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Calcium Input Potentiates the Transforming Growth Factor (TGF)- β 1-dependent Signaling to Promote the Export of Inorganic Pyrophosphate by Articular Chondrocyte*

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Transforming growth factor (TGF)- β 1 stimulates extracellular PP_i (ePP_i) generation and promotes chondrocalcinosis, which also occurs secondary to hyperparathyroidism-induced hypercalcemia. We previously demonstrated that ANK was up-regulated by TGF- β 1 activation of ERK1/2 and Ca²⁺-dependent protein kinase C (PKC α). Thus, we investigated mechanisms by which calcium could affect ePP_i metabolism, especially its main regulating proteins ANK and PC-1 (plasma cell membrane glycoprotein-1). We stimulated articular chondrocytes with TGF- β 1 under extracellular (eCa²⁺) or cytosolic Ca²⁺ (cCa²⁺) modulations. We studied ANK, PC-1 expression (quantitative RT-PCR, Western blotting), ePP_i levels (radiometric assay), and cCa²⁺ input (fluorescent probe). Voltage-operated Ca²⁺-channels (VOC) and signaling pathways involved were investigated with selective inhibitors. Finally, *Ank* promoter activity was evaluated (gene reporter). TGF- β 1 elevated cCa²⁺ and ePP_i levels (by up-regulating *Ank* and PC-1 mRNA/proteins) in an eCa²⁺ dose-dependent manner. TGF- β 1 effects were suppressed by cCa²⁺ chelation or L- and T-VOC blockade while being mostly reproduced by ionomycin. In the same experimental conditions, the activation of Ras, the phosphorylation of ERK1/2 and PKC α , and the stimulation of *Ank* promoter activity were affected similarly. Activation of SP1 (specific protein 1) and ELK-1 (Ets-like protein-1) transcription factors supported the regulatory role of Ca²⁺. SP1 or ELK-1 overexpression or blockade experiments demonstrated a major contribution of ELK-1, which acted synergistically with SP1 to activate *Ank* promoter in response to TGF- β 1. TGF- β 1 promotes input of eCa²⁺ through opening of L- and T-VOCs, to potentiate ERK1/2 and PKC α signaling cascades, resulting in an enhanced activation of *Ank* promoter and ePP_i production in chondrocyte.

The balance between extracellular inorganic pyrophosphate (ePP_i)³ and extracellular inorganic phosphate (eP_i) is critical for

homeostasis of articular cartilage. Indeed, an increase in ePP_i supports calcium pyrophosphate dihydrate crystal (CPPD) depositions responsible for articular chondrocalcinosis (ACC) (1). Production of ePP_i by articular chondrocytes is dependent on PC-1 (plasma cell membrane glycoprotein-1; also known as ENPP1), an ectoenzyme that produces ePP_i from nucleotide triphosphates (2), and on the transporter ANK, which exports PP_i across the cell membrane (3). Although ePP_i level can be controlled by tissue-nonspecific alkaline phosphatase, which hydrolyzes ePP_i into eP_i, no expression of this enzyme was detected in healthy articular cartilage compared with osteoarthritic cartilage (4). Therefore, CPPD formation only depends on the expression level of PC-1 and ANK in mature articular cartilage.

Transforming growth factor (TGF)- β 1 is a well known inducer of ePP_i production by articular chondrocytes (1). We demonstrated previously that ANK was the major contributor of this TGF- β 1-induced ePP_i production; meanwhile, PC-1 played a minor role (5). Furthermore, these effects of TGF- β 1 relied on an ERK1/2-calcium-dependent protein kinase C (PKC) signaling mechanism, which was independent from Smad activation. TGF- β 1 levels were also described to be gradually increased in a rat model of hyperparathyroidism consisting in a daily injection of parathyroid hormone (PTH) for 3 months (6). PTH levels are classically elevated in patients with hyperparathyroidism, and an increase in circulating PTH was also reported in patients with idiopathic CPPD depositions (7). Altogether, these observations support a potential role for TGF- β 1 in the formation of CPPD crystal formation in the context of primary hyperparathyroidism.

ACC linked to hyperparathyroidism reaches a prevalence of 21% (8). Besides development of ACC, patients with hyperparathyroidism have a chronic elevation of free calcium levels in their extracellular fluids. Although no direct link was reported between hypercalcemia and ACC (9), a very recent study demonstrated that patients with familial hypocalciuric hypercalcemia developed ACC (10). However, the *modus operandi* by which calcium may influence CPPD formation remains unclear and is therefore worthy of study.

The higher calcium levels found in patients with hyperparathyroidism could influence chondrocyte metabolism by

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³ The abbreviations used are: ePP_i, extracellular inorganic pyrophosphate; eP_i, extracellular inorganic phosphate; CaSR, calcium-sensing receptor; ACC, artic-

ular chondrocalcinosis; cCa²⁺, cytosolic Ca²⁺; eCa²⁺, extracellular Ca²⁺; CPPD, calcium pyrophosphate dihydrate; PTH, parathyroid hormone; RBD, Ras binding domain; RLU, relative luminescence units; RFU, relative fluorescence unit; VOC, voltage-operated channel; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester.

Calcium Entrance Stimulates TGF- β 1-induced PP_i Export

stimulating calcium input through voltage-operated channels (VOCs). Indeed, several VOCs were described as being expressed in chondrocytes, such as N-VOCs (11) and L- and T-VOCs (12). Another possible mechanism could be related to the activation of the calcium-sensing receptor (CaSR), a G protein-coupled receptor (13). Furthermore, calcium was described as stimulating the activity of the transcription factor SP1 (specific protein 1), which increased the basal transcription of β 1,3-glucuronosyltransferase I, a key enzyme responsible for the completion of cartilage proteoglycans (14). Interestingly, SP1 activity was also shown to be implicated both in the TGF- β 1-induced expression of tissue inhibitor of metalloproteinase-3 (15) and β 1,3-glucuronosyltransferase I (16) in chondrocytes. These findings suggest a possible regulatory interplay between calcium and TGF- β 1 through the SP1 transcription factor, which could result possibly in CPPD crystal deposition in articular cartilage.

The present work investigated whether the variation in either extracellular or cytosolic calcium levels could influence the stimulating effects of TGF- β 1 on ePP_i production by articular chondrocytes. Particular care was taken in deciphering the contribution of VOCs and the signaling events regulating the expression of the main ePP_i-regulating genes *PC-1* and especially *Ank* with a study of its promoter region.

EXPERIMENTAL PROCEDURES

Chondrocyte Isolation and Culture—Articular cartilage was obtained from 6-week-old healthy male Wistar rats (130–150 g) killed under dissociative anesthesia (ketamine (Merial, Lyon, France) and acepromazine (Sanofi-Aventis, Libourne, France)) in accordance with our institutional local ethics committee and the national animal care guidelines. Cartilage pieces were dissected aseptically from femoral head caps, and chondrocytes were obtained by sequential digestion with Pronase and collagenase B (Roche Applied Science), as described previously (17). Cells were washed twice in PBS and cultured to confluence in 75-cm² flasks at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were maintained in calcium-free DMEM (Invitrogen), supplemented with 1.25 mM of CaCl₂ (Sigma, France), L-glutamine (2 mM), gentamicin (50 μ g/ml), amphotericin B (0.5 μ g/ml), and 10% heat-inactivated FCS (Invitrogen). Unless specified, we used first passage chondrocytes plated at 4 \times 10⁵ cells/well in 6-well plates throughout the study. Except for the dose-ranging studies on eCa²⁺ level, experiments were performed in calcium-free DMEM, supplemented with 1.25 mM Ca²⁺ to mimic the physiological free circulating Ca²⁺ levels.

Chemicals—All chemical reagents were from Sigma, unless otherwise specified. All of the compounds used in this study were dissolved in DMSO (final concentration 0.1%), used as vehicle in control experiments. At the concentrations used, no cytotoxicity of either compound was detected in a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay after 72 h of exposure (data not shown). Final concentrations were chosen after preliminary experiments demonstrating the selectivity of signal transduction inhibitors (immunoblotting of the phosphorylation level of the concerned signaling pathway; data not shown). TGF- β 1 (PeproTech, Neuilly-Sur-Seine, France) was used at a final concentration of 10 ng/ml throughout the study.

RNA Extraction and Reverse Transcription—Total RNA was isolated using the RNeasyplus[®] minikit (Qiagen), which allows total removal of genomic DNA with an on-column DNA elimination step. Five hundred ng of total RNA were reverse-transcribed for 90 min at 37 °C in a 20- μ l reaction mixture containing 2.5 mM dNTP, 5 μ M random hexamer primers, 1.5 mM MgCl₂, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). cDNA production was performed in a Mastercycler gradient thermocycler (Eppendorf, France).

Real-time Quantitative PCR—To check our chondrocyte phenotype and measure mRNA levels of target genes, real-time quantitative PCR was performed using the Lightcycler[®] (Roche Applied Science) technology. PCR was done with SYBR Green master mix system (Qiagen). The gene-specific primer pairs are provided in Table 1. A melting curve was performed to determine the melting temperature of the specific PCR products, and after amplification, the product size was checked on a 1% agarose gel stained with ethidium bromide (0.5 μ g/ml). Each run included positive and negative reaction controls. The mRNA level of the gene of interest and of S29, chosen as housekeeping gene, was determined in parallel for each sample. The S29 gene, which codes for a ribosomal protein, was shown previously to be invariable in TGF- β 1-challenged chondrocytes (5). Quantification was determined using a standard curve made from a purified PCR product for each gene tested, with concentrations ranging from 10⁻³ to 10⁻⁹ ng/ μ l. Results were expressed as the ratio of the mRNA level of each gene of interest over the S29 gene.

Western Blot Analysis—Chondrocytes were harvested and lysed in 1 \times Laemmli buffer. Samples were run on SDS-PAGE (10%) and transferred onto a polyvinylidene fluoride membrane (Immobilon[™], Waters, France). After 2 h in blocking buffer (TBS, 0.1% Tween (TBST) supplemented with 5% nonfat dry milk), membranes were washed three times with TBST and incubated overnight at 4 °C with primary antibodies. The antibodies against ANK and PC-1 (Eurogentec, Angers, France) were used at a 1:500 dilution as we described previously (5, 18). Antibodies directed against native and phosphorylated ERK1/2 and PKC α (Cell Signaling Technology) as well as antibodies against native (Santa Cruz Biotechnology) and phosphorylated ELK-1 (Ets-like protein-1) (Millipore) were used at 1:500. Antibodies against total (Millipore) and phosphorylated SP1 (Abcam) were used at 1:1000, and the anti- β -actin (Sigma) was used at 1:4000. After three washings with TBST, each blot was incubated for 1 h at room temperature with anti-rabbit IgG conjugated with HRP (Cell Signaling Technology) at 1:2000 dilution in blocking buffer. After four washings in TBST, protein bands were detected by chemiluminescence with the Phototope Detection[™] system according to manufacturer's recommendations (Cell Signaling Technology). Band intensities were quantified by densitometry with the Gnome[™] computerized image processing system (Syngene, Cambridge, UK).

ePP_i Assays—ePP_i levels were measured in culture supernatants, using the differential adsorption between UDP-[6-³H]-glucose (PerkinElmer Life Sciences) and its reaction product 6-phospho-[6-³H]gluconate on activated charcoal, as described previously (19). The standards, ranging from 10 to 400 pmol of PP_i, were included in each assay. After adsorption of the reaction mixture on charcoal and centrifugation at 16,000 \times g for 10

min, 100 μ l of the supernatant were counted for radioactivity in 5 ml of Bio-Safe II (Research Products International). Results were expressed as pmol of ePP_i/ μ g of total cell proteins (quantified by bicinchoninic acid assay).

