Na⁺/Ca²⁺ Exchanger Activity Modulates Connective Tissue Growth Factor mRNA Expression in Transforming Growth Factor β1- and Des-Arg¹⁰-kallidin-stimulated Myofibroblasts*

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Transforming growth factor (TGF)-β and des-Arg¹⁰-kallidin stimulate the expression of connective tissue growth factor (CTGF), a matrix signaling molecule that is frequently overexpressed in fibrotic disorders. Because the early signal transduction events regulating CTGF expression are unclear, we investigated the role of Ca²⁺ homeostasis in CTGF mRNA expression in TGF-β1- and des-Arg¹⁰-kallidin-stimulated human lung myofibroblasts. Activation of the kinin B1 receptor with des-Arg¹⁰-kallidin stimulated a rise in cytosolic Ca²⁺ that was extracellular Na⁺-dependent and extracellular Ca²⁺-dependent. The des-Arg¹⁰-kallidin-stimulated increase of cytosolic Ca²⁺ was blocked by KB-R7943, a specific inhibitor of Ca²⁺ entry mode operation of the plasma membrane Na⁺/Ca²⁺ exchanger. TGF-β1 similarly stimulated a KB-R7943-sensitive increase of cytosolic Ca²⁺ with kinetics distinct from the des-Arg¹⁰-kallidin-stimulated Ca²⁺ response. We also found that KB-R7943 or 2′,4′-dichlorobenzamid, an amiloride analog that inhibits the Na⁺/Ca²⁺ exchanger activity, blocked the TGF-β1- and des-Arg¹⁰-kallidin-stimulated increases of CTGF mRNA. Pretreatment with KB-R7943 also reduced the basal and TGF-β1-stimulated levels of αI(I) collagen and α smooth muscle actin mRNAs. These data suggest that, in addition to regulating ion homeostasis, Na⁺/Ca²⁺ exchanger acts as a signal transducer regulating CTGF, αI(I) collagen, and α smooth muscle actin expression. Consistent with a more widespread role for Na⁺/Ca²⁺ exchanger in fibrogenesis, we also observed that KB-R7943 likewise blocked TGF-β1-stimulated levels of CTGF mRNA in human microvascular endothelial and human osteoblast-like cells. We conclude that Ca²⁺ entry mode operation of the Na⁺/Ca²⁺ exchanger is required for des-Arg¹⁰-kallidin- and TGF-β1-stimulated fibrogenesis and participates in the maintenance of the myofibroblast phenotype.

Connective tissue growth factor (CTGF),¹ a matrix signaling molecule of the Cyr61/connective tissue growth factor/ephrinstoma-overexpressed (CCN) family (1), promotes extracellular matrix production by fibroblasts. The CCN family of matrix-cellular proteins is distinguished by a high degree of amino acid homology (50–90%) and conservation of 38 cysteine residues. Like other CCN family members, CTGF is comprised of a secretory signal and four distinct protein modules: an insulin-like growth factor-binding domain, a von Willebrand factor type C repeat, a thrombospondin type 1 repeat, and a C-terminal module (1). TGF is a cysteine-rich, heparin-binding, 349-amino acid protein (2–4) expressed at high levels during wound repair and at sites of connective tissue formation in a variety of fibrotic disorders (5–9). Recombinant CTGF stimulates fibroblast proliferation and extracellular matrix protein synthesis (10, 11). Transforming growth factor β (TGF-β) stimulates CTGF transcription in normal rat kidney fibroblasts, which is not observed following epidermal growth factor, fibroblast growth factor, or platelet-derived growth factor stimulation (11). We have shown that TGF-β stimulates an increase in CTGF transcription in human myofibroblasts, suggesting a role for CTGF in the pathogenesis of fibrosia (12).

