TGF-β-induced Ca\(^{2+}\) influx involves the type III IP\(_3\) receptor and regulates actin cytoskeleton

TRACY A. McGOWAN,1 MUNISWAMY MADESH,2 YANQING ZHU,1 LEWEI WANG,1 MARK RUSSO,1 LEO DEELMAN,3 ROB HENNING,3 SURESH JOSEPH,2 GYORGY HAJNOCZKY,2 AND KUMAR SHARMA1

1Dorrance Hamilton Laboratories, Division of Nephrology, Department of Medicine, and 2Department of Anatomy, Pathology, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and 3Department of Clinical Pharmacology, University of Groningen, 9713 AV Groningen, The Netherlands

Received 15 August 2001; accepted in final form 14 November 2001

McGowan, Tracy A., Muniswamy Madesh, Yanqing Zhu, Lewei Wang, Mark Russo, Leo Deelman, Rob Henning, Suresh Joseph, Gyorgy Hajnoczky, and Kumar Sharma. TGF-β-induced Ca\(^{2+}\) influx involves the type III IP\(_3\) receptor and regulates actin cytoskeleton. Am J Physiol Renal Physiol 282: F910–F920, 2002. First published November 20, 2001; 10.1152/ajprenal.00252.2001.—Ca\(^{2+}\) influx has been postulated to modulate the signaling pathway of transforming growth factor-β (TGF-β); however, the underlying mechanism and functional significance of TGF-β-induced stimulation of Ca\(^{2+}\) influx are unclear. We show here that TGF-β stimulates Ca\(^{2+}\) influx in mesangial cells without Ca\(^{2+}\) release. The influx of Ca\(^{2+}\) is prevented by pharmacological inhibitors of inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) as well as specific antibodies to type III IP\(_3\)R (IP\(_3\)RIII) but not to type I IP\(_3\)R (IP\(_3\)RI). TGF-β enhances plasma membrane localization of IP\(_3\)RIII, whereas the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) preferentially translocates to the nucleus. Untreated mesangial cells exhibit actin filamentous protrusions on the cell surface, and treatment with TGF-β dramatically reduces this pattern. The alterations in the actin cytoskeleton by TGF-β are dependent on TGF-β-induced Ca\(^{2+}\) influx. These studies identify a novel pathway by which TGF-β regulates Ca\(^{2+}\) influx and induces cytoskeletal alterations.

mesangial cells; signaling; filipodia; inositol 1,4,5-trisphosphate; transforming growth factor-β

TRANSFORMING GROWTH FACTOR-β (TGF-β) has been implicated in disease processes as diverse as cancer, inflammatory diseases, and chronic fibrotic disorders. This is partly due to the observation that TGF-β is a multifunctional cytokine that can inhibit cellular proliferation, enhance matrix accumulation, and suppress inflammation. The signaling pathway is initiated by binding of ligand to the TGF-β type II receptor (TβRII), and the complex then interacts with the type I receptor (TβRI). Cross phosphorylation of TβRI by the constitutive serine-threonine kinase of TβRII activates the serine-threonine kinase of TβRI. The activated TβRII phosphorylates the receptor-regulated Smads (Smad2 and Smad3), thus enhancing association with Smad4 and migration to the nucleus (24). Although the role of the Smad pathway is critical in mediating inhibition of cell proliferation in many cell types, the role of Smads in mediating other characteristics of TGF-β such as matrix production is less clear (38). Other pathways that have been implicated in TGF-β signaling include the mitogen-activated protein kinase pathway (10) and the protein kinase A pathway (40), although their respective roles are less well defined.

An alternative pathway that may be involved in TGF-β signaling is regulation of intracellular Ca\(^{2+}\). Ca\(^{2+}\) regulation likely plays an important role in modulating TGF-β signaling as calmodulin, in the presence of Ca\(^{2+}\), binds Smad2, Smad3, and Smad4 (43). Furthermore, overexpression of calmodulin reduced the effect of TGF-β on 3TP-Lux activity (43). The binding of calmodulin to Smad2 occurs at specific sites in the Mad homology (MH)1 domain and inhibits Smad2-dependent activity (33). Additionally, activated Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaM kinase II) phosphorylates Smad2 and Smad4 and may inhibit their activity (41). The basis for activation of calmodulin may be due to an increase in intracellular cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_c\) induced by TGF-β. Previously it was established that TGF-β stimulates the [Ca\(^{2+}\)]\(_c\) increase in fibroblasts (1, 29); however, the mechanisms underlying the TGF-β-induced [Ca\(^{2+}\)]\(_c\) increase have not been described. In addition, a direct functional role for the TGF-β-induced [Ca\(^{2+}\)]\(_c\) increase independent of the Smad pathway has not been identified.

