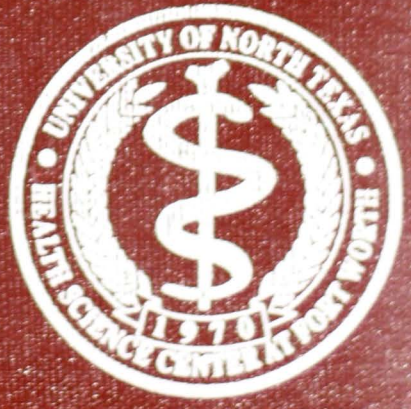

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Sours-Brothers, Sherry. Store-operated Calcium Entry in Glomerular Mesangial Cells. Doctor of Philosophy (Integrative Physiology, Molecular Cardiovascular Science Track), April, 2008, 181 pp; 1 table, 25 figures, bibliography, 241 titles.

Mesangial cells (MCs) are found within the glomerulus, where they contribute to the regulation of glomerular filtration rate (GFR). Their contractile function is similar to that of vascular smooth muscle cells, regulated by a number of different Ca^{2+} release and entry mechanisms in response to vasoactive substances. Among these are store-operated channels (SOC), which have been identified in MC, but whose molecular components are unknown. Deficiency of store-operated Ca^{2+} entry (SOCE) has also been associated with loss of MC contractile function found during early diabetic renal hyperfiltration. For these reasons, it is imperative to clarify the mechanisms underlying SOCE in MCs.

Members of the canonical transient receptor potential (TRPC) family of proteins have been identified as candidates for SOC function in a number of cell types. The distribution of TRPC subtypes, and their combination to form heterotetrameric channels is cell-type specific, possibly allowing for variation SOCE mechanisms in different cells. Recently, the endoplasmic reticulum (ER) resident protein stromal interaction molecule 1 (STIM1) has been identified as a regulator of SOCs, including TRPCs. With this in mind, the following studies were carried out to identify the distribution and function of TRPC proteins in MCs, including their role in the mediation of MC contractile function and potential regulation by STIM1.

In the first study, TRPC1, -3, -6, and -7 were identified in cultured human MCs as well as rat and human kidney sections. TRPC1 was found to associate with TRPC4 and

TRPC6 by co-immunoprecipitation and colocalization by immunocytochemistry. Overexpression of TRPC1 by transient transfection increased, while knockdown of TRPC1 expression by RNAi decreased thapsigargin-mediated SOCE. These results indicate a role for TRPC1 in SOCE in MCs.

In the second study, the contribution of TRPC1-mediated SOCE to Ang II-stimulated MC contractile function was examined. Ang II-mediated SOCE was attenuated by TRPC1-RNAi or by treatment with a TRPC1 antibody known to block channel activity. TRPC1-RNAi and antibody blockade also inhibited Ang II-stimulated single channel activity as measured by cell-attached patch clamp, while TRPC1-RNAi attenuated Ang II-mediated MC contraction. This effect was also examined *in vivo* in rats. Infusion of TRPC1 antibody blocked Ang II-induced decline in GFR.

In the final study, the formation of SOC by TRPC heteromultimerization was assessed. Both TRPC1 and TRPC4 were found to contribute to TG-stimulated SOCE and single-channel activity in cultured MCs. The interaction between these two subtypes increased upon store-depletion with TG, while translocation of TRPC1 but not TRPC4 to the plasma membrane was induced by TG. STIM1 was also found to contribute to regulation of SOC, but co-immunoprecipitation demonstrated an interaction with TRPC1 but not TRPC4. These data suggest that SOC activity is mediated by interaction between TRPC1 and TRPC4, and translocation of TRPC1 to the plasma membrane may be responsible for increasing channel activity upon store depletion. STIM1 may play a regulatory role by activating channel complexes via TRPC4.

Taken together, these studies indicate an important role for TRPC function in MCs. Not only do these studies further understanding of SOC function in MCs specifically, they also contribute to the delineation of TRPC channel activity, complex formation, and regulation by STIM1. Futures studies are needed to further examine TRPC activation mechanisms and their potential role in other physiological and pathophysiological MC functions.

STORE-OPERATED CALCIUM ENTRY IN GLOMERULAR MESANGIAL CELLS

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Sherry Sours-Brothers, B.S., M.S.