We have previously shown that activation of the kinin B1 receptor by des-Arg¹⁰-kallidin enhances CTGF mRNA stability in human myofibroblasts (12). In contrast, activation of the kinin B2 receptor by bradykinin or kallidin does not alter CTGF mRNA levels. The B1 receptor is a G protein-coupled receptor that is expressed following injury or exposure to pro-inflammatory agents such as interleukin-1β (13–17). Des-Arg¹⁰-kallidin, the carboxypeptidase metabolite of kallidin, is the only known natural ligand for the human kinin B1 receptor (Kp = 0.2 nM), whereas des-arg⁹-bradykinin activates the B1 receptor in rodents, cows, sheep, guinea pigs, dogs, and cats (18). The B2 receptor agonists kallidin and bradykinin bind to the B1 receptor with relatively low affinity (Kp > 100 nM) (18). B1 receptor activation induces a rise in cytosolic Ca²⁺ with kinetics distinct from those following B2 receptor activation (6); however, the mechanisms for these transient increases of cytosolic Ca²⁺ are unclear, and the relationship between the Ca²⁺ transients and CTGF mRNA stability has not been determined.

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¹ The abbreviations used are: CTGF, connective tissue growth factor; TGF, transforming growth factor; DCB, dichlorobenzamil; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; AM, acetoxymethyl ester; TPA, 12-O-tetradecanoylphorbol 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Cytosolic Ca\(^{2+}\) levels are maintained by various transporters in the plasma membrane and intracellular organelles. Among the different Ca\(^{2+}\) transporters, the electrogenic Na\(^{+}/Ca^{2+}\) exchanger is unique because it moves Ca\(^{2+}\) across the membrane bidirectionally depending upon the Na\(^{+}\), Ca\(^{2+}\), and K\(^{+}\) gradients and the membrane potential (19). In most cells, the stoichiometry for this transporter is three Na\(^{+}\) for one Ca\(^{2+}\), although this point is controversial (20, 21). Major regulatory events and the membrane potential (19). In most cells, the stoichiometry for this transporter is three Na\(^{+}\) for one Ca\(^{2+}\), although this point is controversial (20, 21). Major regulatory events and the membrane potential (19).

**MATERIALS AND METHODS**

**Tissue Culture**—Human embryonic lung myofibroblasts (IMR-90; Institute for Medical Research, Camden, NJ) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium bicarbonate (0.57 g/liter), 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (10 \(\mu\)g/ml), sodium pyruvate (1 mmol/l), and nonessential amino acids (0.1 mmol/l). The experiments were performed on myofibroblasts with population doubling less than 34.

Human microvascular endothelial cells (HMEC-1), a kind gift from Dr. Thomas Lawley (Emory University), were maintained on modified Eagle's medium (DMEM) containing physiologic saline solution at 37 °C. Fura-2 fluorescence was assessed by ethidium bromide staining of ribosomal bands and by autoradiography at several different times to ensure that the bands could be quantified by densitometry. The \(\alpha1\) collagen probe came from a rat \(\alpha1\) collagen cDNA that specifically binds human \(\alpha1\) collagen mRNA (12). The CTGF probe was generated by reverse transcription-PCR as described (12).

**RESULTS**

**Role of Plasma Membrane Na\(^{+}/Ca^{2+}\) Exchanger in B1 Receptor-mediated Ca\(^{2+}\) Transients**—We have previously reported that kinin B1 receptor activation increases cytosolic Ca\(^{2+}\) in a manner that is kinetically distinct from the Ca\(^{2+}\) increase following B2 receptor activation (12). However, the mechanisms that mediate the kinin-induced Ca\(^{2+}\) responses remain uncharacterized. To determine the contribution of extracellular Ca\(^{2+}\) to the kinin-stimulated increase of cytosolic Ca\(^{2+}\), myofibroblasts were assayed for changes in cytosolic Ca\(^{2+}\) in medium prepared without Ca\(^{2+}\) (nominally Ca\(^{2+}\)-free).