The two sources of increasing [Ca\(^{2+}\)]\(_c\) are release of Ca\(^{2+}\) from internal stores and influx of Ca\(^{2+}\) from the extracellular space via channels located in the plasma membrane. In response to a variety of growth factors (e.g., platelet-derived, epidermal, and hepatocyte...
growth factors) and vasoactive agonists (e.g., angiotensin II and endothelin) there is an initial rise in \([\text{Ca}^{2+}]_c\) via the inositol 1,4,5-trisphosphate (IP_3)-gated intracellular \(\text{Ca}^{2+}\) channel (IP_3R) (2). The generation of IP_3 is mediated by the activation of phospholipase C isoforms by tyrosine kinase-linked receptors or G protein linked receptors. The COOH-terminal domain of the IP_3R forms a channel in the membrane of intracellular \(\text{Ca}^{2+}\) storage sites and upon IP_3 binding to the NH_2-terminal region of the IP_3R allows stored \(\text{Ca}^{2+}\) to enter the cytosol (30). Consequent to the release of stored intracellular \(\text{Ca}^{2+}\), there is a secondary influx of \(\text{Ca}^{2+}\) from the extracellular space. This influx of \(\text{Ca}^{2+}\) occurs via store-operated \(\text{Ca}^{2+}\) channels (SOC; Ref. 31) and appears to involve IP_3Rs, possibly in association with another \(\text{Ca}^{2+}\) channel of the transient-receptor-potential channel family (TRP; Refs. 22, 32). The IP_3Rs exist as three isoforms, and although they have different IP_3 binding affinities and may exist in different spatial compartments (30), their distinct functional roles with respect to influx of \(\text{Ca}^{2+}\) are unclear.

The involvement of IP_3Rs in TGF-\(\beta\) signaling was suggested by our prior study (36), wherein we found that short-term TGF-\(\beta\) treatment (5–30 min) induced phosphorylation of the type I IP_3R (IP_3RI) in mesangial cells. In the present study, we evaluated the roles of the IP_3RI and type III IP_3R (IP_3RIII) in mediating TGF-\(\beta\)-induced \(\text{Ca}^{2+}\) influx. Furthermore, we assessed the impact of TGF-\(\beta\)-induced \(\text{Ca}^{2+}\) influx in relation to cytoskeletal rearrangements in mesangial cells.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** SV40 transformed murine mesangial cells (MMCs) were grown in DMEM with 10% serum and were rested overnight in serum-free DMEM before being exposed to 10 ng/ml of TGF-\(\beta\) (R&D Systems, Minneapolis, MN). All reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted.

**Antibodies.** The following antibodies were used: rabbit polyclonal anti-IP_3RI (Affinity Bioreagents, Golden, CO), murine monoclonal anti-NH_2-terminal IP_3RIII (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-COOH-terminal IP_3RIII (15), murine monoclonal anti-sarcoplasmic-endoplasmic reticulum \(\text{Ca}^{2+}\) ATPase (SERCA; Affinity Bioreagents), and murine monoclonal anti-\(\beta\)-actin (Sigma).

**\(\text{Ca}^{2+}\) measurements.** To measure \([\text{Ca}^{2+}]_c\) fluorescence, imaging measurements of \([\text{Ca}^{2+}]_c\), in MMCs were performed as previously described (35). After cells were grown on coverslips and loaded with fura 2-AM, TGF-\(\beta\)-1 was added in the presence of 2 mM CaCl_2 or in the absence of \(\text{Ca}^{2+}\) in the extracellular medium. Time courses of \([\text{Ca}^{2+}]_c\) in individual cells were calculated from fluorescence imaging pairs obtained using 340- and 380-nm excitation (10-nm bandwidth) with a broadband emission filter passing 460–600 nm. To ensure that intracellular stores of \(\text{Ca}^{2+}\) were not depleted in the low-\(\text{Ca}^{2+}\) media, thapsigargin (2 \(\mu\)M; Alexis Biochemicals, San Diego, CA) was added to cells in the presence of 2 mM CaCl_2 or in the absence of \(\text{Ca}^{2+}\) in the extracellular medium, and \([\text{Ca}^{2+}]_c\) was measured. Studies were carried out using 3 different cell cultures during 3 parallel experiments on each occasion, and 30–50 cells were monitored in each experiment.