Cytosolic Calcium Analyses—Calcium levels were measured in a fluorimeter (FLX-Xenius, SAFAS, Monaco) using the Fluo-4 NWTM calcium assay kit (Invitrogen) according to manufacturer's protocol (20). This assay is based on a dye solution containing the Fluo-4AM fluorescent probe and probenecid. This drug limits the extrusion of Fluo-4AM and reduces the base-line signal. Chondrocytes were plated in 96-well plates at 3×10^4 cells/well and then cultured for 24 h until 90% confluence ($\sim 4 \times 10^4$ cells). Briefly, cells were washed with PBS and then incubated with 100 μ l/well of dye solution for 1 h at 37 °C, in the presence or not of the different selective modulators of cytosolic Ca²⁺ level and signaling inhibitors. Afterward, cells were placed back in culture medium containing TGF- β 1 for 1 min, which corresponded to the maximal cytosolic Ca²⁺ level measured (time course over 30 min; data not shown). Fluorescence was read after PBS washing. Results were expressed as mean relative fluorescence units (RFU)/10⁴ per 4×10^4 cells.

Ras Activation Assay—The amount of Ras-GTP (active form) was evaluated using a Ras activation assay kit (Millipore) according to the manufacturer's protocol. This ELISA is based on a selective trapping of Ras-GTP using the Ras binding domain (RBD) of Raf-1 (a kinase downstream Ras) that fails to bind Ras-GDP (inactive form). The coupling of RBD to GST (RBD-GST) allows immobilization of the complex on microplate wells precoated with glutathione. The trapped Ras-GTP is further detected with a monoclonal antibody against Ras. Briefly, chondrocytes were lysed in the Mg²⁺ lysis/wash buffer from the kit, supplemented with one tablet of complete mini protease inhibitor mixture (Roche Applied Science), before protein quantification by a Bradford assay. Cellular lysates containing 50 μ g (in 100 μ l) of proteins were incubated for 1 h in the RBD-GST-coated wells. After washing three times with TBST, wells were incubated for 1 h with the primary anti-Ras mouse antibody. After TBST washings, wells were incubated for 1 h with an HRP-conjugated goat anti-mouse secondary antibody. After four additional TBST washings, wells were finally rinsed three times with TBS, before incubation for 10 min with a chemiluminescent substrate. All incubation steps were performed at room temperature under gentle rocking. Luminescence was read with a CentroLB960 microplate luminometer (Berthold Technologies). Results were expressed as relative luminescence units (RLU)/10⁶.

siRNA Assays—RNA silencing was processed with either a control siRNA-A or a mix of three siRNA sequences against Sp1 from Santa Cruz Biotechnology. The final concentration used was 10 nM, and transfections were performed using INTERFERinTM (Polyplus Transfection). Briefly, siRNA were diluted in serum-free medium, and INTERFERinTM was then added to this mixture (12 μ l of INTERFERinTM/200- μ l mixture) for a short incubation at room temperature. During that time, cells were washed with PBS and then placed in serum-free medium. The siRNA-INTERFERinTM mix was then added to the culture for 24 h (200 μ l of mix/2 ml of medium).

Ank Promoter Cloning in pGL3—Human *Ank* promoter sequence (GenBankTM accession number AC016575) was cloned from a mix of genomic DNA from different organs (brain, heart, liver, and kidney) (Clinisciences) in the pGL3 luciferase reporter vector (Promega), using the KpnI and XhoI sites. Primers used for the amplification are described in Table 1. We cloned a -720 bp fragment containing two putative Sp1-responsive elements (-125/15 bp and -532/22 bp) and a -2715 bp fragment containing the Sp1-responsive elements and one putative Elk-1-responsive element (-1860/44 bp). These responsive elements were identified using the TFSearch 1.3 algorithm (21).

Plasmid Electroporation—Cells were electroporated as described previously (5), using the Chondrocyte Nucleofector[®] kit (Lonza) according to the manufacturer's protocol, with the Nucleofector[®] (Lonza) program U-28. In one set of experiments, we transfected either the -720 bp or the -2715 bp *Ank* promoter fragment (1 μ g/3 $\times 10^5$ cells) in chondrocytes exposed or not to TGF- β 1. In another set of experiments, cells were co-transfected with either one of these constructs, along with 1 μ g/3 $\times 10^5$ cells of either pCMV-Sp1 (overexpressing wild-type human SP1 protein) or pCMV-Elk-1 (overexpressing wild-type human ELK-1 protein) or both vectors. These constructs were a generous gift from Dr. M. Ouzzine (UMR7561 CNRS-Nancy University (14)). In each experiment, chondrocytes were transfected with pTK-RL expressing *Renilla* luciferase (500 ng/3.10⁵ cells) to normalize the results. Plasmid pmaxGFPTM (Lonza), encoding a GFP, was used to assess transfection efficiency.

Reporter Gene Assay—Luciferase activities were measured using the Dual-LuciferaseTM reporter assay kit (Promega). Briefly, chondrocytes electroporated with reporter gene constructs were stimulated or not for 48 h with TGF- β 1. After two washings in PBS, cells were lysed in 1 \times passive lysis buffer for 15 min at room temperature. Cell lysate was placed in LAR II (firefly luciferase substrate), and luminescence was read with a Sirius luminometer (Berthold Technologies). Afterward, Stop & Glo reagent (*Renilla* luciferase substrate) was added to the sample, and luminescence was acquired in the same way. Results were expressed as the mean ratio of firefly/*Renilla* luciferase activity, in -fold induction over control.

Statistical Analysis—Results are expressed as the mean \pm S.D. of at least three independent assays. Comparisons were made by analysis of variance, followed by Fisher's *t post hoc* test, using the StatviewTM 5.0 software (SAS Institute Inc.). A value of $p < 0.05$ was considered significant.

RESULTS

Effects of eCa²⁺ Levels on TGF- β 1-stimulated Metabolism of PP_i and Calcium Mobilization in Chondrocytes—First, we confirmed that the articular phenotype of chondrocyte was maintained throughout the study. Indeed, a strong expression of type II collagen was observed, whereas collagens type I and X, Runx2, and tissue-nonspecific alkaline phosphatase remained undetectable (data not shown). This confirmed our previous report (5) and underlined that no dedifferentiation or switch toward hypertrophy occurred in our culture conditions.

To determine the influence of eCa²⁺ level on the TGF- β 1-stimulated metabolism of ePP_i, chondrocytes were incubated in

TABLE 1
Gene-specific primer pairs used

Gene	Sense	Antisense	Amplicon length bp	GenBank™ accession number
<i>Ank</i>	5'-CAA GAG AGA CAG GGC CAA AG-3'	5'-AAG GCA GCG AGA TAC AGG AA-3'	173	NM_053714
<i>Ank</i> promoter -720 bp	5'-CGG GGT ACC CCT GGA AAC CCT GGG GGA CA-3'	5'-CCG CTC GAG GGG GCC CGG AAA TAA ATA AC-3'	720	AC016575
<i>Ank</i> promoter -2715 bp	5'-CGG GGT ACC AGC GGA CCA AAC ATG AAG AG-3'	5'-CCG CTC GAG GGG GCC CGG AAA TAA ATA AC-3'	2715	AC016575
<i>PC-1</i>	5'-TAT GCC CAA GAA AGG AAT GG-3'	5'-GCA GCT GGT AAG CAC AAT GA-3'	165	NM_053535
<i>Runt-2</i>	5'-TAT TCC CGT AGA TCC GAG CA-3'	5'-GCT CAC GTC GCT CAT TT G-3'	82	NM_053470
<i>S29</i>	5'-AAG ATG GGT CAC CAG CAG CTC TAC TG-3'	5'-AGA CGC GGC AAG AGC GAG AA-3'	67	NM_012876
<i>TNAP</i>	5'-GAA CGT CAA TTA ACG GCT GA-3'	5'-CAG ATG GGT GGG AAG AGG TT-3'	50	NM_013059
Type IA2 collagen	5'-TTG ACC CTA ACC AAG GAT GC-3'	5'-CAC CCC TTC TGC GTT GTA TT-3'	197	NM_053356
Type II collagen	5'-TCC CTC TGG TTC TGA TGG TC-3'	5'-CTC TGT CTC CAG ATG CAC CA-3'	161	NM_012929
Type X collagen	5'-ATA TCC TGG GGA TCC AGG TC-3'	5'-TGG GTC ACC CTT AGA TCC AG-3'	241	A131848