Fort Worth, Texas

April, 2008

Acknowledgements

First, I acknowledge my mentor, Rong Ma, PhD. I first joined Dr. Ma's laboratory as a Senior Research Assistant, and assisted him in setting up as a new faculty member in the Department of Integrative Physiology. After three semesters, he encouraged me to continue my doctoral studies, which I did under his guidance. As his first graduating doctoral student, I am thankful for Dr. Ma mentorship and support. The work included in this dissertation was supported by a Scientist Development Grant from American Heart Association National Center and a Young Investigator Grant from the National Kidney Foundation, both with Dr. Ma as Principle Investigator. I also thank the other members of the lab, Juan Du, Sarabeth Graham, and especially Min Ding, whose collaborative efforts make these works possible. In addition, I thank the members of my committee, Drs. Joan Carroll, Glenn Dillon, Peter Koulen, and Robert Mallet for their input and assistance with my final dissertation.

Finally, I acknowledge my husband, R. Matthew Brothers, PhD. It was also with his encouragement that I continued my studies to complete my doctoral degree. Not only has he steadfastly supported me when I needed it most, his expertise in human physiology also helped me to succeed in a department in which I, as a molecular biologist, may have otherwise struggled.

Articles and abstracts resulting from this dissertation

- **Sours S**, Du J, Chu S, Ding M, Xhou XJ, Ma R. Expression of canonical transient receptor proteins (TRPC) proteins in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 290(6): F1507–F1515, 2006.

- **Sours S**, Oberdorfer J, Ding M, Du J, Ma R. Function of TRPC1/TRPC4 complex in mesangial cells. *FASEB J* 20: A767, 2006.

- Du J, **Sours-Brothers S**, Coleman R, Ding M, Graham S, Kong DH, Ma R. Canonical transient potential 1 channel is involved in contractile function of glomerular mesangial cells. *J Am Soc Nephrol* 18(5): 1437-1445, 2007.

- **Sours-Brothers S**, Ding M, Oberdorfer J, Graham S, Ma R. Store-operated Ca^{2+} entry through TRPC1/TRPC4 complexes interacting with STIM1 protein in glomerular mesangial cells. *Submitted manuscript under revision.*

- **Sours-Brothers S**, Ding M, Graham S, Ma R. Interaction between STIM1 and TRPC1/TRPC4 complexes, a mechanism for store-operated calcium entry in glomerular mesangial cells. *FASEB J* 22: 1158.15, 2008.

Other published works

- Ma R, Du J, **Sours S**, Ding M. Store-operated Ca^{2+} channel in renal microcirculation and glomeruli. *Exp Biol Med* 231(2): 145-153, 2006.

- Duncan RS, **Sours-Brothers S**, Koulen P. Calcium signaling and neurodegeneration. *Cellscience Rev* 3(3): 121, 2007.

- Graham S, Ding M, **Sours-Brothers S**, Yorio T, Ma JX, Ma R. Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca^{2+} signaling in glomerular mesangial cells in diabetes. *Am J Physiol, Renal Physiol* 293(4):F1381-F1390, 2007.

- Du J, Ding M, **Sours-Brothers S**, Graham S, Ma R. Mediation of Angiotensin II-induced Ca^{2+} signaling by polycystin 2 in glomerular mesangial cells. *Am J Physiol, Renal Physiol* 294(4): F909-F918, 2008.

- **Sours-Brothers S**, Ma R, Koulen P. Ca^{2+}-sensitive transcriptional regulation: direct DNA interaction by DREAM. Front Biosci. *In press.*

TABLE OF CONTENTS

LIST OF TABLES

Page

CHAPTER III

LIST OF FIGURES

Page

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER I

INTRODUCTION

Background

Physiological function of glomerular mesangial cells

The glomerulus, the site of renal filtration, is composed of a network of capillaries, known as the glomerular tuft, contained within Bowman's capsule (**Figure 1**). These capillaries are lined with a fenestrated endothelial cell layer surrounded by the glomerular basement membrane followed by specialized epithelial cells called podocytes. The cell bodies of the podocytes have projections known as foot processes which interlace to form the slit diaphragm. These three layers, the fenestrated endothelium, glomerular basement membrane, and podocyte slit diaphragm, form the barrier through which the plasma is filtered from the glomerular capillaries to the proximal end of the nephron (89). The glomerular filtration rate (GFR) through this filtration barrier is regulated by a number of factors, including renal blood flow, glomerular capillary hydrostatic pressure, glomerular capillary permeability, as well as filtration surface area (24). For example, Angiotensin II (Ang II) can elicit vasoconstriction in both the afferent and efferent arterioles supplying and draining the glomerular capillaries, resulting in decreased renal blood flow with a subsequent decrease in GFR (97).

