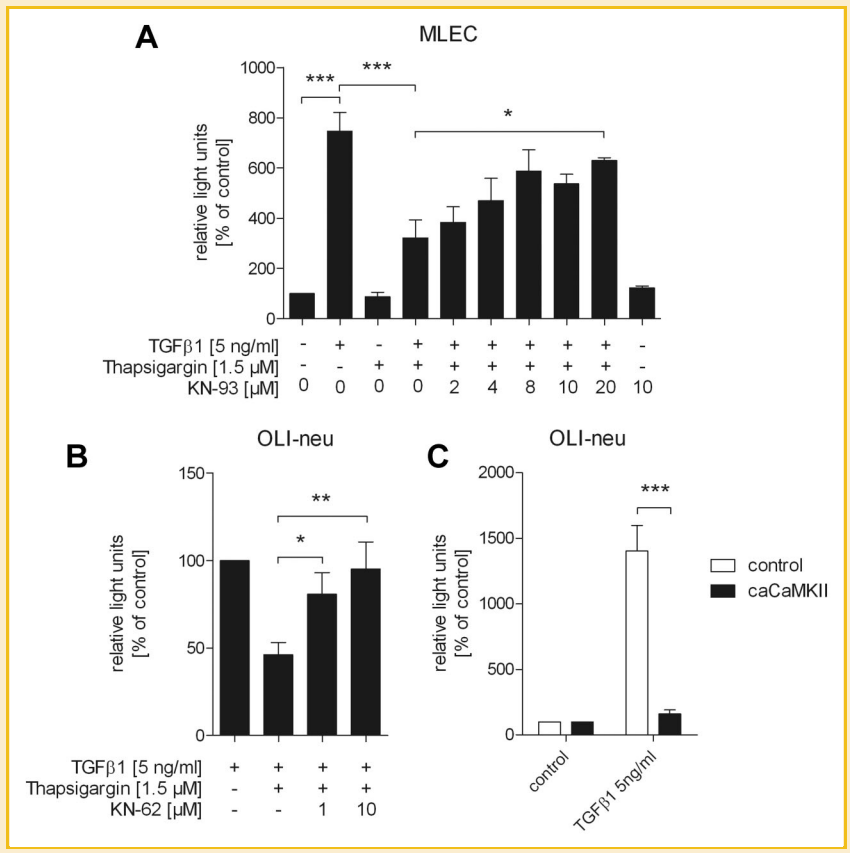

Fig. 6. Thapsigargin-mediated inhibition of Smad transcriptional activity was CaMKII dependent. A: The CaMKII inhibitor KN-93 restored Smad-mediated transcription in the presence of thapsigargin in MLEC in a dose-dependent manner. Cells were treated for 24 h and protein lysates were used to determine luciferase activity. Data are given as means of four independent experiments ± SEM. B: OLI-neu cells were treated with the CaMKII inhibitor KN-62 and Smad activity was monitored after TGFβ1 treatment. Blocking CaMKII activity abrogated thapsigargin-induced inhibition of Smad transcriptional responses in OLI-neu cells. Data are given as means of four independent experiments ± SEM. C: Overexpression of constitutively active CaMKII blocks Smad transcriptional activity after TGFβ1 treatment in OLI-neu cells. Cells were transfected with the plasmid pJPA7-CaMKIIca coding for a constitutively active His-282-Arg mutant. 24 h after transfection cells were treated with TGFβ1 (5 ng/ml) for 8 h and protein lysates were used for analysis of luciferase activity. Data are given as means of ten independent experiments ± SEM. *P*-values derived from one-way ANOVA are $^{*}<0.05$, $^{**}<0.01$ and $^{***}<0.001$.

concentrations of 1 and 10 μM and were able to significantly increase the TGFβ1-induced Smad response in the presence of thapsigargin (Fig. 6B). Pharmacological inhibitors are in most cases not very specific and might also interfere with functions of several other cellular proteins. Thus, we overexpressed a caCaMKII in OLI-neu cells using the plasmid pJPA7-CaMKIIca coding for a constitutively active His-282-Arg mutant to confirm our previous results. Figure 6C shows that overexpression of caCaMKII resulted in a significant loss of TGFβ1-induced Smad transcriptional responses. This result shows that overexpression of caCaMKII alone is able to abrogate TGFβ1-induced Smad-mediated transcriptional responses, thereby mimicked the effects of thapsigargin treatment in OLI-neu cells.