As we previously reported, B1 receptor activation with des-Arg\(^{10}\)-kallidin induced a rise in cytosolic Ca\(^{2+}\) (Fig. 1B). In nominally Ca\(^{2+}\)-free medium, activation of the B1 receptor did not increase cytosolic Ca\(^{2+}\) levels (Fig. 1A). These data strongly suggest that the des-Arg\(^{10}\)-kallidin-stimulated increase of cytosolic Ca\(^{2+}\) is dependent on entry of extracellular Ca\(^{2+}\). In contrast, B2 receptor activation with kallidin stimulates an increase of cytosolic Ca\(^{2+}\) in the presence or absence of extracellular Ca\(^{2+}\) (Fig. 1B).

Many mechanisms may mediate entry of extracellular Ca\(^{2+}\) such as Ca\(^{2+}\) channels and Na\(^{+}/Ca^{2+}\) exchange. We initially examined the activity of L-type channels and found that the des-Arg\(^{10}\)-kallidin-stimulated increase of cytosolic Ca\(^{2+}\) was not sensitive to inhibition of L-type Ca\(^{2+}\) channels with verapamil (data not shown). The Na\(^{+}\) dependence of the des-Arg\(^{10}\)-kallidin-stimulated Ca\(^{2+}\) response was examined in myofibroblasts incubated in nominally Na\(^{+}\)-free medium in which the Na\(^{+}\) content was iso-osmotically replaced with N-methyl-D-glucamine (NMDG). RNA was quantified by absorbance at 260 nm, and purity was determined by absorbance at 280 and 310 nm. RNA (2 \(\mu\)g) was converted to cDNA in a 20-\(\mu\)l reaction volume containing 50 \(\mu\)l Tris-HCl (pH 8.3), 7.5 mm KCl, 3 mm MgCl\(_2\), 10 mm diethiothreitol, 0.5 mm dNTP mix, 500 ng of oligo(dT)\(_{20}\) and 10 units of RNAsin (Promega), and 200 units of reverse transcriptase (Promega) at 42 °C for 60 min followed by 93 °C for 5 min. An aliquot (0.2 \(\mu\)l) of the cDNA was then amplified in an ABI Prism 7000 total real time PCR unit, using SYBR green dye and primers for human \(\alpha1\) smooth muscle actin (GenBank\(^\text{TM}\) accession number NM_000260), for \(\kappa1\) collagen (GenBank\(^\text{TM}\) accession number NM_000260) using the forward primer (5'-ctgtatcctctgccct-3') and the reverse primer (5'-agttgtagctgccacct-3'), corresponding to nucleotides 959–979 and 988–1008, respectively, and for CTGF (GenBank\(^\text{TM}\) accession number BC087839) using the forward primer, 5'-atgtagctccgagccg-3', and the reverse primer 5'-cggtgagctaccttgc-3', corresponding to nucleotides 994–912 and 937–955, respectively. The results were normalized to real time PCR of human GAPDH (GenBank\(^\text{TM}\)) accession number NM_002046 and the product was amplified with the primers 5'-ctcaagagatggagacctc-3' and the reverse primer 5'-tgctgctgagcagacc-3', corresponding to nucleotides 1113–1136 and 1145–1163, respectively.

Luciferase Assay—IMR-90 myofibroblasts were plated in 6-well plates (350,000 cells/well) in DMEM supplemented with 10% FBS. After 24 h the cultures were washed with phosphate-buffered saline and incubated with the 3TP-LUX reporter (1 \(\mu\)g/well) mixed with Lipofectamine (1 \(\mu\)l/ml) according to the manufacturer's instructions. After 4 h, DMEM supplemented with 10% FBS without antibiotics (3 ml/well) was added to the transfection medium, and the cultures were incubated for an additional 20 h. Before experimentation, the cultures were incubated in DMEM supplemented with 0.4% FBS without antibiotics for 16 h.
glucamine and immediately assayed for changes in cytosolic Ca\(^{2+}\). The rise in cytosolic Ca\(^{2+}\) following B1 receptor activation by des-Arg\(^{10}\)-kallidin was abrogated in myofibroblasts incubated in nominally Na\(^{+}\)-free medium (Fig. 2A). In contrast, B2 receptor activation in nominally Na\(^{+}\)-free medium induces a rapid and sustained rise in cytosolic Ca\(^{2+}\) (Fig. 2B). Altogether, these observations indicate that des-Arg\(^{10}\)-kallidin stimulates a Na\(^{+}\)-dependent Ca\(^{2+}\) influx, suggesting that des-Arg\(^{10}\)-kallidin activates Ca\(^{2+}\) entry mode operation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger. Expression of Na\(^{+}\)/Ca\(^{2+}\) exchanger isoforms 1 and 2 in IMR-90 myofibroblasts was determined by reverse transcription-PCR (data not shown).