calcium-free DMEM containing 1% FCS, which was supplemented or not with 1.25, 2.5, or 5 mM CaCl₂ for 24 h. Then cells were exposed to TGF-β1 for either 24 h (mRNA expression), 48 h (protein level), or 72 h (ePP_i production). Of note, all results were analyzed in comparison with the “physiological” concentration of 1.25 mM eCa²⁺. Basal ePP_i level was reduced from 65 to 42 pmol/μg protein (-35%) in the calcium-free medium while being increased to 89 pmol/μg protein (1.4-fold) at 5 mM eCa²⁺ (Fig. 1A). TGF-β1 increased the ePP_i production from 65 to 274 pmol/μg protein (4.2-fold) at 1.25 mM eCa²⁺. This effect was limited to 94 pmol/μg protein (2.3-fold) in the absence of eCa²⁺, while increasing gradually with eCa²⁺ to peak at 389 pmol/μg protein (4.4-fold) at 5 mM eCa²⁺. Compared with the 1.25 mM eCa²⁺ condition, basal Ank mRNA level (evaluated by real-time quantitative PCR) was reduced from 3.8 to 0.8 (-79%) in the absence of eCa²⁺, whereas PC-1 expression was decreased from 2.5 to 1.2 (-52%) (Fig. 1B). Conversely, supplementation of culture medium with 5 mM eCa²⁺ up-regulated the expression of Ank from 3.8 to 5.4 (1.4-fold) and of PC-1 from 2.5 to 5 (2-fold) (Fig. 1B). TGF-β1 increased Ank and PC-1 mRNA expression to 19.2 (5-fold) and 9.6 (3.8-fold), respectively, at 1.25 mM eCa²⁺. In the absence of eCa²⁺, TGF-β1 elevated the expression of Ank only from 0.8 to 2.1 (2.6-fold) and of PC-1 from 1.2 to 2.5 (2.1-fold). However, the effect of TGF-β1 reached 32 (8.4-fold) for Ank and 22.5 (9-fold) for PC-1 at 5 mM eCa²⁺. Interestingly, the articular phenotype of chondrocytes was affected neither by changes in eCa²⁺ levels nor by TGF-β1 challenge because type I and type X collagen remained undetectable, whereas a high mRNA level of type II collagen was maintained (data not shown). Western blot analysis (Fig. 1C) confirmed the influence of eCa²⁺ on the inducing effect of TGF-β1 on Ank and PC-1 mRNA levels because the progressive increase in eCa²⁺ boosted the TGF-β1 up-regulation of ANK and PC-1 protein levels. Finally, we checked whether TGF-β1 and/or eCa²⁺ could initiate calcium mobilization in chondrocytes. For that goal, cytosolic calcium (cCa²⁺) level was evaluated in cells exposed for 24 h to the different eCa²⁺ concentrations before being challenged or not with TGF-β1. *Per se*, eCa²⁺ variation was unable to modify cCa²⁺ level after 1 min of exposure (Fig. 1D). However, after 5 min of exposure, the cCa²⁺ level was increased from 0.5 to 0.8 RFU (1.6-fold) and from 0.5 to 1 RFU (2-fold) in cells incubated with either 2.5 or 5 mM eCa²⁺, respectively (Fig. 1D). After 1 min, TGF-β1 was ineffective in the absence of eCa²⁺, whereas it increased the cCa²⁺ level from 0.5 to 2.7 RFU (5.4-fold) at 1.25 mM eCa²⁺ (Fig. 1D). At higher eCa²⁺ concentrations, this increase in cCa²⁺ level was amplified to 3.6 RFU (7.2-fold, at 2.5 mM) and 5 RFU (10-fold, at 5 mM). Interestingly, after 5 min of exposure to the same culture conditions, TGF-β1 remained ineffective in the absence of eCa²⁺ while producing lower effects than at 1 min for the other eCa²⁺ concentrations (Fig. 1D). This was consistent with the time course analysis showing that TGF-β1 influence was maximal after 1 min (data not shown). Taken together, these data demonstrate that the stimulating effect of TGF-β1 on Ank and PC-1 was enhanced by eCa²⁺ in a dose-related fashion, which suggests a key regulatory role for calcium on the modulation of ePP_i metabolism by TGF-β1 in chondrocyte.

Effect of Selective Ca²⁺-VOC Blockers and CaSR Agonist on TGF- β 1-activated Metabolism of PP_i and Calcium Mobilization in Chondrocytes—We next investigated the mechanisms involved in the cCa²⁺ mobilization and stimulation of ePP_i metabolism by TGF- β 1. With that goal, we examined the consequence of a 1-h preincubation of chondrocytes with either a selective CaSR agonist, GdCl₃ (100 μ M), or the following selective VOCs blockers (Merck, Darmstadt, Germany): ω -agatoxin (1 μ M, for P/Q-type), ω -conotoxin (1 μ M, for N-type), lercanidipin (10 μ M, for L-type), and NiCl₂ (50 μ M, for a selective blockade of T-VOCs (22)). Their effects were then assessed after either 24 h (mRNA expression), 48 h (protein level), or 72 h (ePP_i production) of stimulation with TGF- β 1 in the presence of 1.25 mM eCa²⁺.

The basal ePP_i production was unaffected by any of the compounds listed above (Fig. 2A). Moreover, neither activation of CaSR nor blockade of N-VOCs affected the TGF- β 1-stimulated ePP_i production, whereas ω -agatoxin slightly decreased it from 274 to 235 pmol/ μ g protein (–14%) (Fig. 2A). In contrast, lercanidipin and NiCl₂ markedly diminished the TGF- β 1 effect to 162 pmol/ μ g protein (–41%) and 93 pmol/ μ g protein (–66%) respectively (Fig. 2A). In accordance with these findings, none of these compounds modified the basal mRNA expression of Ank and PC-1 (Fig. 2B). Once again, neither GdCl₃ nor ω -conotoxin modulated the stimulating effect of TGF- β 1 on Ank and PC-1 mRNA levels, whereas P/Q-VOC blockade by ω -agatoxin diminished it from 19.2 to 15.3 (–20%) for Ank and from 9.6 to 7.9 (–18%) for PC-1 (Fig. 2B). More importantly, blockade of L- or T-VOCs by lercanidipin or NiCl₂ reduced the impact of TGF- β 1 on Ank mRNA level to 9.9 (–49%) and 6.6 (–66%), respectively (Fig. 2B). Similarly, PC-1 expression was reduced to 6.1 (–36%) and 5 (–48%), respectively. Western blot analysis (Fig. 2C) corroborated the assets obtained at the mRNA level because only lercanidipin and NiCl₂ significantly reduced the increase in ANK and PC-1 protein level induced by TGF- β 1 (Fig. 2C), whereas GdCl₃ and ω -conotoxin remained ineffective (data not shown). Finally, to evaluate the possible involvement of these VOCs in the TGF- β 1-induced calcium mobilization, we measured the cCa²⁺ level in the same experimental conditions as above. None of the compounds affected the basal cCa²⁺ level (Fig. 2D). Interestingly, only the blockade of L- and T-VOCs lowered the TGF- β 1-stimulated calcium mobilization, from 2.7 RFU to 1.7 RFU (–37%) and 1.1 RFU (–60%), respectively (Fig. 2D). Although ω -agatoxin displayed a moderate inhibitory effect on TGF- β 1-stimulated metabolism of ePP_i, it remained ineffective on the cCa²⁺ level. These results demonstrate a major role for calcium entrance through L- and T-type VOCs in the modulation of the TGF- β 1-stimulating effect.

Effect of Calcium Chelation or Release from Internal Stores on TGF- β 1-induced Metabolism of PP_i and Ca²⁺ Mobilization in Chondrocytes—To evaluate the respective contribution of cCa²⁺ level and calcium located in internal stores on the stimulating effect of TGF- β 1 on ePP_i production, we examined the consequence of a 1-h preincubation of cells with the three following compounds (Merck): a calcium chelator, BAPTA-AM (1 μ M); a calcium ionophore, ionomycin (1 μ M); and an inhibitor of phospholipase C (PLC)-dependent calcium release from

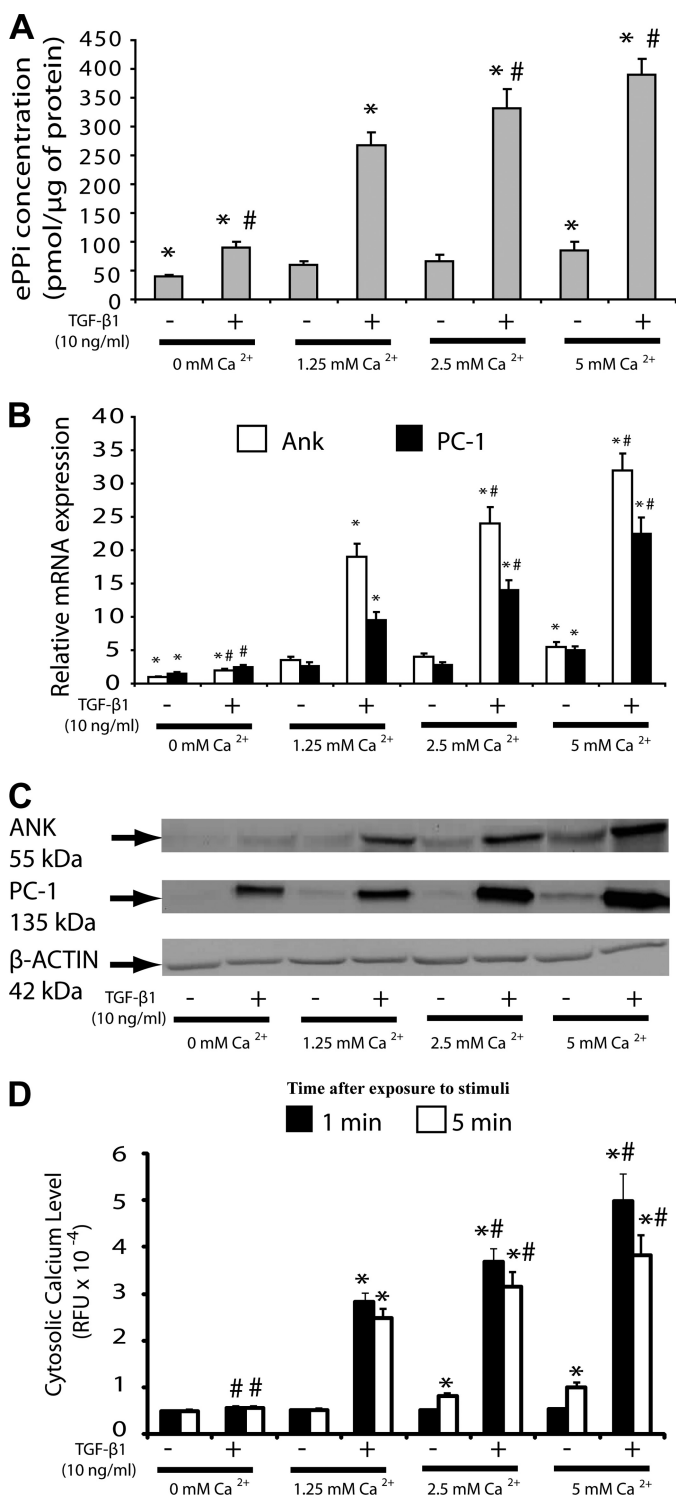


FIGURE 1. Effects of the eCa²⁺ levels on TGF- β 1-stimulated metabolism of PP_i and calcium mobilization in chondrocytes. A, ePP_i production. ePP_i level was assayed in culture supernatants and normalized to total cell proteins. Data (n = 6) are expressed as mean \pm S.D. (error bars) in pmol/ μ g protein. B, Ank and PC-1 mRNA expression. mRNA levels were normalized to S29 (reference gene) (n = 3). Results are presented as mean \pm S.D. over the S29 value. C, ANK and PC-1 protein level. Their relative abundance was normalized to β -actin. Images are representative of three independent experiments. D, cCa²⁺ levels. Data (n = 6) are expressed as mean \pm S.D. in RFU/ 10^4 per 40,000 cells. Statistically significant differences versus cells without TGF- β 1 at 1.25 mM eCa²⁺ are indicated as p < 0.05 (*), and significant differences versus TGF- β 1-stimulated cells at 1.25 mM eCa²⁺ are shown as p < 0.05 (#).