DISCUSSION

In this study, we demonstrated that increases in $[Ca^{2+}]_i$ have significant effects on TGFβ signalling in cells of glial origin. Using OLI-neu cells we showed that thapsigargin treatment is sufficient to impair TGFβ1-induced Smad2 nuclear translocation without affecting its phosphorylation at serine residues 465/467. Moreover, we identified CaMKII to be the effector protein of Ca^{2+}-mediated blockade of Smad2-driven transcriptional activation of TGFβ target genes.

In OLI-neu cells, thapsigargin and caffeine, both of which triggering increase of $[Ca^{2+}]_i$, inhibit TGFβ1-induced Smad-mediated transcription of the firefly luciferase reporter gene. However, treatment with the voltage-dependent Na^+ channel activator, veratridine, which also increases $[Ca^{2+}]_i$, only resulted in slight reduction of Smad-mediated transcription. These effects could be explained by the different mechanisms of the used chemicals. Thapsigargin functions as a specific inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase and raises cytosolic calcium concentrations by blocking the ability of the cell to pump calcium into the sarcoplasmic or endoplasmic reticula [Inesi et al., 1998]. Caffeine is an adenosine receptor antagonist and an inhibitor of adenosine 3′,5′-cyclic monophosphate (cAMP) phosphodiesterase [Schwabe et al., 1985] which increases the levels of cAMP. Further, it has been reported that caffeine releases calcium from intracellular stores [Islam et al., 1998]. Veratridine acts by opening voltage-dependent Na^+ channels subsequently increasing $[Ca^{2+}]_i$ via

voltage-activated calcium channels [Dobrev et al., 1998]. Interestingly, it has been reported that functional Na^+ channels are not expressed in the majority of immature oligodendrocytes from the corpus callosum [Mallon et al., 2002]. OLI-neu cells are immortalized from murine oligodendroglial precursor cells [Jung et al., 1995], and therefore might have no or very limited levels of reactive Na^+ channels that response to veratridine. Recent reports demonstrated that calcium-dependent proteins are involved in the regulation of TGFβ signalling by interacting with Smad proteins [Zimmerman et al., 1998; Scherer and Graff, 2000; Wicks et al., 2000]. Using COS-1 cells Wicks et al. [2000] demonstrated that Smad2 is a substrate of activated CaMKII. In COS-1 cells, CaMKII blocked the nuclear translocation of Smad2 by phosphorylation at Ser240 and prevention of Smad2/Smad3 interactions. However, the phosphorylation of Smad2 at the TGFβ-specific serine residues 465/467 was not analysed in this study. Here, we demonstrate that in OLI-neu cells the phosphorylation of Smad2 at Ser465/467 was not impaired after thapsigargin treatment, but the TGFβ1-mediated nuclear translocation of Smad2 was dramatically reduced in the presence of thapsigargin. This indicates that increased Ca^{2+} levels in OLI-neu cells have no effect on TGFβ-induced phosphorylation of Smad2 at Ser465/467, whereas the shuttling of phosphorylated Smad2 to the nucleus is inhibited.

One must be aware that CaMKII-mediated effects are contributed by a family of isoforms derived from four closely related yet distinct genes (α, β, γ and δ) [Hudmon and Schulman, 2002]. Further, different isoforms have different intracellular localizations which change depending on the activity of the kinase [Schulman, 2004]. The antibody used in this study is able to detect all different CaMKII isoforms, thus, we cannot determine which isoform is mediating the effects in OLI-neu cells.

Calcium signalling plays crucial roles in the central nervous system including synaptic transmission and plasticity [Catterall and Few, 2008], axon guidance [Dent et al., 2003], neuronal migration [Komuro and Kumada, 2005] and excitotoxicity-mediated neurodegeneration [Arundine and Tymianski, 2003]. However, many aspects of calcium signalling take place in neurons. Thus, it would be of major interest if increased $[Ca^{2+}]_i$ also interferes with TGFβ-mediated Smad2 nuclear translocation and subsequent inhibition of TGFβ target gene transcription in neurons under physiological or pathophysiological conditions.

In summary, this study clearly shows that the TGFβ1-induced Smad2 nuclear translocation is negatively regulated by intracellular Ca^{2+} in OLI-neu cells and that increased $[Ca^{2+}]_i$ blocks Smad2-mediated transcription of TGFβ target genes. These results might be of particular interest to understand the regulation of TGFβ signalling in the central nervous system and underline the importance of intracellular Ca^{2+} for the regulation of TGFβ signalling in cells of neural origin.

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