We investigated the role of the Na\(^{+}/Ca\(^{2+}\) exchanger in the kinin-stimulated Ca\(^{2+}\) transients using KB-R7943, a novel iso-thiourea derivative that inhibits Ca\(^{2+}\) entry mode operation of the Na\(^{+}/Ca\(^{2+}\) exchanger by interacting with an extracellular region of the exchanger (30–32) but does not modulate Na\(^{+}\)/H\(^{+}\) exchanger activity (33). In KB-R7943-treated myofibroblasts, basal Ca\(^{2+}\) levels are unchanged; however, des-Arg\(^{10}\)-kallidin does not induce an increase of cytosolic Ca\(^{2+}\) (Fig. 3). In contrast, B2 receptor activation with kallidin in the presence of KB-R7943 induces a rapid and sustained cytosolic Ca\(^{2+}\) transient (data not shown). These results suggest that the increase of cytosolic Ca\(^{2+}\) observed in des-Arg\(^{10}\)-kallidin-stimulated myofibroblasts is mediated by Ca\(^{2+}\) entry mode operation of the Na\(^{+}/Ca\(^{2+}\) exchanger.

12-O-Tetradecanoylphorbol 13-Acetate (TPA) Attenuates B1 Receptor-mediated Ca\(^{2+}\) Transients—we previously reported that agonist-stimulated levels of CTGF mRNA are reduced in TPA-treated IMR-90 myofibroblasts (12) presumably through modulation of PKC activity. Direct phosphorylation of Na\(^{+}/Ca\(^{2+}\) exchanger isoform 1 by PKC has recently been reported in rat myocytes (22). We now report that basal Ca\(^{2+}\) levels are not changed, and B1 receptor activation with des-Arg\(^{10}\)-kallidin does not induce an increase of cytosolic Ca\(^{2+}\) in TPA-treated myofibroblasts (Fig. 4A). In contrast, the B2 receptor-mediated increase of cytosolic Ca\(^{2+}\) is only slightly diminished in TPA-treated myofibroblasts (Fig. 4B). We also examined the effects of the PKC inhibitors calphostin C and chelerythrine on the des-Arg\(^{10}\)-kallidin-stimulated increase of cytosolic Ca\(^{2+}\). Fifteen-minute preincubation with either 500 nM calphostin C or 1 \(\mu M\) chelerythrine does not alter basal cytosolic Ca\(^{2+}\) levels and does not affect the B1 receptor-mediated increases of cytosolic Ca\(^{2+}\) (data not shown). These findings suggest that PKC modulates Na\(^{+}/Ca\(^{2+}\) exchanger activity and are consistent with other observations demonstrating down-regulation of Na\(^{+}/Ca\(^{2+}\) exchanger activity by PKC (34, 35). Thus, it appears that PKC regulates the exchanger, which in turn regulates CTGF production. However, at the moment we do not know which PKC isoform(s) regulate the exchanger nor which Na\(^{+}/Ca\(^{2+}\) exchanger isoform(s) mediate the observed Ca\(^{2+}\) fluxes.