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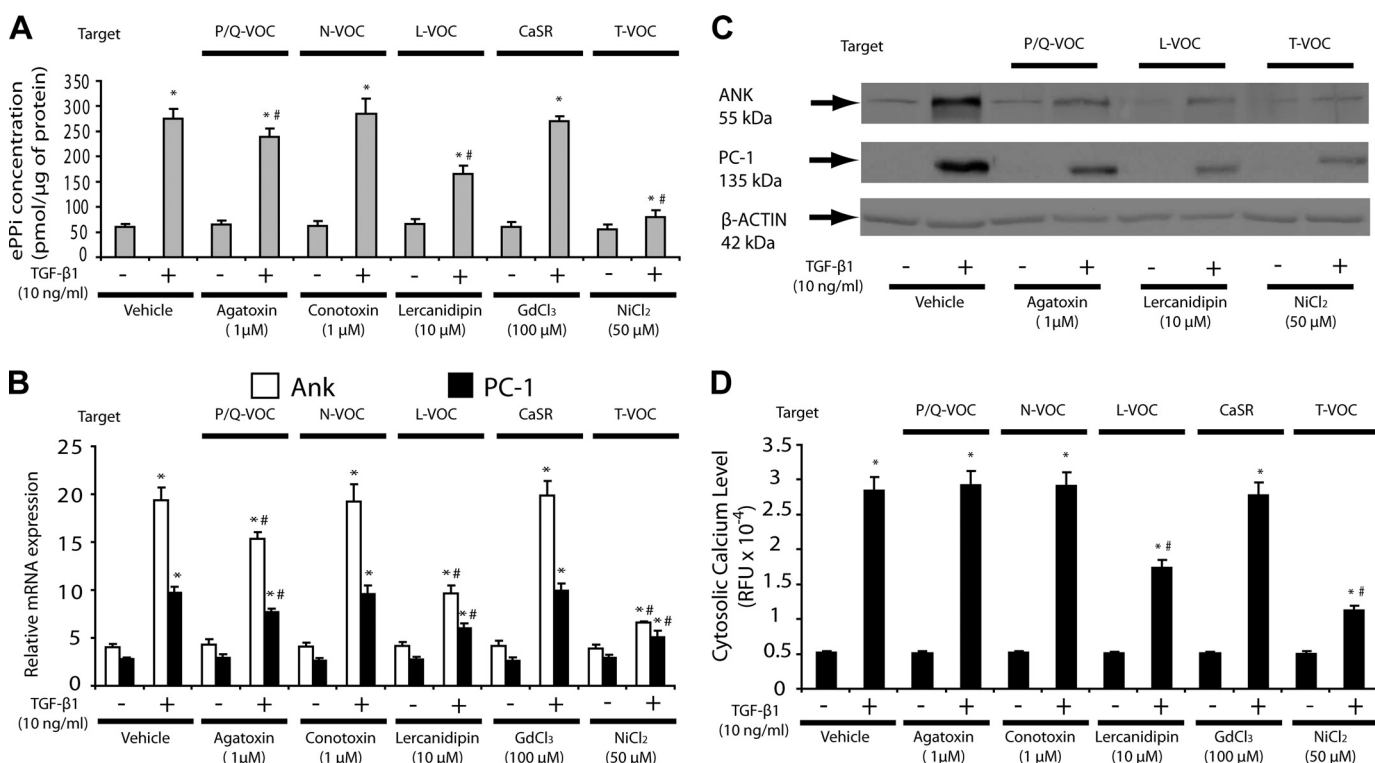


FIGURE 2. Effect of selective calcium VOC blockers and CaSR agonist on TGF- β 1-stimulated metabolism of PP_i and calcium mobilization in chondrocytes cultured with 1.25 mM eCa²⁺. *A*, ePP_i production. ePP_i level was determined in culture supernatants and normalized to total cell proteins. Data ($n = 6$) are expressed as mean \pm S.D. (error bars) in pmol/ μ g protein. *B*, Ank and PC-1 mRNA expression. mRNA levels were normalized to S29 (reference gene) ($n = 3$). Results are presented as mean \pm S.D. over the S29 value. *C*, ANK and PC-1 protein level. Their relative abundance was normalized to β -actin. Blots are representative of three independent experiments. *D*, cCa²⁺ levels. Data ($n = 6$) are expressed as mean \pm S.D. in RFU/ 10^4 per 40,000 cells. Cells were maintained for 24 h in medium containing 1.25 mM eCa²⁺ before starting these experiments. Statistically significant differences versus vehicle are indicated as $p < 0.05$ (*) and versus TGF- β 1 condition as $p < 0.05$ (#).

endoplasmic reticulum, U73122 (10 μ M). Their effects were then assessed after either 24 h (mRNA expression), 48 h (protein level), or 72 h (ePP_i production) of stimulation with TGF- β 1 in the presence of 1.25 mM eCa²⁺.

None of the compounds listed above modified the basal ePP_i production (Fig. 3A). Moreover, PLC inhibition did not affect the TGF- β 1-stimulated production of ePP_i (Fig. 3A). Interestingly, although BAPTA significantly decreased the TGF- β 1-stimulated ePP_i level from 274 to 145 pmol/ μ g protein (-49%), ionomycin raised it to 442 pmol/ μ g protein (1.6-fold). None of the compounds significantly modified the basal mRNA expression of Ank and PC-1 (Fig. 3B). Chelation of cCa²⁺ by BAPTA reduced the effect of TGF- β 1 from 19.2 to 7.9 (-59%) for Ank and from 9.6 to 4.9 (-49%) for PC-1 mRNA levels. In contrast, cCa²⁺ elevation induced by ionomycin boosted the effect of TGF- β 1 from 19.2 to 36.4 (1.9-fold) for Ank and from 9.6 to 17.8 (1.9-fold) for PC-1. Once again, U73122 remained ineffective (Fig. 3B). Western blot analysis (Fig. 3C) confirmed the opposite influence of BAPTA and ionomycin on the inducing effect of TGF- β 1 on ANK and PC-1 protein levels (Fig. 3C). Interestingly, although ionomycin alone failed to modify Ank mRNA level, it strongly induced ANK expression. This apparent discrepancy may come from the ability of ionomycin to stabilize mRNA, as demonstrated previously for the interleukin-3 transcript (23), therefore allowing an increase in the translation step. Finally, neither the basal nor the TGF- β 1-induced increase in cCa²⁺ level was affected by PLC inhibition

(Fig. 3D). In contrast, although BAPTA did not modify the basal cCa²⁺ level, it lowered the TGF- β 1-induced influx from 2.7 RFU to 1 RFU (-63%). In an opposite fashion, ionomycin increased the basal cCa²⁺ level to 3.3 RFU (6.6-fold) and potentiated the stimulating effect of TGF- β 1 to 5.5 RFU (1.7-fold) (Fig. 3D). Taken together, these results demonstrate that an increase in cCa²⁺, independent of PLC-dependent calcium release from the endoplasmic reticulum, is critical for the stimulating effect of TGF- β 1 on ePP_i metabolism.

Study of the Cross-talk between the eCa²⁺ Level, cCa²⁺ Level, and the TGF- β 1-activated Signaling Pathways and Influence on ANK and PC-1 Expression—To evaluate the influence of calcium on the signaling pathways activated by TGF- β 1 and implicated in the regulation of ePP_i metabolism, we assessed, by immunoblotting, the activation of ERK1/2 and Ca²⁺-dependent PKC. For that purpose, chondrocytes were challenged with TGF- β 1 for 5 min (PKC) or 15 min (ERK), as described elsewhere (5). During these experiments, we modulated either the eCa²⁺ level or modified the eCa²⁺ entrance (with the only efficient VOC blockers, lercanidipin and NiCl₂) as well as the cCa²⁺ level (using BAPTA and ionomycin). We completed this study by evaluating the consequence of ERK1/2 or PKC α inhibition on the increase in ANK and PC-1 protein level induced by high eCa²⁺ levels. With that goal, cells were preincubated for 1 h with the following selective inhibitors (Merck): PD98059 (10 μ M; a MEK 1 inhibitor that prevents ERK1/2 activation) or Gö6976 (5 μ M; a selective calcium-dependent PKC inhibitor).

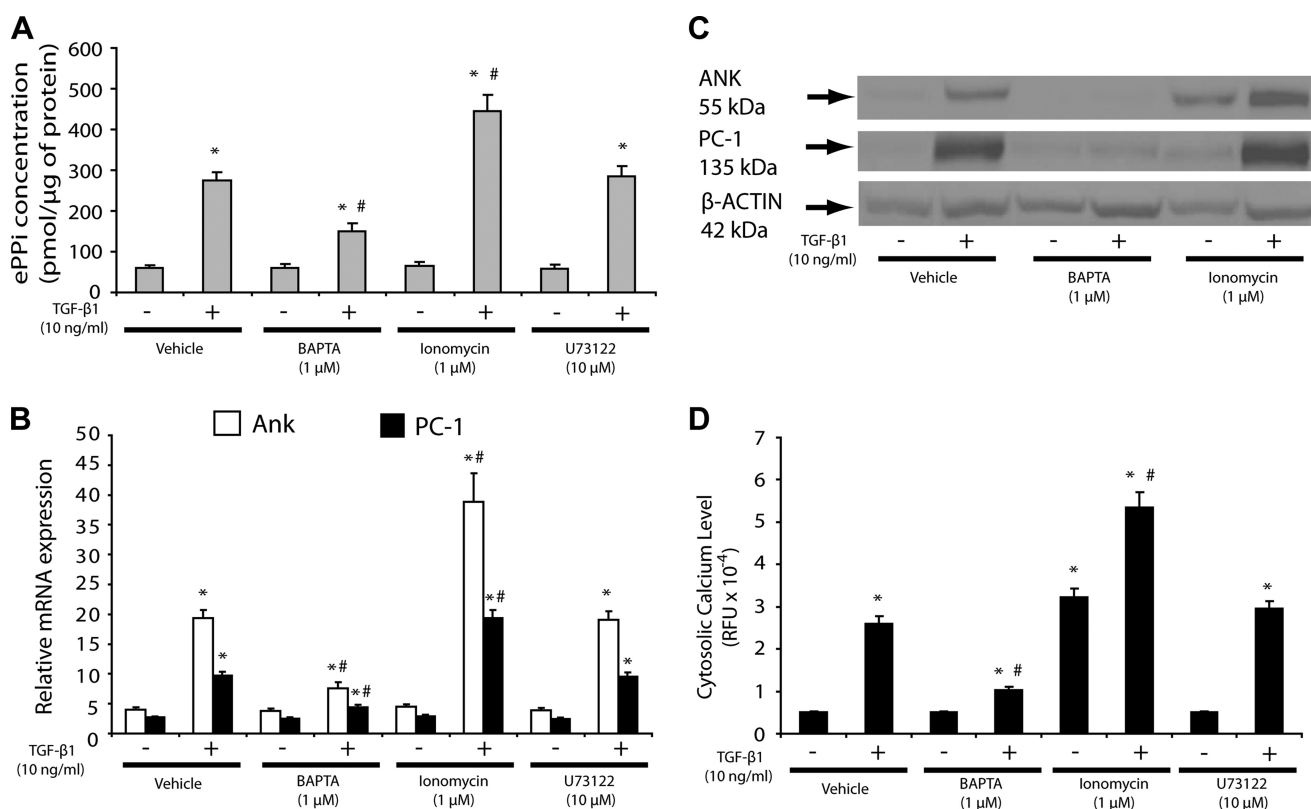


FIGURE 3. Effect of calcium chelation or release from internal stores on TGF- β 1-induced metabolism of PP_i and calcium mobilization in chondrocytes cultured with 1.25 mM eCa²⁺. *A*, ePP_i production. ePP_i level was determined in culture supernatants and normalized to total cell proteins. Data ($n = 6$) are expressed as mean \pm S.D. (error bars) in pmol/ μ g protein. *B*, Ank and PC-1 mRNA expression. mRNA levels were normalized to S29 (reference gene) ($n = 3$). Results are presented as mean \pm S.D. over the S29 value. *C*, ANK and PC-1 protein level. Their relative abundance was normalized to β -actin. Images are representative of three independent experiments. *D*, cCa²⁺ levels. Data ($n = 6$) are expressed as mean \pm S.D. in RFU/ 10^4 per 40,000 cells. Cells were maintained for 24 h in medium containing 1.25 mM eCa²⁺ before starting these experiments. Statistically significant differences versus vehicle are indicated $p < 0.05$ (*), and significant differences versus TGF- β 1 condition are shown as $p < 0.05$ (#).