**Fig. 1.** Transforming growth factor (TGF-\(\beta\)) stimulates \(\text{Ca}^{2+}\) entry in mesangial cells. A: a representative group of fura 2-AM-loaded murine mesangial cells under control conditions and with TGF-\(\beta\) treatment (10 ng/ml) showing increased intracellular cytosolic \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}]_c\)) after TGF-\(\beta\) treatment. Elevation in \([\text{Ca}^{2+}]_c\), appears as green-to-red shift in the green/red overlay images (380: 340 nm excitation; width of frame corresponds to 121 \(\mu\)m). B: time course of TGF-\(\beta\)-induced rise in \([\text{Ca}^{2+}]_c\), was evaluated from 3 sets of experiments with fura 2-AM loaded cells. Top: a rise in \([\text{Ca}^{2+}]_c\), was noted after 5 min of TGF-\(\beta\)-treatment and continued for 30 min. Bottom: in the absence of extracellular \(\text{Ca}^{2+}\), TGF-\(\beta\) treatment failed to evoke an increase in \([\text{Ca}^{2+}]_c\). [\(\text{Ca}^{2+}]_c\), extracellular \(\text{Ca}^{2+}\) concentration; R_fura, 340/380 ratio.

To measure \(\text{Ca}^{2+}\) influx, cells were grown on six-well plates, washed with \(\text{Ca}^{2+}\)-free physiological saline solution (PSS) buffer that contained (in mM) 145 NaCl, 5 KCl, 1 MgCl_2, 10 glucose, and 5 HEPES, pH 7.4, and were then placed in PSS with 0.12 mM CaCl_2 as previously described (1). Cells were treated with TGF-\(\beta\) for various amounts of time. For experiments to study the effects of heparin, xestospongin, or antibodies, the cells were pretreated with TGF-\(\beta\) for 30 min before addition of IP_3R inhibitors or antibodies for the last 5 min. Cells were then labeled with 1 \(\mu\)Ci of \(^{45}\text{Ca}^{2+}\) well for the final 30 s of incubation before being washed and harvested in lysis buffer. \(^{45}\text{Ca}^{2+}\) was counted using a liquid scintillation counter. Experiments were performed three times, and samples were analyzed in triplicate.
Confocal microscopy. MMCs grown on coverslips were exposed to TGF-β1 for 5–30 min. Immunostaining and confocal analysis for IP₃RI and IP₃RIII were performed as previously described (35). For SERCA, a double-antibody method with AlexaFluor was utilized to amplify staining. Slides were visualized via confocal microscopy, and representative regions were photographed. Confocal analysis was performed with 1-μm sections and sequential z-images to ascertain intracellular locations of proteins. As a negative control, cells stained only with secondary antibody showed minimal background fluorescence. The actin cytoskeleton was visualized using rhodamine-phalloidin (Molecular Probes) staining on control and TGF-β-treated cells according to the manufacturer’s instructions.

Biotinylation and analysis of plasma membrane proteins. Confluent MMCs grown on 100-mm dishes under control conditions or with TGF-β treatment were biotinylated with 10 ml of 0.5 mg/ml of the membrane-impermeant biotinylating reagent sulfo-NHS-biotin (sulfo-NHS-biotin; Pierce Chemical, Rockford, IL) for 30 min at 4°C. After excess NHS-biotin was washed off, cells were lysed with 1 ml of 1% (wt/vol) Triton X-100 in 10 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A, and 10 μM leupeptin for 30 min at 4°C. The lysates were incubated with streptavidin-treated beads for 6–12 h at 4°C to recover biotinylated proteins. The recovered proteins were then run on a 7% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with the IP₃RI (1:2,000 dilution), IP₃RIII (1:5,000 dilution), or SERCA 2 antibodies. To control for an overestimate in cellular protein levels of IP₃RI and IP₃RIII, total cell lysates were obtained from control and TGF-β-treated cells with lysis buffer containing 1% Triton X-100. Protein (20 μg) was analyzed as above and standardized by immunoblotting the membrane with anti-β-actin antibody.