Regulation of CTGF and α1(I) Collagen mRNAs by Extracellular Ca\(^{2+}\) —The involvement of Ca\(^{2+}\) influx pathways in TGF-β1 signaling pathways has been proposed. Nesti et al. (36) described TGF-β1-stimulated, nifedipine-sensitive Ca\(^{2+}\) influx in human osteoblasts. McGowan et al. (37) reported that TGF-β1 stimulated a Ca\(^{2+}\) influx in SV40-transformed murine mesangial cells via cell membrane-associated inositol 1,4,5-trisphosphate receptors. We hypothesized that Ca\(^{2+}\) metabolism modulates TGF-β1 signaling in human lung myofibroblasts. In Fig. 5, we demonstrate that TGF-β1 induces a gradual rise in cytosolic Ca\(^{2+}\) that continues to increase for as long as 40 min after stimulation (filled triangles), at which time the data accumulation was terminated. In contrast, there is no increase of cytosolic Ca\(^{2+}\) in untreated myofibroblasts (filled diamonds). The rise in cytosolic Ca\(^{2+}\) induced by TGF-β1 is abrogated in the absence of extracellular Ca\(^{2+}\) (data not shown). Although the kinetics of the TGF-β1-induced increase in cytosolic Ca\(^{2+}\) are distinct from those observed following B1 receptor activation, we examined the effects of KB-R7943 treatment on TGF-β1-induced increase of cytosolic Ca\(^{2+}\). KB-R7943 blocked the rise in cytosolic Ca\(^{2+}\) in response to TGF-β1 (open circles). These data strongly suggest that the TGF-β1-stimulated increase of cytosolic Ca\(^{2+}\) is also mediated by Ca\(^{2+}\) entry mode operation of the Na\(^{+}/Ca\(^{2+}\) exchanger.

We then examined the role of extracellular Ca\(^{2+}\) in the TGF-β1-stimulated increase of CTGF mRNA. Myofibroblasts were incubated in nominally Ca\(^{2+}\)-free DMEM and stimulated with TGF-β1. Basal expression of CTGF is not altered in Ca\(^{2+}\)-free DMEM; however, both the des-Arg\(^{10}\)-kallidin and TGF-β1-stimulated increases of CTGF mRNA are attenuated (Fig. 6). These findings are consistent with a role for the Na\(^{+}/Ca\(^{2+}\) exchanger mediating CTGF mRNA stimulation by both des-Arg\(^{10}\)-kallidin and TGF-β1. We also examined the role of extracellular Ca\(^{2+}\) in α1(I) collagen mRNA expression by incubating the myofibroblasts in nominally Ca\(^{2+}\)-free DMEM. As observed with CTGF mRNA, the TGF-β1-stimulated increase of α1(I) collagen mRNA is attenuated in myofibroblasts incubated in nominally Ca\(^{2+}\)-free DMEM (Fig. 7).

To determine the effect of removal of extracellular Ca\(^{2+}\) on TGF-β-stimulated responses, we transfected IMR-90 myofibroblasts with the TGF-β-responsive, Smad-mediated luciferase reporter 3TP-LUX (38). The myofibroblasts were stimulated with TGF-β1 in DMEM or in nominally Ca\(^{2+}\)-free DMEM.
Fig. 2. Effect of extracellular Na\(^+\) in kinin-stimulated cytosolic Ca\(^{2+}\) increase. Myofibroblasts were grown to confluence on coverslips, incubated with DMEM supplemented with 0.4% FBS for 24 h, loaded with fluo-2 (as described), and stimulated with 10 nM des-Arg\(^{10}\)-kallidin (A, added at arrow) or 1 nM kallidin (B, added at arrow) in nominally Na\(^+\)-free medium or complete medium as indicated. The values are 340/380 nm emission ratios. The increase in cytosolic Ca\(^{2+}\) is a reflection of the increase in fluorescence emission ratio (expressed in arbitrary units) when cells are excited at 340/380 nm as described. The tracings are representative of three independent experiments.

TGF-β1 stimulated an increase of luciferase expression in both DMEM and Ca\(^{2+}\)-free DMEM (Fig. 8). These findings suggest that removal of extracellular Ca\(^{2+}\) does not alter Smad-mediated TGF-β1 signaling. We conclude that the attenuation of the TGF-β1-stimulated increase of CTGF mRNA by the absence of extracellular Ca\(^{2+}\) is not Smad-mediated.