Then ANK and PC-1 protein levels were determined after an additional 48-h incubation in culture medium containing either 1.25 or 5 mM Ca²⁺. As shown in Fig. 4A, eCa²⁺ alone was sufficient to stimulate the phosphorylation cascades, from 1.25 mM for ERK1/2 and 2.5 mM for PKC α . Interestingly, although TGF- β 1 was able to induce ERK1/2 phosphorylation in the absence of calcium, this activation increased gradually with eCa²⁺ level. Likewise, the phosphorylation of PKC α by TGF- β 1 was also related to eCa²⁺ level (Fig. 4A). When chondrocytes cultured at 1.25 mM Ca²⁺ were preincubated with L-VOC blocker lercanidipin, the TGF- β 1-induced ERK1/2 phosphorylation was weakened, whereas the activation of PKC α was totally suppressed (Fig. 4B). The same results were observed for T-VOC blockade by NiCl₂, but with a more marked reduction of ERK1/2 phosphorylation. As shown in Fig. 4C, BAPTA totally prevented ERK1/2 and PKC α phosphorylation by TGF- β 1. In contrast, ionomycin was sufficient *per se* to enhance the basal phosphorylation of PKC α and ERK1/2. However, in response to TGF- β 1, only the phosphorylation of PKC α was enhanced by the cCa²⁺ burst, suggesting that activation of the ERK1/2 pathway was still maximal under ionomycin challenge. As demonstrated in Fig. 4D, the increase in ANK and PC-1 protein levels by 5 mM Ca²⁺ was almost totally suppressed by both PD98059 and G66976. Finally, to assess how calcium input could regulate TGF- β 1 signaling, we checked the activation status of Ras, which was previously shown to be activated secondary to an

increase in cCa²⁺ level (24). With that goal, chondrocytes at 90% confluence were incubated in calcium-free DMEM containing 1% FCS, which was supplemented or not with 1.25 mM eCa²⁺ for 24 h. Then cells cultured without eCa²⁺ were preincubated for 1 h with vehicle, whereas cells exposed to 1.25 mM eCa²⁺ were preincubated with either vehicle or calcium modulators (*i.e.* lercanidipin, NiCl₂, or BAPTA). Afterward, chondrocytes were exposed to TGF- β 1 for 5 min, and the amount of Ras-GTP was estimated in total cell lysates. As shown in Fig. 4E, no increase in Ras-GTP level was detected in cells stimulated with TGF- β 1 in the absence of eCa²⁺. In contrast, in the presence of 1.25 mM eCa²⁺, TGF- β 1 induced a 2.8-fold increase in Ras-GTP level (from 1.1 to 3.1 RLU). Interestingly, the blockade of VOCs, of L-type by lercanidipin and T-type by NiCl₂, reduced this effect, from 3.1 to 2.2 RLU (-43%) and 1.4 RLU (-85%), respectively (Fig. 4E). In these experimental conditions, BAPTA reduced the TGF- β 1-induced increase in Ras-GTP level to those observed in the absence of eCa²⁺.

These results demonstrate that the stimulatory effect of TGF- β 1 on Ras, ERK1/2, and PKC α activation is critically regulated by eCa²⁺ entry. ERK1/2 and PKC α directly contribute to the increase in ANK and PC-1 protein level provoked by high calcium levels.

Contribution of SP1 to the Regulatory Role of Calcium in the Chondrocyte Response to TGF- β 1—Because SP1 was reported to relay the effect of TGF- β 1, we assessed its possible role in the

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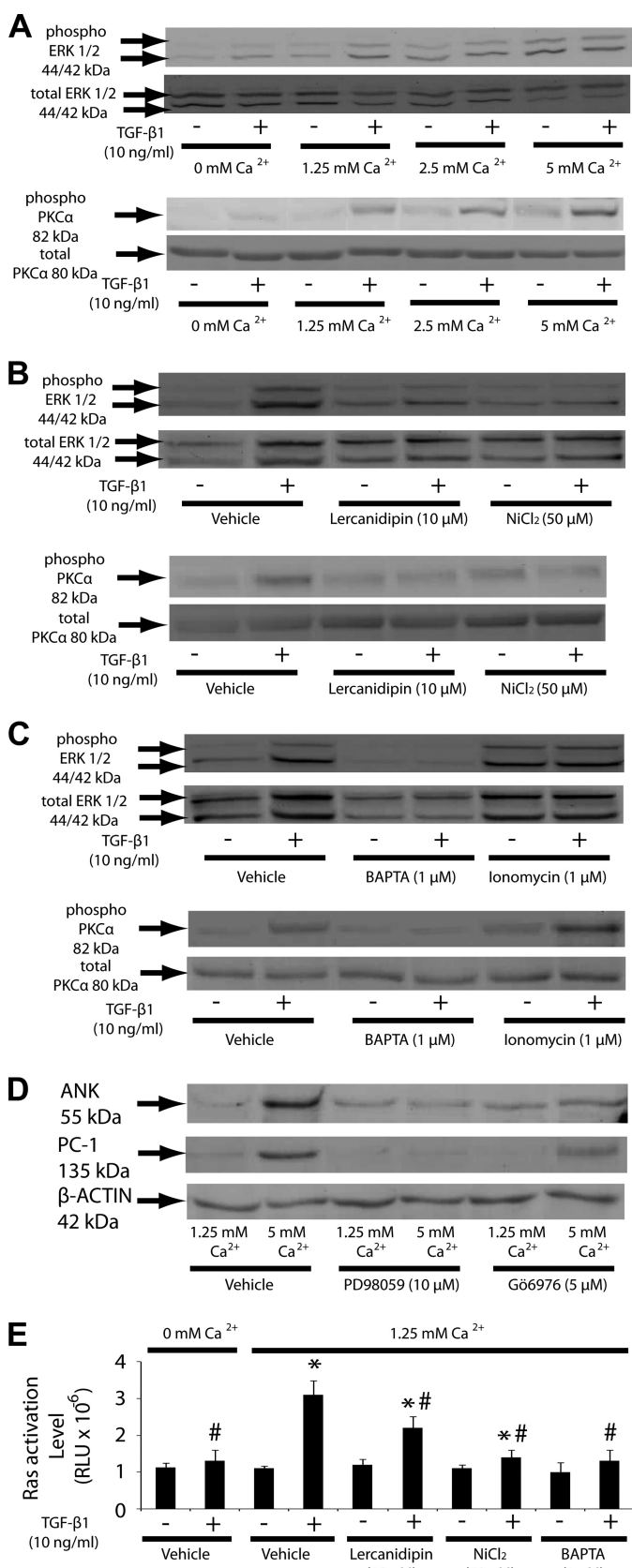


FIGURE 4. Effect of eCa^{2+} level and selected Ca^{2+} modulators on TGF- β 1-activated signaling pathways. *A*, influence of eCa^{2+} concentrations on the TGF- β 1-mediated activation of ERK1/2 and PKC α . *B*, influence of L- and T-VOCs blockers on TGF- β 1-mediated activation of ERK1/2 and PKC α at an eCa^{2+} concentration of 1.25 mM. *C*, influence of BAPTA and ionomycin on

modulation of TGF- β 1-stimulated metabolism of ePP_i in regard to calcium. With that goal, we investigated the influence of eCa^{2+} level on SP1 protein expression by immunoblotting. Thus, chondrocytes were challenged with TGF- β 1 for 24 h while modulating either the eCa^{2+} level, the eCa^{2+} entrance (preincubation of 1 h with the efficient VOC blockers lercanidipin and NiCl₂), or the cCa^{2+} level (1 h of pre-exposure with BAPTA or ionomycin). We completed this study using chondrocytes either pretreated for 1 h with WP631 (Merck; 1 μ M; inhibitor of the DNA-binding ability of SP1) or transfected with a control siRNA or a mix of siRNA targeting Sp1 (Santa Cruz Biotechnology; 10 nM). We then exposed cells to TGF- β 1 for either 24 h (mRNA expression), 48 h (protein level), or 72 h (ePP_i production) in the presence of 1.25 mM eCa^{2+} . As shown in Fig. 5A, a physiological eCa^{2+} level alone was sufficient to elevate SP1 protein level compared with the no calcium condition. Interestingly, although TGF- β 1 was able to induce SP1 expression by itself, this effect increased gradually with eCa^{2+} concentration. In chondrocytes pretreated with lercanidipin or NiCl₂, the TGF- β 1-induced increase of SP1 was totally suppressed, as was also the case for BAPTA (Fig. 5A). Ionomycin increased the total expression of SP1 by itself but blunted the stimulatory effect of TGF- β 1 on this protein (Fig. 5A). When we assessed the phosphorylation of SP1 after 30 min of stimulation, we showed that TGF- β 1 induced SP1 phosphorylation (Fig. 5A). This activation of SP1 was totally suppressed by BAPTA. In contrast, ionomycin enhanced both the basal and the TGF- β 1-induced increase in SP1 phosphorylation (Fig. 5A). Neither WP631 nor Sp1 silencing modulated the basal ePP_i production (Fig. 5B). In contrast, WP631 significantly decreased the TGF- β 1-stimulated ePP_i production from 274 to 99 pmol/ μ g protein (-64%). Similarly, Sp1 silencing reduced the TGF- β 1-induced ePP_i production to 87 pmol/ μ g protein (-68%) (Fig. 5B). Once again, neither WP631 nor Sp1 silencing modified the basal mRNA expression of Ank and PC-1 (Fig. 5C). However, WP631 reduced the TGF- β 1 induction from 19.2 to 7.5 (-61%) for Ank and from 9.6 to 5 (-48%) for PC-1 mRNA expression. Similarly, Sp1 silencing decreased the TGF- β 1-induced Ank expression to 5.7 (-70%) as well as decreasing the PC-1 level to 4.8 (-50%). Western blot analysis (Fig. 5D) validated the efficiency of Sp1 silencing and confirmed its major impact on the stimulatory effects of TGF- β 1 on ANK and PC-1 protein level. These results show that SP1 contributes to the modulatory influence of calcium on the TGF- β 1-induced production of ePP_i .