Mesangial cells (MCs) are found among the glomerular capillary loops where they form a support network for the glomerular tuft and help to regulate capillary surface

area and thereby GFR (**Figure 1**). These smooth muscle-like pericytes respond to vasoactive substances, such as Ang II and endothelin (ET). The contractile apparatus of MCs is similar to that of vascular smooth muscle cells, including actin, myosin and tropomyosin (18). However, isoform expression is not identical to smooth muscle, and may be altered in disease states or under cell culture conditions (30; 35; 61). MCs also exert paracrine activity through the secretion of growth factors and inflammatory mediators. For example, MCs can produce ET-1 in response to a number of stimuli, including Ang II, insulin, transforming growth factor β_1 (TGF-β_1) and platelet derived growth factor (PDGF), as well as ET-1 itself (81). ET-1 secreted by MCs can then interact with ET receptors A and B, both found on MCs (29). ET receptor activation can then lead to cellular hypertrophy, proliferation, and extracellular matrix secretion, in addition to cellular contraction (7; 26). Growth factors such as TGF-β and PGDF also stimulate MC hypertrophy and proliferation in a paracrine and autocrine fashion (1).

The development of the micropuncture technique to directly measure filtration in the glomerulus allowed researchers to confirm the regulation of GFR within the glomerulus, independent of changes in afferent and efferent arteriolar tone(82). This regulation can be attributed at least in part to the contractile function of MCs which regulates glomerular capillary surface area and blood flow. This is a result of the functional coupling between the mesangial extracellular matrix and the basement membrane surrounding the glomerular capillaries via fibronectin(78; 82). In addition, MC proliferation and extracellular matrix secretion during inflammation can also alter GFR (53). This MC expansion occurs in response to glomerular injury and can lead to a

limitation of glomerular filtration, such as that seen in glomerulosclerosis and diabetic nephropathy (4).

MC contraction has been studied extensively *in vivo* in Munich Wistar rats and *in vitro* in MC culture (34; 51). Like vascular smooth muscle cells, their contraction is dependent on influx of Ca^{2+} from the extracellular space. This Ca^{2+} influx can also result in upregulation of MC proliferation and extracellular matrix secretion. A number of ion channels contribute to the regulation of MC function, including voltage-operated Ca^{2+} channels, receptor-operated, and store-operated channels (VOCC, ROC, and SOC respectively). VOCC function was the first to be described in MC culture (**Figure 2a**) (63; 99). These channels can be blocked by nifedipine, suggesting that they are L-type channels, and are activated by membrane depolarization in response to vasoactive peptides. Activation of VOCC also contribute to MC growth (39; 83). ROCs present in MCs contribute to regulation of growth factor-mediated responses (**Figure 2b**). In this case, tyrosine kinase receptors, such as epidermal growth factor (EGF) and PDGF receptors, directly couple to ROCs to activate Ca^{2+} influx, resulting in MC growth (40). ROC-mediated Ca^{2+} influx may also be activated by a G_q-coupled mechanism, including ET (64).

Characteristics of store-operated Ca^{2+} entry

Activation of SOCs in MC response to growth factors and vasoactive peptides has also been described. (50; 54; 64). These channels are characterized as non-specific, cation permeable channels with a very low single-channel conductance of 1-2.1 pS. SOC

influx occurs in response to depletion of intracellular Ca^{2+} stores resulting from activation of the G_q-protein coupled receptor, phospholipase C, inositol triphosphate (IP_3) cascade (2; 60). This mode of Ca^{2+} entry was originally identified as capacitative Ca^{2+} entry, as the amount of Ca^{2+} influx indicates the capacity of Ca^{2+} lost from intracellular stores (70; 71). PKC activation resulting from this same cascade may also directly activate SOC (**Figure 2c**) (46; 70; 71) . Receptor-independent activation of SOC can also be studied by passively inducing store-depletion with the sarco/endoplasmic reticulum ATPase (SERCA) inhibitors thapsigargin or cyclopiazonic acid (**Figure 2d**) (74).