Effect of Na\(^+\)/Ca\(^{2+}\) Exchanger Inhibitors on CTGF mRNA in Human Myofibroblasts—To demonstrate that the B1 receptor-induced stabilization of CTGF mRNA is mediated through the Na\(^+\)/Ca\(^{2+}\) exchanger, we treated MG-63 cells with KB-R7943. In KB-R7943-treated osteoblasts, basal expression of CTGF mRNA did not change. However, the TGF-β1-stimulated increase of CTGF mRNA was attenuated in KB-R7943-treated cells (Fig. 10). Because CTGF was originally described in endothelial cells, we examined changes in CTGF mRNA in TGF-β1-stimulated human microvascular endothelial cells that were treated with KB-R7943. As with the osteoblasts, treatment with KB-R7943 did not alter basal expression of CTGF mRNA, but the TGF-β1-stimulated increase of CTGF was attenuated (data not shown).

Effect of Na\(^+\)/Ca\(^{2+}\) Exchanger Inhibitors on α(I) Collagen and a Smooth Muscle Actin Expression—We also examined the effect of inhibiting Ca\(^{2+}\) entry mode operation of the Na\(^+\)/Ca\(^{2+}\) exchanger on α(I) collagen mRNA. Northern blot analyses indicate that, in KB-R7943-treated myofibroblasts, basal expression of α(I) collagen mRNA and the TGF-β1-stimulated increase of α(I) collagen mRNA were strongly down-regulated (Fig. 11). To further characterize this modulation of the myofibroblast phenotype, we examined the expression of α smooth muscle actin mRNA in the presence of KB-R7943. IMR-90 myofibroblasts preincubated with KB-R7943 were stimulated with TGF-β1, and changes in α smooth muscle actin mRNA were monitored by real time PCR (Fig. 12) and confirmed by Northern blot analyses (data not shown). In untreated myofibroblasts, TGF-β1 stimulated an increase of expression of α smooth muscle actin mRNA. In KB-R7943-treated myofibroblasts, basal levels and TGF-β1-stimulated levels of α smooth muscle actin mRNA were strongly down-regulated. These results strongly suggest that inhibition of Na\(^+\)/Ca\(^{2+}\) exchanger activity down-regulates the myofibroblast phenotype.

The mechanisms by which Na\(^+\)/Ca\(^{2+}\) exchanger activity regulates CTGF, α(I) collagen, and α smooth muscle actin mRNA levels are unclear. The Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, calcineurin, has been shown to participate in TGF-β1-stimulated extracellular matrix accumulation (42). In turn, calcineurin has been shown to regulate transcription of Na\(^+\)/Ca\(^{2+}\) exchanger isofoms (43). However, we did not observe changes in CTGF, α(I) collagen, and α smooth muscle actin mRNA levels in quiescent and TGF-β1-stimulated myofibroblasts that were treated for 4 or 16 h with the calcineurin inhibitor, cyclosporin A (data not shown). Further experimentation is required to definitively conclude that calcineurin does not participate in these responses.

**DISCUSSION**

In this report, we characterize the role of the Na\(^+\)/Ca\(^{2+}\) exchanger in fibrogenesis. Using des-Arg\(^{10}\)-kallidin- and TGF-
The tracings are representative of three independent experiments.

Myofibroblasts were grown to confluence on coverslips, incubated in DMEM supplemented with 0.4% FBS for 24 h, and stimulated with 1 ng/ml TGF-β for 16 h in nominally Ca²⁺-free DMEM (−Ca²⁺) or complete DMEM as indicated. Total RNA was harvested and Northern blotted (10 μg/lane) with probes for α1(I) collagen and GAPDH as described under “Materials and Methods.” The values are representative of two experiments.

Fig. 8. Effect of nominally Ca²⁺-free medium on TGF-β-stimulated luciferase activity. Myofibroblasts were transfected with 3TP-LUX using Lipofectamine 2000 as described under “Materials and Methods.” The medium was replaced with Ca²⁺-free DMEM (−Ca²⁺), and the cultures were stimulated with 1 ng/ml TGF-β1 for 6 h as indicated. The results are the means of triplicates ± standard deviation; the values are representative of two experiments.