*Effect of a Selective Inhibition of TGF- β 1-induced Signaling Pathways on cCa^{2+} Level and Transcription Factor Activation—*To evaluate further the respective roles of calcium mobilization and TGF- β 1 in the activation of signaling pathways, we

TGF- β 1-mediated activation of ERK1/2 and PKC α at an eCa^{2+} concentration of 1.25 mM. Relative abundance of phosphoproteins was normalized to the corresponding non-phosphoproteins. *D*, influence of PD98059 and G66976 on the basal and calcium-induced expression of ANK and PC-1. Their relative abundance was normalized to β -actin. *E*, influence of L- and T-VOC blockers and BAPTA on TGF- β 1-mediated activation of Ras. Data ($n = 3$) are expressed as mean \pm S.D. (error bars) in RLU/ 10^6 per 50 μ g of total cell lysates. Statistically significant differences versus cells without TGF- β 1 at 1.25 mM eCa^{2+} are indicated as $p < 0.05$ (*), and significant differences versus TGF- β 1-stimulated cells at 1.25 mM eCa^{2+} are shown as $p < 0.05$ (#). Blots are representative of four independent experiments (A–D).

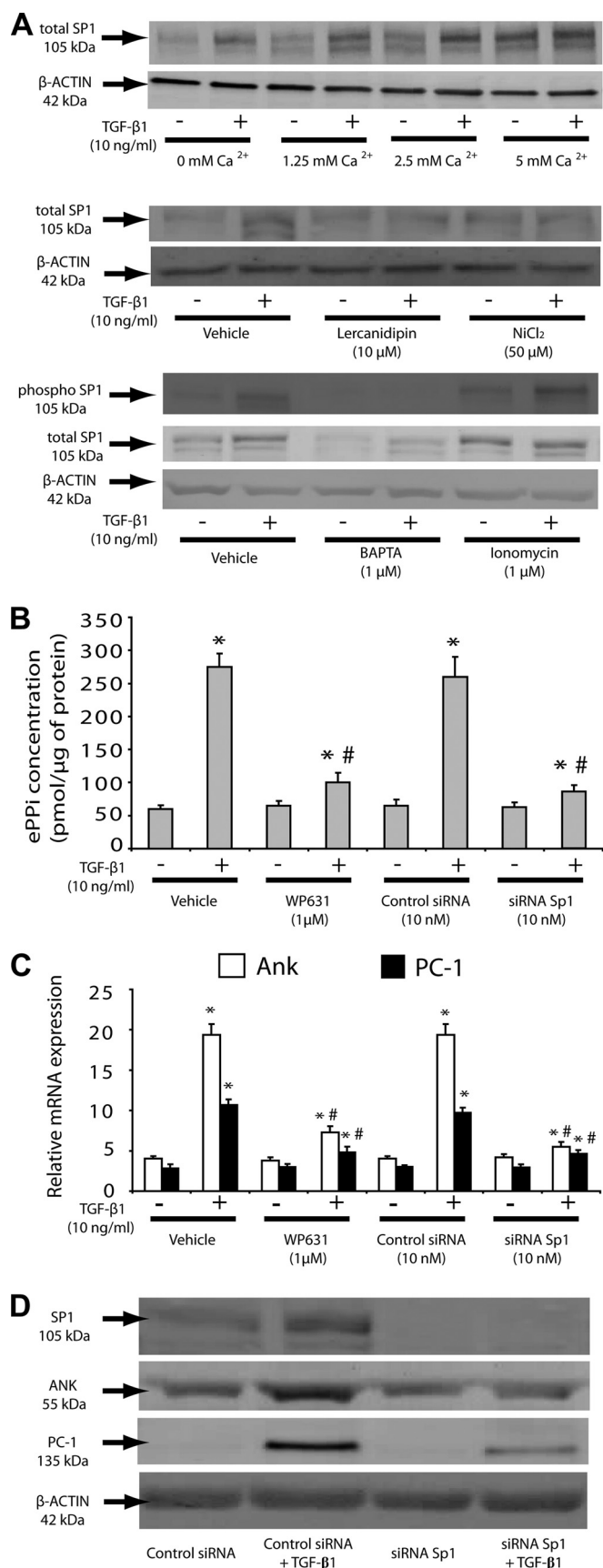


FIGURE 5. Contribution of SP1 to the effect of eCa²⁺ level and selected Ca²⁺ modulators in the chondrocyte response to TGF- β 1. *A*, effect of eCa²⁺ level, selected VOC blockers, and cCa²⁺ modulators on SP1 expression

assessed the consequence of a selective inhibition of ERK1/2 and PKC α . First, cells were preincubated for 1 h in the presence of 1.25 mM eCa²⁺ with one of the following selective inhibitors: PD98059, Gö6976, and WP631. Then cCa²⁺ levels cells were determined after 1 min of stimulation with TGF- β 1. As shown in Fig. 6*A*, neither the basal nor the TGF- β 1-induced increase in cCa²⁺ level was affected by the inhibition of either signaling pathway. Second, we assessed whether inhibition of ERK1/2 and PKC α activation could alter SP1 expression and phosphorylation status because cross-talks between ERK1/2 and PKC α (25) and between ERK2 and SP1 (26) pathways have been reported. For that purpose, in the same preincubation conditions as above, chondrocytes were stimulated with TGF- β 1 for 30 min (SP1 phosphorylation) or 24 h (total SP1 expression level) in the presence of 1.25 mM eCa²⁺. As demonstrated in Fig. 6*B*, the TGF- β 1-induced phosphorylation of SP1 was totally suppressed by MEK1 inhibition (PD98059 treatment), whereas Gö6976-induced blockade of PKC α activation had no effect. Third, we assessed whether the blockade of ERK1/2 and PKC α activation could influence the phosphorylation of the transcription factor ELK-1, described as a downstream effector of ERK1/2 activation (27). With that goal, in the same preincubation conditions as above, ELK-1 phosphorylation was evaluated after 30 min of stimulation of chondrocytes with TGF- β 1 in the presence of 1.25 mM eCa²⁺. As seen in Fig. 6*C*, the TGF- β 1-induced phosphorylation of ELK-1 was totally suppressed by PD98059 treatment, whereas Gö6976-induced blockade of PKC α activation remained ineffective.

These results demonstrated that TGF- β 1-induced mobilization of cCa²⁺ is an early event independent of the activation of ERK1/2, PKC α , and SP1. Moreover, TGF- β 1 is responsible for a ERK1/2-dependent activation of SP1 and ELK-1, independently of PKC α activation.

Regulation of Ank Promoter by cCa²⁺ Level, Sp1, and Elk-1 in Chondrocytes—Because we demonstrated previously that *Ank* contributed much more than *PC-1* to the TGF- β 1-induced production of ePP_i by chondrocytes (5), we therefore focused our investigation on the *Ank* promoter sequence. For that purpose, we challenged chondrocytes, electroporated with either the -720 bp or the -2715 bp *Ank* promoter construct (Fig. 7*A*), with TGF- β 1 for 48 h in the presence of 1.25 mM eCa²⁺ while modulating the eCa²⁺ entrance (pretreatment of 1 h with lercanidipin or NiCl₂) or the cCa²⁺ level (1 h of pre-exposure with BAPTA or lonomycin).

As depicted in Fig. 7*B*, neither the VOCs blockade nor the dramatic changes in cCa²⁺ modified the basal firefly/*Renilla*

and SP1 phosphorylation status. Phosphorylated SP1 relative abundance was normalized to both total SP1 and β -actin. Blots are representative of three independent experiments. *B*, influence of Sp1 blocker WP631 and Sp1 silencing on ePP_i production. ePP_i level was determined in culture supernatants and normalized to total cell proteins. Data ($n = 6$) are expressed as mean \pm S.D. (error bars) in pmol/ μ g protein. *C*, influence of WP631 and Sp1 silencing on *Ank* and *PC-1* mRNA expression. mRNA levels were normalized to *S29* (reference gene) ($n = 3$). Results are presented as mean \pm S.D. over the *S29* value. *D*, influence of Sp1 silencing by a pool of three siRNA sequences on SP1, ANK, and PC-1 protein level. Their relative abundance was normalized to β -actin. Images are representative of three independent experiments. Except for *A*, cells were maintained for 24 h in medium containing 1.25 mM eCa²⁺ before starting these experiments. Statistically significant differences versus vehicle are indicated as $p < 0.05$ (*), and significant differences versus TGF- β 1 condition are shown as $p < 0.05$ (#).

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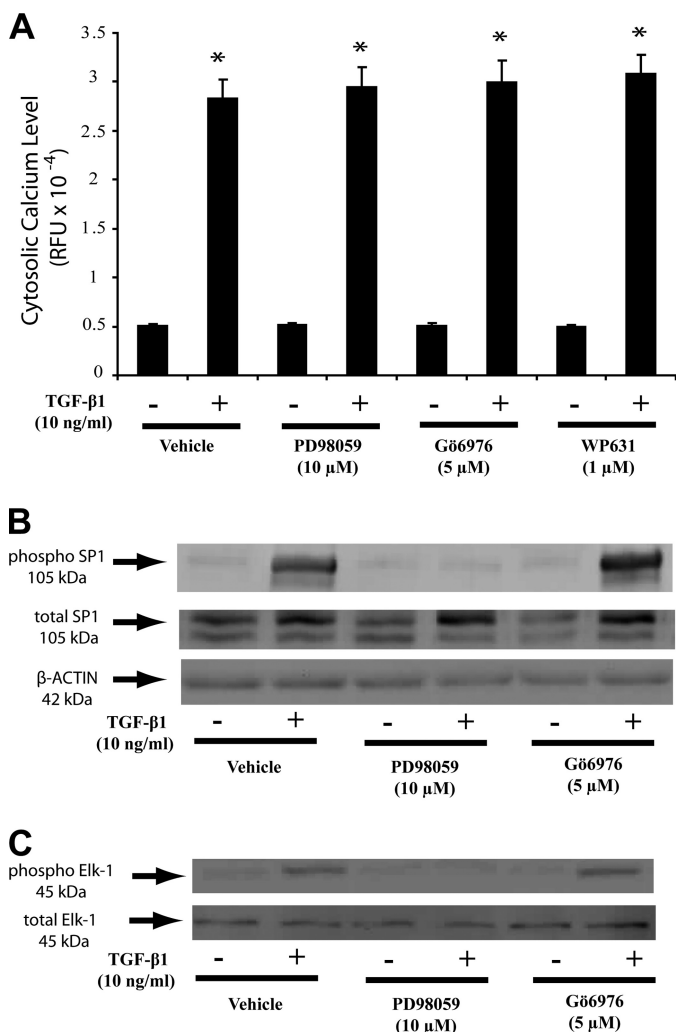