The current associated with capacitative Ca^{2+} entry is termed the Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) (32). This is a non-voltage-gated, inwardly rectifying current with a reverse potential greater than +60 mV (67). While the single-channel conductance of I_{CRAC} is very low, less than 1 pS, it is highly selective for Ca^{2+}, while also being permeable to Na^+ (67). Interestingly, I_{CRAC} also exhibits anomalous mole fraction-effect, such that when Ca^{2+} is removed from the extracellular space, permeability to Na^+ is greatly increased, generating a channel conductance that is even greater than that for Ca^{2+} (72). I_{CRAC} with these typical characteristics have been identified in a number of cell types, including mast cells, hepatocytes, dendritic cells, megakaryocytes, and Jurkat T cells (68).

Another current associated with store-depletion is known as the store-operated current (I_{SOC}). While this current has also been identified in a number of cell types, its characteristics such as single-channel conductance and permselectivity are less discreet. For example, I_{SOC} in endothelial cells has a conductance of 11 pS with a selectivity for

Ca^{2+}:Na^+ of 10:1 (90), while I_{SOC} in aortic myocytes exhibits a lower conductance of 2.7 pS, and is not selective for Ca^{2+} (Ca^{2+}:Na^+:K^+ = 1:1:1) (88). SOC has been characterized in MCs using thapsigargin-stimulated store-depletion (50). Treatment with thapsigargin produces a single-channel conductance of 2.1 pS with Ba^{2+} and 0.7 pS with Ca^{2+} that can be inhibited by La^{3+}. These channels are slightly more selective for Ca^{2+} ($Ca^{2+} < Ba^{2+} << K^+$).

The variability of currents produced by store-depletion is accompanied by an array of proposed activation mechanisms for store-operated Ca^{2+} entry (SOCE). Included among these are: 1) vesicular fusion to transport SOCs to the plasma membrane; 2) direct conformational coupling between active IP_3 receptors on the ER with SOCs at the plasma membrane; 3) a messenger molecule that is released from the ER which then diffuses to the plasma membrane to activate SOCs (**Figure 3**) (67).

In the vesicular fusion model, SOCs are stored in the cytosol in vesicles which are transported to the plasma membrane upon store-depletion (**Figure 3a**). This would be a similar mechanism to that of the glucose transporter GLUT4, which is translocated to the plasma membrane of skeletal muscle cells and adipocytes upon stimulation by insulin (13). Evidence for this mechanism has been demonstrated in Xenopus oocytes, where a dominant negative mutant of SNAP-25, a vesicular docking molecule that is the target of botulinum toxin, was shown to inhibit I_{SOC} activation (98). This evidence was supported by a study by Alderton *et al.* in which injection of botulinum neurotoxin A, which targets SNAP-25, inhibits SOCE in HEK-293 cells (3). Bezzerides *et al.* also demonstrated that TRPC5 specifically is transported to the plasma membrane upon growth factor

stimulation in HEK-293 cells expressing TRPC5 (see discussion of TRPC proteins below), as well as natively expressed protein in hippocampal neurons (11). This translocation, which the authors termed "rapid vesicular insertion of TRP" (RiVIT), occurs in a phosphatidylinositol 3-kinase-dependent manner.

The conformational coupling model suggests that IP_3 receptors on the ER directly activate SOCs at the plasma membrane via protein-protein interaction (9; 10). This direct interaction should allow for very rapid activation of SOC after binding of IP_3. In most cells, however, SOC activation occurs slowly, over minutes rather than seconds (68). A similar model proposed by Gill *et al.* instead suggests a secretion-like coupling in which coupling occurs in a reversible manner upon activation, and is mediated by cytoskeletal modification (69).

The final model currently under investigation involves the diffusion of an unknown messenger from the ER to the plasma membrane to activate SOC upon store-depletion. Candidates include protein kinase C (PKC), tyrosine kinase, lysophospholipids, GTP-binding proteins, and calmodulin (6; 6; 22; 47; 68; 80; 91). Yet another poorly characterized messenger known as the Ca^{2+}-influx factor (CIF) has also been identified. Randriamampita and Tsien first extracted CIF from Jurkat T cells (75), and characterization and purification has been continued by other groups (17; 85; 87). However, the molecular identity of this messenger is still unknown.