To determine whether extracellular IP₃R antibodies would recognize their respective antigens, MMCs were plated on 100-mm dishes in DMEM that contained 10% fetal calf serum. After reaching 90% confluence, cells were rested overnight in serum-free DMEM. The rabbit polyclonal anti-IP₃RI (Affinity Bioreagents) or murine monoclonal anti-NH₂-terminal IP₃RIII (Transduction Laboratories) antibodies were added to cells at a 1:500 dilution in 5 ml of serum-free DMEM for 30 min at 37°C. Unbound antibodies were washed off three times with ice-cold PBS, and 1 ml of 0.25 M Tris·HCl (pH 7.8) was added before cells were harvested with a cell scraper into 1.5-ml Eppendorf tubes. Subsequently cells were subjected to three cycles of freeze-thaw lysis. Protein lysate (600 μg) was then added to 70 μl of protein A-agarose and rotated overnight at 4°C. After centrifugation at 3,000 rpm for 5 min at 4°C, the pellet was washed 4× with wash buffer containing 0.1% (wt/vol) Triton X-100, 50 mM Tris·HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, and 0.02% (wt/vol) sodium azide. The pellet was then resuspended in sample buffer, boiled for 5 min, and loaded onto a 7% SDS-PAGE gel. Immunoblotting was performed as described.

Immunoelectron microscopy for IP₃RIII. MMCs were fixed in wells by removing the medium and adding a mixture of freshly prepared 2% paraformaldehyde and 1% glutaraldehyde.
hyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature. After washing and incubation for 2 h with the mouse monoclonal antibody for IP3RIII (1:2,000 dilution), the cells were incubated using the ABC method (Vectastain Elite ABC kit, Vector Laboratories). Subsequent to reacting with diaminobenzidine, a gold-substituted silver peroxidase enhancement reaction was performed to improve the visibility of the reaction product. After the incubation procedure, the cells were osmicated and dehydrated in a graded series of ethanol and propylene oxide and embedded in epon. The epon blocks were cut into ultrathin microsections of 60–75 nm and contrasted with uranyl acetate and lead citrate before being visualized.

Quantification of IP3RIII staining was performed by computerized quantification of staining of the peroxidase enhancement reaction product (ImagePro Plus 4.1, Media Cybernetics). For each group, at least six photographs taken from different cells were digitized at a resolution of 300 dpi (Agfa Snapscan 600 scanner). IP3RIII staining was quantified as the area of staining relative to the cell area investigated. Photographic material was quantified by an observer blinded to the experimental protocol. Computerized selection of stained areas was carefully checked by the observer to exclude any artifacts.

Statistical analysis. Results are expressed as means ± SE. Significance was determined by Student’s t-test or by ANOVA for cases with multiple comparisons. The variability within the groups was random. P < 0.05 was considered significant.

RESULTS

TGF-β increases Ca2+ influx without Ca2+ release in mesangial cells. To determine the time frame in which TGF-β has its maximal effect on Ca2+ influx, [Ca2+]c transients were measured in MMCs using fluorescence imaging with fura 2-AM loading. After 1 min of TGF-β treatment, there was no observable change in [Ca2+]c; however, after 25 min of TGF-β treatment, the majority of cells exhibited an increase in [Ca2+]c (Fig. 1A). The increase in [Ca2+]c began gradually, 5 min after the addition of TGF-β, and reached a plateau after 25 min (Fig. 1B). This pattern of a TGF-β-induced rise in [Ca2+]c is unique among growth factors and contrasts with the typical tyrosine kinase-linked or G protein-linked receptors that induce an IP3-mediated rapid rise in [Ca2+]c (2). In the absence of extracellular Ca2+,
there was no increase in [Ca\(^{2+}\)]\(_{c}\) with TGF-\(\beta\) (Fig. 1B), which suggests that Ca\(^{2+}\) was not being released from intracellular stores. To further confirm that TGF-\(\beta\) induced Ca\(^{2+}\) influx, MMCs were treated with TGF-\(\beta\) for increasing amounts of time and the cells were exposed to \(^{45}\)Ca\(^{2+}\) for the last 30 s of treatment (Fig. 2A). There was a trend for TGF-\(\beta\)-induced \(^{45}\)Ca\(^{2+}\) influx after 5 and 15 min; however, this did not reach statistical significance. TGF-\(\beta\)-induced \(^{45}\)Ca\(^{2+}\) influx was significant after 30 min.