FIGURE 6. Effect of the selective inhibitors of the TGF- β 1-activated signaling pathways on calcium mobilization and transcription factor activation in chondrocytes cultured with 1.25 mM eCa^{2+} . A, influence of PD98059, Gö6976, and WP631 on the TGF- β 1-mediated calcium mobilization. Data ($n = 6$) are expressed as mean \pm S.D. (error bars) in RFU/ 10^4 per 40,000 cells. Statistically significant differences versus vehicle are indicated as $p < 0.05$ (*). B, influence of ERK1/2 and PKC α activation blockers on TGF- β 1-mediated activation of SP1. Phospho-SP1 relative abundance was normalized to both total SP-1 and β -actin. Images are representative of three independent experiments. C, influence of ERK1/2 and PKC α activation blockers on TGF- β 1-mediated activation of ELK-1. Phospho-ELK-1 relative abundance was normalized to non-phospho-ELK-1. Their relative abundance was normalized to β -actin. Blots are representative of four independent experiments. Cells were maintained for 24 h in medium containing 1.25 mM eCa^{2+} before starting these experiments.

ratio in chondrocytes transfected with the -720 bp *Ank* construct. In contrast, TGF- β 1 increased the luciferase activity by 1.3-fold. This weak induction was totally suppressed by the blockade of VOCs as well as by cCa^{2+} chelation. In contrast, ionomycin increased the effect of TGF- β 1 from 1.3- to 1.6-fold. In chondrocytes electroporated with the -2715 bp fragment, the basal firefly/*Renilla* ratio was increased by 2.7-fold by TGF- β 1 (Fig. 7B). When L- and T-VOCs were blocked, the stimulatory effect of TGF- β 1 was reduced from 2.7-fold to 2- and 1.3-fold, respectively. Similarly, BAPTA decreased the TGF- β 1 effect from 2.7- to 1.3-fold. In contrast, ionomycin alone was sufficient to induce a 1.4-fold activation of *Ank* promoter but also boosted the TGF- β 1-stimulated activation from

2.7- to 4.4-fold (Fig. 7B). Besides highlighting the regulatory role of cCa^{2+} level, the highest response to TGF- β 1 obtained with the long promoter construct suggested a critical and major contribution of ELK-1. Because we showed in the present study that TGF- β 1 increased SP1 and ELK-1 phosphorylation, we focused on the possible regulatory role of both SP1 and ELK-1 on the promoter constructs. In the presence of 1.25 mM eCa^{2+} , we analyzed the effects of a 48-h overexpression of either Sp1 or Elk-1 or both in chondrocytes, electroporated with either the -720 bp or the -2715 bp fragment. As depicted in Fig. 7C, in chondrocytes transfected with the -720 bp fragment, overexpression of Elk-1 remained ineffective. In contrast, overexpression of Sp1 alone and in combination with Elk-1 induced the basal firefly/*Renilla* ratio by 1.2-fold. Moreover, in chondrocytes electroporated with the -2715 bp construct, overexpression of Sp1 raised the basal luciferase ratio by 1.3-fold, whereas overexpression of Elk-1 elevated it by 1.8-fold (Fig. 7C). Likewise, simultaneous overexpression of Sp1 and Elk-1 increased the basal firefly/*Renilla* ratio until 2.6-fold. Finally, to further evaluate the respective contribution of SP1 and ERK1/2, we assessed the activity of *Ank* promoter constructs in chondrocytes challenged or not with TGF- β 1 for 48 h after a 1-h pretreatment with WP631 or PD98059. As depicted in Fig. 7D, the basal firefly/*Renilla* ratios were unaffected by either inhibitor. In chondrocytes transfected with the -720 bp construct, the stimulating effect of TGF- β 1 on the *Ank* promoter activity was reduced from 1.3- to 1.2-fold in the presence of WP631 or PD98059 (Fig. 7D). This result confirmed that activation of SP1 was a downstream event of ERK1/2 activation, consistent with the data provided in Fig. 6B. In chondrocytes transfected with the -2715 bp construct, the stimulating effect of TGF- β 1 on the reporter activity was reduced from 2.7-fold to 2- and 1.55-fold in the presence of WP631 and PD98059, respectively (Fig. 7D). The greater inhibitory potency of PD98059 supported the dual contribution of SP1 and ELK-1 to the control of the *Ank* promoter by TGF- β 1, consistent with the data obtained with overexpressing plasmids (Fig. 7C).

These results demonstrate that calcium is a key regulator of activation of the *Ank* promoter by TGF- β 1 in chondrocytes. This activation requires the phosphorylation of ERK1/2, which in turn activates the SP1 and ELK-1 transcription factors, where SP1 seems to behave as a transcriptional co-activator for ELK-1.

DISCUSSION

Although the clinical association between ACC and primary hyperparathyroidism was established 40 years ago (28), very few studies have addressed the relationship between CPPD deposition and calcium levels in extracellular fluids. Recently, Volpe *et al.* (10) demonstrated the high frequency of ACC in patients with familial hypocalciuric hypercalcemia and ACC. However, the molecular mechanisms linking high Ca^{2+} levels to ACC still needed to be elucidated.

Because an increase in PTH-mid-region fragments was noticed in patients with CPPD crystal deposition disease (7), the possible influence of PTH on *Ank* and PC-1 expression was studied previously in our laboratory.⁴ At that time, we failed to

⁴ A. Bianchi, S. Sebillaud, J.-Y. Jouzeau, and P. Netter, unpublished observations.

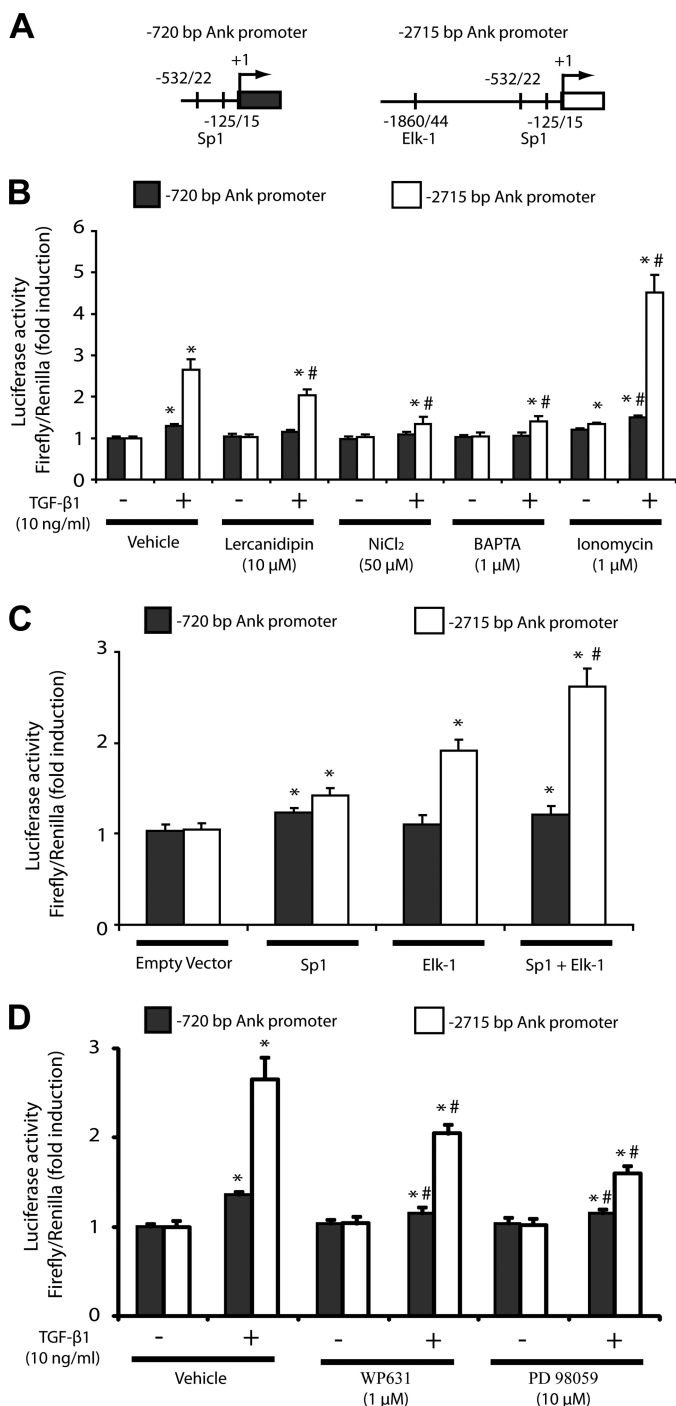


FIGURE 7. Influence of cCa^{2+} level, Sp1, and Elk-1 on the regulation of Ank promoter in chondrocytes cultured with 1.25 mM eCa^{2+} . A, representation of both promoter constructs. B, influence of selected VOCs blockers and cCa^{2+} modulators on Ank promoter activity in TGF- β 1-stimulated chondrocytes. Cells were electroporated either with the -720 or -2715 bp construct (firefly luciferase as reporter gene). Normalization was performed using the TK-Renilla reporter gene. Data are expressed as the mean-fold induction of firefly/Renilla luciferase activity ratio \pm S.D. (error bars) ($n = 4$). Statistically significant differences versus vehicle are indicated as $p < 0.05$ (*), and significant differences versus TGF- β 1 condition are shown as $p < 0.05$ (#). C, influence of Sp1, Elk-1, and Sp1 + Elk-1 overexpression on the basal activity of Ank promoter. Chondrocytes were transfected either with the -720 or -2715 bp construct and co-transfected with either pCMV-Sp1 or pCMV-Elk-1 or with both. Normalization was performed using the TK-Renilla reporter gene. Data are expressed as the mean-fold induction of firefly/Renilla luciferase activity ratio \pm S.D. ($n = 4$). Statistically significant differences versus empty vector-transfected cells are indicated as $p < 0.05$ (*), and significant differences

observe any difference between controls and PTH-stimulated chondrocytes. This finding is consistent with a previous study that described the ineffectiveness of PTH on PC-1 enzymatic activity in human osteoblast-like cells (29). Therefore, we focused our work on the regulation of the ePP_i/P_i balance by TGF- β 1, whose elevated synovial fluid levels were reported to be associated with CPPD formation in OA patients (30).