β1-stimulated human myofibroblasts, we investigated the role of the Na⁺/Ca²⁺ exchanger in early agonist-stimulated signal transduction events. Our results demonstrate that activation of the kinin B1 receptor induces an increase of cytosolic Ca²⁺ that is mediated through Ca²⁺ entry mode operation of the Na⁺/Ca²⁺ exchanger. We previously reported that activation of the B1 receptor (but not the B2 receptor) increases the stability of CTGF mRNA (12). The present results indicate that the B1 receptor-induced increase of CTGF mRNA is blocked by KB-R7943. From these results, we conclude that Ca²⁺ entry mode operation of the Na⁺/Ca²⁺ exchanger is required for the B1 receptor-mediated increase in CTGF mRNA stability. These results suggest that the Na⁺/Ca²⁺ exchanger is functionally coupled to B1 receptor activation and that CTGF mRNA stability is modulated by cation homeostasis. It is important to note that a rise in cytosolic Ca²⁺ per se is not sufficient to induce an increase in CTGF mRNA. Although increased cytosolic Ca²⁺ is required for the TGF-β1- and the B1 receptor-stimulated increases of CTGF mRNA, kallidin, which also mobilizes intracellular Ca²⁺, does not induce an increase in CTGF mRNA levels.
**Fig. 9. Effect of Na+/Ca2+ exchange inhibitors on CTGF mRNA levels.** Confluent human myofibroblasts were incubated in DMEM supplemented with 0.4% FBS for 24 h and pretreated for 5 min with 10 μM amiloride, 15 μM DCB, or 20 μM KB-R7943 in DMEM supplemented with captopril (10 μM), phosphoramidon (1 μM), and DL-2-mercaptopropionic acid (1 μM). The cultures were subsequently stimulated with 100 nM des-Arg10-kallidin, 100 nM kallidin, or 1 ng/ml TGF-β1 for 3 h as indicated. Total RNA was harvested and Northern blotted (10 μg/lane) with probes for CTGF and GAPDH as described under “Materials and Methods.” The values are representative of three independent experiments.

**Fig. 10. Effect of KB-R7943 on CTGF mRNA in osteoblasts.** Confluent human myofibroblasts were incubated in DMEM supplemented with 0.4% FBS for 24 h and pretreated with 20 μM KB-R7943 for 5 min. The cultures were stimulated with 1 ng/ml TGF-β1 for 4 h as indicated. The results are the relative fold change of CTGF mRNA transcripts obtained by real time PCR analysis of three independent experiments normalized with GAPDH as described. The values are the averages of relative fold changes plus standard error. *, p < 0.05 comparing KB-R7943 + TGF-β1-treated levels with TGF-β1-treated levels.

**Fig. 11. Effect of KB-R7943 on α1(I) collagen mRNA.** Confluent human myofibroblasts were incubated in DMEM supplemented with 0.4% FBS and pretreated with 20 μM KB-R7943 for 5 min in DMEM. The cultures were then stimulated with 1 ng/ml TGF-β1 for 16 h as indicated. Total RNA was harvested and Northern blotted (10 μg/lane) with probes for α1(I) collagen and GAPDH as described under “Materials and Methods.” The values are representative of two experiments.

**Fig. 12. Effect of KB-R7943 on α smooth muscle actin mRNA.** Confluent human myofibroblasts were incubated in DMEM supplemented with 0.4% FBS and pretreated with 20 μM KB-R7943 for 5 min. The cultures were stimulated with 1 ng/ml TGF-β1 for 16 h as indicated. The results are the relative fold change of α smooth muscle actin (SMA) mRNA transcripts obtained by real time PCR analysis of three independent experiments normalized with GAPDH as described. The values are the averages of relative fold changes plus standard error. *, p < 0.001 compared with untreated control levels.