The roles of SOC and voltage-gated Ca\(^{2+}\) channels were evaluated after TGF-\(\beta\) exposure. TGF-\(\beta\) did not stimulate IP\(_3\)-mediated Ca\(^{2+}\) release, as an increase in [Ca\(^{2+}\)]\(_{c}\) did not occur in the absence of extracellular Ca\(^{2+}\) (Fig. 1B). To ensure that internal stores of Ca\(^{2+}\) were not depleted in the Ca\(^{2+}\)-free condition, thapsigargin-induced Ca\(^{2+}\) release was monitored and found not to be significantly affected under either medium (340:380 ratio of 0.64 \(\pm\) 0.06 at baseline in 2 mM Ca\(^{2+}\) bath increasing to 1.18 \(\pm\) 0.30 with thapsigargin, and 340:380 ratio of 0.49 \(\pm\) 0.04 at baseline in Ca\(^{2+}\)-free bath increasing to 1.02 \(\pm\) 0.25 with thapsigargin). In addition, we previously found that IP\(_3\) levels did not increase after TGF-\(\beta\) treatment in MMCs (35). The role of voltage-gated Ca\(^{2+}\) channels has been implicated in mediating TGF-\(\beta\)-induced Ca\(^{2+}\) influx (12); however, [Ca\(^{2+}\)]\(_{c}\) was not increased in MMCs with exposure to 20 or 40 mM KCl as measured by \(^{45}\)Ca\(^{2+}\) or by fura 2 imaging (data not shown), which suggests that classic voltage-gated channels were not operating in our cells.

Ca\(^{2+}\) influx induced by TGF-\(\beta\) involves IP\(_3\)RIII. Plasma membrane-bound IP\(_3\)Rs have been considered to play a role in Ca\(^{2+}\) influx (17, 20); therefore, we studied the effects of various inhibitors of IP\(_3\)Rs in mediating TGF-\(\beta\)-induced \(^{45}\)Ca\(^{2+}\) influx. Addition of heparin, which interferes with IP\(_3\) binding and activation of the IP\(_3\)Rs (8, 19), inhibited TGF-\(\beta\)-induced Ca\(^{2+}\) influx (Fig. 2B). However, heparin may also inhibit Ca\(^{2+}\) channels on the cell surface (27) and may interact with TGF-\(\beta\) itself (21). Xestospongin has been described as being a highly specific, cell-permeable inhibitor of Ca\(^{2+}\) flux via the IP\(_3\)R (7). Addition of a low concentration of xestospongin (2 \(\mu\)M) also led to attenuation of TGF-\(\beta\)-induced Ca\(^{2+}\) influx (Fig. 2B). Results of these studies suggest that IP\(_3\)Rs play a critical role in TGF-\(\beta\)-induced Ca\(^{2+}\) influx.

Mesangial cells have a predominance of IP\(_3\)RI and IP\(_3\)RIII isoforms (28, 35, 37), and both of these isoforms have been described to be located at or near the plasma membrane in various cell types (6, 17, 39). Therefore, we focused on IP\(_3\)RI and IP\(_3\)RIII as candidate proteins that may facilitate Ca\(^{2+}\) influx on stimulation with TGF-\(\beta\). To determine whether an isoform of IP\(_3\)R at the cell surface is required for the effects of TGF-\(\beta\) on Ca\(^{2+}\) influx, the \(^{45}\)Ca\(^{2+}\)-influx studies were repeated in the presence or absence of extracellular antibodies specific for IP\(_3\)RI or IP\(_3\)RIII (Fig. 2C). As seen in Fig. 2C, TGF-\(\beta\)-mediated Ca\(^{2+}\) influx is prevented by an antibody directed against either the COOH or NH\(_2\) terminus of IP\(_3\)RII but not with an antibody to IP\(_3\)RI.