As previously mentioned, SOCs have been identified in MCs, and SOCE may be significant in some renal diseases characterized by MC dysfunction. For example, dysregulation of MC contraction can result in glomerular hyperfiltration during early

diabetic nephropathy, and can be attributed to down-regulation of SOCE in response to vasoactive peptides (52; 55). SOCE also contributes to MC proliferation and promotes glomerular inflammation, which are both complications also associated with diabetic nephropathy (38; 38; 48; 56). SOC activation can be mediated by PKC in MCs. For example, EGF activates PKC by tyrosine kinase-mediated diacylglycerol (DAG) production (48). This can lead to activation of SOC by PKC that is independent of store-depletion. On the other hand, SOC can be stimulated by Ang II as a result of IP_3-mediated store-depletion (58). Both EGF and Ang II are important mediators of MC function *in vivo*. Importantly, EGF is involved in diabetic proliferation of mesangial cells and Ang II-mediated MC contractile response is impaired in diabetes, contributing to glomerular dysfunction. Thus, SOC impairment may account for these responses in MC dysfunction in diabetes.

Transient receptor potential proteins function as store-operated channels

In light of the potential role in glomerular pathophysiology, mechanisms of SOCE in MCs warrant continued study. This is complicated, however, by controversy over the molecular components of the SOC themselves. Members of the canonical transient receptor potential (TRPC) family of proteins have been identified as candidates for SOC channel activity (62; 68). TRPCs are members of the transient receptor potential superfamily first identified in *Drosophila melanogaster* (57). There are 7 subtypes, designated TRPC1-7, of which TRPC2 is a pseudogene in humans. The remaining six proteins can be divided phylogenetically based on their structure (**Figure 4**) (94). TRPCs

are structurally similar to voltage-gated K^+ channels (K_V), with six trans-membrane spanning regions and a pore-forming domain between trans-membrane spanning regions 5 and 6. Also like K_V channels, TRPCs are thought to form tetramers centered around the pore-forming domains (16). Other conserved regions among the TRPC family include a series of ankyrin repeats, a coiled-coil domain and caveolin binding region found in the cytosolic N-terminal region (93). The C-terminus is characterized by a TRP motif (EWKFAR), another coiled-coil domain, a proline-rich motif, and a calmodulin/IP_3 receptor binding region (93).

TRPC expression varies among cell types, and heteromultimerization of different TRPC isoforms also appears to be cell-type specific. This may allow for specificity of SOC function and activation mechanisms in different cells. The coiled-coil domains found on the C- and N- termini have both been found to regulate this multimerization (20; 27; 79). The C-terminal caveolin-binding domain is also involved in the co-localization of various TRPCs and their targeting to the plasma membrane (12; 44; 45).

TRPC1 currently has the strongest evidence for function as an SOC in a variety of cell types (14; 43; 59; 76). Although TRPC1 is known to heteromultimerize with other TRPC (15; 42; 96), it has also been demonstrated to be a pore-forming subunit itself(43). TRPC1-mediated SOCE can be activated by each of the mechanisms described above, including passive store-depletion, IP_3-mediated store-depletion, and channel phosphorylation by PKC. Direct interaction with endoplasmic IP_3 receptors has also been demonstrated to activate TRPC1 (100). In vascular smooth muscle cells, TRPC1 contributes to contractile function by regulating SOCE in response to vasoconstrictors

such as ET (8; 37). TRPC1 is also involved in neointimal hyperplasia of vascular smooth muscle (36). TRPC1 is expressed in the rat glomerulus and proximal tubule, as well as in the rat renal microcirculation where it contributes to Ang II-mediated arteriolar vasoconstriction (21; 25; 84).

Other TRPC isoforms have less concrete evidence of SOC function, and may instead act as ROCs. For example, TRPC3 can be activated by store-depletion in both a receptor-dependent and receptor-independent manner (86; 92). However, TRPC3, 6, and 7 have also been shown to be directly activated in a DAG-dependent manner, independent of PKC or store-depletion (31; 65). In contrast, TRPC1, 4, and 5 are not activated by DAG, and activation of TRPC4 and 5 can even be inhibited by DAG (31; 95).