**Na+/Ca2+ Exchanger Regulates CTGF mRNA Expression**

TGF-β1 also induces KB-R7943-sensitive increases in cytosolic Ca2+ and CTGF mRNA, suggesting that TGF-β1 induces an increase of CTGF mRNA stability in addition to the previously reported increase in CTGF transcription. However, regulation of TGF-β1-stimulated CTGF transcription by Ca2+ entry mode operation of the Na+/Ca2+ exchanger cannot be ruled out at this moment. Furthermore, basal expression of CTGF mRNA is not sensitive to inhibition of Ca2+ entry mode operation of the Na+/Ca2+ exchanger. Taken together, these results suggest that in quiescent human lung myofibroblasts expression of CTGF may be mediated through both TGF-β-dependent and TGF-β-independent mechanisms. These results are important because they show that two divergent agonists modulate CTGF mRNA levels via convergence on Ca2+ entry mode operation of the Na+/Ca2+ exchanger, suggesting a commonality for this mechanism in CTGF mRNA stability. Furthermore Na+/Ca2+ exchanger activity appears to play a central role in the TGF-β-stimulated increase of CTGF mRNA in human lung myofibroblasts, microvascular endothelial cells, and osteoblast-like cells.

We previously reported that TGF-β1 stimulates CTGF transcription (12), and others have shown this process to be mediated via Smad signaling (40, 41). The luciferase experiments demonstrate that, in TGF-β1-stimulated myofibroblasts, Smad-mediated responses are not significantly affected by removal of extracellular Ca2+, suggesting that Smad-mediated signaling is not affected by changes in Na+/Ca2+ exchanger activity. In addition, these experiments demonstrate that the myofibroblasts are responsive to TGF-β1 stimulation in the absence of extracellular Ca2+. Thus, it appears that both genomic and nongenomic mechanisms modulate the TGF-β-stimulated increase of CTGF mRNA.

Quiescent IMR-90 myofibroblasts express α1(I) collagen and a smooth muscle actin, genes characterizedly expressed in myofibroblasts. We show that inhibition of Ca2+ entry mode operation of the Na+/Ca2+ exchanger attenuates basal and TGF-β1-stimulated levels of α1(I) collagen and a smooth muscle actin mRNAs, suggesting that Ca2+ entry mode operation of the Na+/Ca2+ exchanger is required for maintenance of the myofibroblasts phenotype. Together, these findings suggest that the Na+/Ca2+ exchanger, functioning as a Ca2+ influx pathway, plays an important and novel role in fibrogenesis. Consistent with this hypothesis, Stains and Gay (39) have recently shown by immunocytochemistry that the secretion of type I collagen and bone sialoproteins is reduced in KB-R7943-treated osteoblasts. A relationship among TGF-β1 fibrogenesis, and the Na+/Ca2+ exchanger has been suggested by
our data demonstrate that regulation of CTGF expression by the Na+/Ca2+ exchanger in hepatic stellate cells. Carrillo et al. (45) showed that TGF-β increases Na+/Ca2+ exchanger mRNA levels via a PKC-sensitive pathway. In vivo induction of fibrosis in rats with CCl4 was also associated with an increase in liver Na+/Ca2+ exchanger mRNA. Our studies extend these observations, highlighting the necessity of the Ca2+ entry mode operation of the Na+/Ca2+ exchanger for CTGF mRNA expression and fibrogenesis in human lung myofibroblasts. Taken together, these results suggest that Na+/Ca2+ exchanger expression is a biomarker for fibrosis and a potential target for intervention.

In conclusion, our data suggest that Ca2+ entry mode operation of the Na+/Ca2+ exchanger plays an integral role in the expression of CTGF, α(I) collagen, and α-smooth muscle actin, implicating an important role for the Na+/Ca2+ exchanger in the maintenance of the myofibroblast phenotype. Furthermore, our data demonstrate that regulation of CTGF expression by the Na+/Ca2+ exchanger is a mechanism shared by various cell types and that calcium metabolism plays an important role in the expression of genes associated with fibrosis. Based on these findings we speculate that targeting Na+/Ca2+ exchanger activity may be an efficacious intervention for the treatment of fibrosis.

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