TGF-\(\beta\) enhances localization of IP\(_3\)RII to plasma membrane. To assess whether TGF-\(\beta\) alters the intracellular distribution pattern of IP\(_3\)R isoforms to promote Ca\(^{2+}\) influx, we analyzed the same cell system by IP\(_3\)R immunostaining. Under control conditions, both IP\(_3\)RI and IP\(_3\)RIII are primarily present in the cytoplasm (Fig. 3, A and B). After TGF-\(\beta\) treatment, IP\(_3\)RI takes on a more vesicular staining pattern at or near the nucleus (Fig. 3A). In contrast, IP\(_3\)RIII exhibits increased intensity at the plasma membrane after TGF-\(\beta\) treatment (Fig. 3B). Interestingly, both IP\(_3\)R
isoforms appeared to have overall greater intensity of immunostaining after TGF-β treatment. The divergence of distribution of the two IP₃R isoforms suggests that the isoforms may be associated with different subcellular structures. We therefore assessed the location of another endoplasmic reticulum Ca²⁺ transport protein, SERCA, under control conditions and with TGF-β treatment (Fig. 3C). In the basal state, SERCA is in a vesicular perinuclear distribution, whereas after TGF-β treatment, SERCA moves into the nucleus.

To confirm that there is an increased amount of IP₃RIII at the plasma membrane after exposure to TGF-β, plasma-membrane proteins were isolated with sulfo-NHS-biotin to biotinylate the cell-surface proteins (6, 39). Plasma membrane proteins were assessed for the presence of IP₃RI, IP₃RIII, and SERCA. At baseline, both IP₃RI and IP₃RIII are present in plasma membrane proteins and are further increased after TGF-β treatment (Fig. 4, A and B). However, SERCA was not found in plasma membrane proteins (Fig. 4C), which suggests that pools of IP₃Rs exist that are spatially distinct from SERCA. Overall levels of IP₃RI and IP₃RIII did not vary in total cell lysates with TGF-β treatment (Fig. 4, D–F).

As previously noted in Fig. 2C, addition of IP₃RIII antibodies to intact cells inhibited TGF-β-induced Ca²⁺ influx. Therefore, it would be presumed that extracellular antibodies are able to recognize the IP₃Rs located on the cell surface. To directly test this concept, MMCs were exposed to anti-IP₃RI or anti-IP₃RIII antibodies and vigorously washed with PBS before lysing of the cells. The cell lysates were then incubated with protein A-agarose beads, and the immunoprecipitate was analyzed by Western blot analysis. As indicated in Fig. 5, only the lysates from MMCs exposed to extracellular antibodies exhibited a band for the respective IP₃R. This result suggests that the extracellular antibodies do indeed recognize their respective epitopes in intact MMCs.

Immunoelectron microscopy for IP₃RIII. To better characterize the spatial intracellular distribution of IP₃RIII in mesangial cells under control and TGF-β treatment conditions, immunoelectron microscopy was performed with antibody to the COOH-terminal region of IP₃RIII (Fig. 6). Under control conditions, IP₃RIII is distributed diffusely in the cytosol with occasional intense immunostaining at the plasma membrane. On TGF-β treatment, there was a marked increase in IP₃RIII at the plasma membrane. Quantitation of
IP3RIII staining revealed a 10-fold increase of plasma membrane staining of IP3RIII with TGF-β treatment (pixels/area ratio: control, 0.05 ± 0.02 vs. TGF-β treated, 0.48 ± 0.08; \(P = 0.0001\)).

**TGF-β-induced Ca\(^{2+}\) influx regulates effect of TGF-β on actin cytoskeleton.** As Ca\(^{2+}\) has been closely associated with regulation of the actin cytoskeleton (34), we questioned whether TGF-β-induced Ca\(^{2+}\) influx was associated with alterations in the actin cytoskeleton. Under control conditions, the majority of rhodamine-phalloidin-stained mesangial cells present exhibited numerous actin filament protrusions, or filopodia, on the plasma membrane (Fig. 7). With administration of TGF-β, there were a reduced number of filopodia present on the cell surface, which was noted in the majority of cells at 15 and 30 min (Fig. 7). To evaluate whether the TGF-β-induced effect on the actin cytoskeleton was related to Ca\(^{2+}\) influx, the studies were repeated in the presence and absence of extracellular Ca\(^{2+}\). Under basal conditions, in the presence of extracellular Ca\(^{2+}\), 92.3% of the cells exhibited filopodia (Figs. 8 and 9). After 15 min of exposure to TGF-β, the percentage of cells exhibiting actin filopodia were markedly reduced (7.4%). This effect of TGF-β was dependent on extracellular Ca\(^{2+}\), as cells incubated in Ca\(^{2+}\)-free media failed to elicit a TGF-β effect on filopodia (91.8% untreated and 84.3% treated with TGF-β). Furthermore, addition of the antibody to IP3RI did not alter the TGF-β effect, whereas the presence of IP3RIII antibody prevented the effect of TGF-β to reduce the number of filopodia. Similar results were obtained when heparin was added extracellularly to block TGF-β-induced Ca\(^{2+}\) influx (data not shown).