In the present study, we paid first special attention to the characterization of the articular phenotype of the chondrocytes. We confirmed the absence of tissue-nonspecific alkaline phosphatase mRNA, thus indicating that our evaluation of the ePP_i levels throughout the study was not biased by its possible hydrolysis into eP_i . As a consequence, no eP_i accumulation could occur in the culture medium. This feature is critical because eP_i was also demonstrated to increase the TGF- β 1-induced expression of Ank in chondrogenic cells (31), a finding that we confirmed recently in articular chondrocytes supplemented with eP_i (32). Moreover, eP_i was previously shown to enhance the expression of matrix γ -carboxyglutamic acid (Gla) protein in growth plate chondrocytes, thus inhibiting the mineralization process (33). This suggests that eP_i alone could orient the chondrocyte phenotype toward a hypertrophic state, whereas we demonstrated previously that ePP_i contributed to the maintenance of the differentiated chondrocyte phenotype (18).

The present work demonstrates clearly that increasing eCa^{2+} concentrations potentiated the TGF- β 1-induced production of ePP_i in a concentration-related fashion by increasing Ank and PC-1 expression. These results are consistent with the very recent work from Oca *et al.* (31), who showed that the up-regulation of Ank by TGF- β 1 was increased in the presence of a higher eCa^{2+} level during chondrogenesis of the ATDC5 cell line. However, neither the dose dependence on eCa^{2+} concentration nor the deciphering of the underlying signaling pathway implicated was determined. A possible regulation by calcium could occur by CaSR activation, which, however, failed to modify the TGF- β 1-mediated ePP_i production in our experimental conditions. This lack of efficiency of $GdCl_3$ was consistent with the differentiated phenotype of chondrocyte because a strong expression of CaSR was reported in growth plate or hypertrophic chondrocytes rather than in articular chondrocytes (34). Altogether, these findings demonstrated that the eCa^{2+} pool modulated the control of the ePP_i/eP_i balance by TGF- β 1, providing a possible mechanistic link between hypercalcemia and secondary CPPD deposition.

In our experimental conditions, TGF- β 1 provoked an entrance of eCa^{2+} in chondrocytes, which depended on L- and T-VOCs. The eCa^{2+} input was consistent with previous works that have demonstrated a similar influx in other cell types, like

versus single construct-transfected cells are shown as $p < 0.05$ (#). D, influence of SP1 (WP631) or ERK1/2 (PD98059) blockade on the basal and calcium-induced activity of Ank promoter. Chondrocytes were transfected with either the -720 or -2715 bp construct. Normalization was performed using the TK-Renilla reporter gene. Data are expressed as the mean-fold induction of firefly/Renilla luciferase activity ratio \pm S.D. ($n = 4$). Statistically significant differences versus cells without TGF- β 1 at 1.25 mM eCa^{2+} are indicated as $p < 0.05$ (*), and significant differences versus TGF- β 1-stimulated cells are shown as $p < 0.05$ (#). Cells were maintained for 24 h in medium containing 1.25 mM eCa^{2+} before starting these experiments.

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fibroblasts (35) or vascular smooth muscle cells (36). In the present study, the contribution of VOCs was evaluated indirectly by their blockade with selective inhibitors, but there was a good correlation between the changes observed in cCa^{2+} levels and ePP_i levels in our study. The contribution of L-VOCs to TGF- β 1-induced calcium entrance was shown recently in ATDC5 cells, although no contribution of T-VOCs was reported (31). This was quite unexpected because T-VOCs were shown to be expressed not only in primary chondrocytes but also in the ATDC5 cell line (12). Our data on cCa^{2+} level support a major role for T-VOCs compared with L-VOCs. In contrast, ω -agatoxin was unable to block the calcium influx in our experimental system, although a modest, probably Ca^{2+} -independent effect was observed on ePP_i levels and expression of Ank and PC-1. This result is in line with the current lack of a demonstration that P/Q-VOCs are expressed in articular chondrocytes or in ATDC5 cells.

The link between IP_3 and calcium is well described in chondrocytes because other studies have suggested (37) or demonstrated (38) an IP_3 -dependent calcium release from the internal stores, using hypotonic shock and fluid flow experiments, respectively. It is noteworthy that, in mesangial cells, TGF- β 1 induced a calcium mobilization that depended on both IP_3 release and the disruption of the actin cytoskeleton organization (39). In our study, we used U73122, an inhibitor of PLC, which cleaves phosphatidylinositol 2-phosphate in IP_3 and diacylglycerol. We demonstrated that U73122 was ineffective on the TGF- β 1-mediated calcium influx, indicating that this phenomenon was independent of the IP_3 pathway in articular chondrocytes. Similar results were reported in chondrocytes in which a calcium influx was generated by an electrical stimulation (40). Thus, the variable implication of IP_3 could depend on the nature of the stimulation used (*i.e.* localized voltage modifications *versus* cell membrane stretches). TGF- β 1 was also shown to modify the cell membrane voltage by modulating K^+ currents in microglia cells (41). Interestingly, rat articular chondrocytes express such K^+ channels (42), suggesting that electrophysiological changes may have contributed to the observed IP_3 -independent mechanism. Altogether, these findings support the hypothesis of a quick TGF- β 1-mediated modification of the K^+ currents, resulting in the opening of L- and T-VOCs, and secondary calcium influx into chondrocytes. This was already demonstrated in pituitary cells, where a variation in K^+ currents induced an L-VOC-dependent Ca^{2+} influx (43).

Although ionomycin alone induced an increase in cytosolic calcium level in chondrocytes (calcium released from internal stores), the addition of TGF- β 1 further increased the Ca^{2+} mobilization in our system. Therefore, the TGF- β 1-induced production of ePP_i is thought to depend on the availability of the eCa^{2+} pool rather than on intracellular stores. Indeed, we showed here that no significant calcium mobilization was detectable in the absence of eCa^{2+} in the culture medium and that cCa^{2+} levels measured were higher in the presence of elevated eCa^{2+} concentrations. Likewise, another work (40) reported an exclusive dependence of the increase in aggrecan and type II collagen mRNA to the eCa^{2+} reservoir in electrically stimulated chondrocytes.

We deciphered previously the signaling pathways activated by TGF- β 1 responsible for Ank induction and thus ePP_i production by articular chondrocytes (5). These were Smad-independent but relied on ERK1/2 and Ca^{2+} -dependent PKCs. Oca *et al.* (31) confirmed recently that the induction of Ank expression depended on activation of Ca^{2+} -dependent PKCs. We demonstrate here that ePP_i production mostly depended upon the synergistic action of TGF- β 1 and eCa^{2+} input, which both controlled the protein level of the transcription factor SP1. We also showed that TGF- β 1 induced the phosphorylation of SP1 in a ERK1/2-dependent mechanism, as demonstrated by others for the regulation of the gastrin promoter (26). TGF- β 1 is often described as a biological agent promoting SP1 activity. For example, TGF- β 1 enhanced the DNA binding capacity of SP1 in trabecular bone-derived cells (44), while promoting the SP1-dependent transcription of type I collagen in fibroblasts (45). Calcium was also demonstrated to be critical for the ability of SP1 to stimulate the basal (14) and TGF- β 1-induced expression of β 1,3-glucuronosyltransferase I (16).

Our experiments underline that the calcium-increased effects of TGF- β 1 on ePP_i metabolism were supported by an enhanced activation of the ERK1/2 and $\text{PKC}\alpha$ pathways. This is consistent with another study (46) that has shown the calcium dependence of ERK1/2 phosphorylation during the induction of the phosphate transporters Glvr-1 and -2 by P_i in odontoblasts. It is noteworthy that we demonstrated that the ability of eCa^{2+} to potentiate the phosphorylation of ERK1/2 by TGF- β 1 was probably supported by the activation of Ras because the blockade of VOCs inhibited both Ras and ERK1/2 activation. These data are consistent with the contribution of the Ras/Raf-1 pathway to the stimulatory effect of TGF- β 1 on Ank expression at a "physiological" (1.25 mM) free eCa^{2+} level (5) and the previous demonstration that eCa^{2+} influxes could activate Ras, which is subsequently able to activate MEK1 and MAPK (47). Such a close relationship was further supported by the inability of TGF- β 1 to activate Ras in the absence of eCa^{2+} or after Ca^{2+} chelation. Additionally, PKCs are well described to directly activate ERK1/2 (48–50), and Ca^{2+} -dependent PKCs are key regulators of Ank expression in chondrocytes. Taken together, these data indicate that a critical cytosolic Ca^{2+} level must be reached to activate Ras, ERK1/2, and $\text{PKC}\alpha$. Such a level is achieved at a physiological free eCa^{2+} level by a TGF- β 1-dependent calcium influx through L- and T-VOCs.

Finally, using two constructs of the Ank promoter, we demonstrated that the presence of the Elk-1-responsive element was indisputable for the inducing effect of TGF- β 1. This finding corroborated the ERK1/2-dependent activation of ELK-1 by TGF- β 1. A comparable signaling cascade was reported for the regulation of type I collagen expression in fibroblasts (51) and after the stimulation of the human chondrosarcoma-derived chondrocytic cell line HCS-2/8 by connective tissue growth factor stimulation (52).

It is noteworthy that a synergistic action of ELK-1 and SP1 was detectable in our experimental conditions. One can suggest the formation of a transcription enhancer complex containing these two transcription factors, as observed during the lipopo-

lysaccharide-induced expression of tumor necrosis factor- α in macrophages (53). Therefore, the cooperative effect between SP1 and ELK-1 could depend upon a direct protein-protein interaction, although Tsai *et al.* (53) are rather in favor of an indirect interaction through a protein scaffold depending on CBP/p300.

To summarize, we show here that high extracellular calcium level strongly increases the TGF- β 1-induced production of ePP_i. This effect is due to an enhancement of Ank and PC-1 mRNA and protein expression. The underlying molecular mechanisms implicated include a TGF- β 1-mediated calcium influx through L- and T-VOCs. This results in an elevation of SP1 level, the activation of Ras, and the phosphorylation of ERK1/2 and PKC α , to enhance finally the transcriptional activity of ELK-1 and SP1 acting synergistically on Ank expression.

Our results provide for the first time a possible mechanistic explanation for the onset of ACC secondary to primary hyperparathyroidism. This could support the research of new therapeutic options, such as the use of calcimimetics. For example, cinacalcet has proven to reduce biochemical parameters such as PTH and hypercalcemia in patients with primary or secondary hyperparathyroidism (54). However, clinical outcomes in ACC still need to be demonstrated. Calcimimetics target the CaSR, which remains very marginally expressed in articular cartilage (34). However, we show here that articular chondrocytes are very responsive to high eCa²⁺ level by producing ePP_i. Therefore, modulation of calcium levels and/or entrance could be an interesting avenue of clinical research aiming at the prevention of secondary ACC.

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Calcium Input Potentiates the Transforming Growth Factor (TGF)- β 1-dependent Signaling to Promote the Export of Inorganic Pyrophosphate by Articular Chondrocyte

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