The effect of TGF-β on the actin cytoskeleton appears to be specifically dependent on TGF-β-induced Ca\(^{2+}\) influx, because the addition of thapsigargin to raise intracellular Ca\(^{2+}\) had no effect on the actin cytoskeleton (Fig. 10). Addition of ionomycin led to cell detachment and was therefore not interpretable (data not shown).

**DISCUSSION**

Based on the above evidence, we propose that TGF-β stimulates transmembrane Ca\(^{2+}\) influx in mesangial cells via the involvement of cell membrane-associated IP3RIII. Furthermore, Ca\(^{2+}\) influx is directly linked to acute cytoskeletal changes induced by TGF-β. Localization of IP3Rs to plasma membrane proteins has been recently documented in several cell types and is not limited to the IP3RIII isoform (39). However, our study indicates for the first time that localization of IP3RIII to the plasma membrane is enhanced by short-term TGF-β treatment. Inhibition of IP3RIII by inhibitors or specific antibodies demonstrates its critical role in facilitating Ca\(^{2+}\) influx.

---

**Fig. 7.** TGF-β-induced reorganization of actin filaments: time course. MMCs cultured on coverslips in serum-free growth medium for 24 h were treated with TGF-β1 (10 ng/ml) at the times indicated (B–D). After fixation, cells were stained with rhodamine-phalloidin and evaluated by confocal microscopy. Under control conditions (A), cells exhibited long, thin filamentous protrusions from the cell surface (filopodia); however, after 15 (C) and 30 min (D) of TGF-β treatment, filopodia were largely absent. Experiments were repeated three times with similar results.
The basis for IP₃RIII to act as a Ca²⁺ channel at the plasma membrane is unclear, although several studies have suggested that plasma-membrane-associated IP₃Rs are intimately associated with Ca²⁺ entry (3, 18). It is conceivable that plasma membrane-bound IP₃RIII associates with other proteins to form an operative Ca²⁺ entry pathway analogous to the association of IP₃R with plasma membrane-associated TRP in SOC entry (22, 32). However, distinct from movement of IP₃Rs to the plasma membrane with SOC, our study suggests that the IP₃RIII isoform translocates to the plasma membrane in response to TGF-β without depletion of internal Ca²⁺ stores. On reaching the plasma membrane, IP₃RIII may promote Ca²⁺ entry via TRP or possibly other Ca²⁺ channels. Therefore, IP₃Rs associated at or near the plasma membrane are critical in mediating Ca²⁺ influx with SOC as well as Ca²⁺ influx in the absence of Ca²⁺ release from internal stores. A specific role for the IP₃RIIH isoform in SOC has not yet been determined. A role for IP₃RIII has been implicated in apoptosis of lymphocytes (3, 18), and this process may be initiated by Ca²⁺ influx. It is tempting to speculate that apoptosis induced by TGF-β may also involve recruitment of IP₃RIII to the plasma membrane.

Fig. 8. TGF-β-induced reorganization of actin filaments: role of Ca²⁺ influx and IP₃RIII. MMCs cultured on coverslips in serum-free growth medium for 24 h were treated with 10 ng/ml of TGF-β1 for 15 min (B, D, F, H) or solvent (A, C, E, G). After fixation, cells were stained with rhodamine-phalloidin and evaluated by confocal microscopy. To test the role of Ca²⁺ influx, cells were washed and incubated in Ca²⁺-free medium (C) before addition of TGF-β (D). To evaluate the role of IP₃Rs, MMCs were preincubated with antibodies to IP₃RI (IP₃RIAb, 1:500 dilution) for 5 min (E and F) or antibodies to IP₃RIII (IP₃RIIIAb, 1:500 dilution; G and H) before addition of TGF-β. Experiments were repeated three times with similar results.
The topology of the plasma membrane-bound IP_3RIII is unclear. Prior topology studies with IP_3RI (25) have found that intracellular IP_3RI has its NH_2- and COOH-terminal sites facing the cytosolic aspect. By analogy, it would be predicted that plasma membrane IP_3RI and other isoforms of IP_3R would similarly have their NH_2- and COOH-terminal sites facing the intracellular aspect. However, there have been no studies to specifically examine plasma membrane-bound IP_3Rs. Based on our results with extracellular heparin and extracellular antibodies to block TGF-β-induced Ca^{2+} influx, it would appear that the NH_2- and COOH-terminal sites of IP_3RIII face the extracellular space. In addition, we demonstrate that extracellular antibodies are able to recognize IP_3R isoforms in intact MMCs (see Fig. 5). However, it is conceivable that heparin (4, 26, 27) and the antibodies (23, 42) may enter the intracellular space and remain active. Therefore, at the present time we cannot distinguish between the possibilities that the IP_3R inhibitors blocked the function of IP_3RIII by binding to intracellular or extracellular NH_2- and COOH-terminal sites. Studies are under way to identify the topology of IP_3RIII in mesangial cells.

Our finding that SERCA is translocated to the nucleus by TGF-β was unexpected. Of note, it has been recently observed that thapsigargin, an inhibitor of SERCA, attenuates the activity of several TGF-β-responsive promoters (41). The presence of SERCA in the nucleus after TGF-β treatment may be related to the activation of nuclear Ca^{2+}-activated proteins (e.g., CaM kinase II) with consequent effects on transcription factors regulating TGF-β-responsive genes.

The overall conclusion of recent studies examining Ca^{2+}, calmodulin (33, 43), and CaM kinase II (41) is that stimulation of Ca^{2+}-regulated pathways leads to a negative modulatory role in Smad-mediated pathways and cross talk with the extracellular signal-regulated (ERK) pathway. However, these studies have not identified a direct role for TGF-β-induced Ca^{2+} influx that may be independent of the Smad and ERK pathways. In our studies, we show that TGF-β-induced Ca^{2+} influx is critical for the effects of TGF-β on the actin cytoskeleton. The role of the Smad pathway in mediating Ca^{2+} influx by TGF-β cannot be excluded based on our results. However, it would appear unlikely that
Smads play a critical role, as Smad-mediated processes generally require gene transcription and are observed hours after TGF-β exposure, whereas Ca²⁺ influx and alteration of the actin filaments occur as early as 15–30 min after the addition of TGF-β.

The actin filamentous protrusions on the plasma membrane of MMCs closely resemble filopodia. Filipodia have been described to be present in neurites (14), ovarian granulosa cells (9), and malignant cells (16) and appear to be most closely associated with cell-cell communication and cell spreading. Although [Ca²⁺]ᵢ has been considered to regulate filopodia formation (34), there have been no reports that TGF-β inhibits filopodia formation. However, cyclopodin-induced membrane filament protrusions in adenocarcinoma cells have been found to be blocked by anti-TGF-β antibodies (11), which suggests that TGF-β may inhibit filopodia in some cell types (the present study) and enhance it in other cell types. The role of filopodia in mesangial cells is unclear but may be relevant to cell-cell communication with adjacent glomerular endothelial cells and matrix interaction. As membrane filopodia are associated with cell proliferation, it is possible that the effect of TGF-β on the actin cytoskeleton may be involved in limiting proliferation of cells and promoting cell hypertrophy. Of note, TGF-β has previously been found to inhibit proliferation (13) and induce cell hypertrophy (5) in mesangial cells. Further studies are required to elucidate the downstream effects of TGF-β-induced actin filament reorganization as well as the impact of Ca²⁺ influx on cross talk with other well-described pathways involved in TGF-β signaling.

The authors thank Stephen R. Dunn for data presentation. This study was funded by National Institutes of Health Grants R01 DK-53867 (to K. Sharma) and GM-58574 (to S. K. Joseph) and an American Diabetes Association research award (to K. Sharma).

REFERENCES

8. Ghosh TK, Eis PS, Mullaney JM, Ebert CL, and Gill DL. Competitive, reversible, and potent antagonist of inositol 1,4,5-