STIM1 regulation of SOC

Recently, the stromal interaction molecule 1 (STIM1) has been identified as a Ca^{2+} sensor in the endoplasmic reticulum (ER), activating SOC at the plasma membrane upon store-depletion (41; 77). STIM1 is ubiquitously expressed, and localized primarily to the ER, although some studies have indicated its presence in the plasma membrane of some cells (**Figure 5**) (19). An EF-hand domain is found on the ER luminal N-terminus of STIM1, indicating that it may act as a Ca^{2+} sensor to signal store-depletion (73; 77). There is evidence that STIM1 translocates to regions of the ER directly apposed to the plasma membrane upon store depletion, where it triggers Ca^{2+} influx via SOC (5; 41). Translocation of STIM1 may even play a role in the rearrangement of the ER itself.

STIM1 is associated with microtubule-plus-end-tracking protein EB1, part of the tip-attachment complex which allows the ER to elongate along microtubules (28). This rearrangement may allow STIM1 to activate SOC in reversible manner similar to the secretion-like coupling model originally proposed for IP_3 receptor-mediated activation described above.

While the role of TRPCs as SOCs has been controversial, the binding of STIM1 to various members of the TRPC family gives new credence to their functionality. Huang *et al.* have demonstrated that STIM1 binds to TRPC1, -2, -4, and -5 through an ERM (ezrin/radixin/moesin) domain which is required for its activation of SOC (33; 101). Overexpressed STIM1 co-immunoprecipitates with TRPC1 in HEK293 cells, but this interaction is not enhanced by store-depletion with thapsigargin. Fluorescence resonance energy transfer experiments however do demonstrate that STIM translocates to distinct punctae associated with TRPC1 after thapsigargin treatment. This subcellular redistribution also occurs in HeLa cells (41). STIM1 has also been shown to form a complex with TRPC1 and Orai1, another potential component of SOCE originally identified by a mutation associated with severe combined immunodeficiency disorder (23; 66). These data suggest that STIM1 may activate SOCE by sensing store-depletion in the ER, followed by translocation and activation of TRPC1 by direct interaction between these two proteins. A new definition of SOC has been proposed by Yuan et al. as "channels that are regulated by STIM1 and require the store depletion-mediated clustering of STIM1" (101). However, SOCs have previously been characterized as having other activation mechanisms including store-independent activation by PKC as

described above. The potential contribution of STIM1 in these other activation mechanisms warrants further study.

Specific Aims

While TRPC1 is thought to be ubiquitously expressed, expression of TRPCs in general varies among cell types and species. This is even true within the kidney. TRPC1, 3, 4, 5 and 6 have been identified in the rat renal microcirculation and glomeruli (21), but another study found only TRPC1, 3, and 6 in rat glomeruli (25). This demonstrates the importance of identifying which TRPC isoforms are expressed in a specific tissue or cultured cell line. Meanwhile, specificity of function and activation mechanisms may result from the cell-type specific expression of TRPCs. For example, TRPC1 in particular has the most evidence for SOC function, but the physiological relevance for this role is undetermined in many cell types, including MCs. Another factor that may determine TRPC specificity of function is the heteromultimerization of different TRPC isoforms, which is also undetermined in MCs. While SOCE has been identified in MCs, it is unclear if TRPCs account for this mode of Ca^{2+} entry, and what their physiological relevance may be. In addition, the potential for STIM1 to act as a regulator or SOC function, including TRPCs, is unknown. The following specific aims have been designed to address the hypothesis that TRPCs do indeed function as SOC in mesangial cells, and that TRPC1 in particular contributes to the regulation of MC contractility. This function is likely regulated by the heteromultimerization of TRPC isoforms and by STIM1 acting as a Ca^{2+} sensor in the ER.

Specific Aim I: Determine the expression profile of TRPC proteins in mesangial cells and if TRPC1 specifically contributes to store-operated Ca^{2+} entry in mesangial cells.

Specific Aim II: Evaluate the role of TRPC1 in contractile function of mesangial cells.

Specific Aim III: Determine if store-operated Ca^{2+} entry is mediated by interaction between TRPC1, TRPC4, and/or STIM1 in mesangial cells.

These specific aims have been addressed in the manuscripts comprising the following three chapters of this dissertation, with the research design and methodology, results, and discussion described therein. These manuscripts are followed by a summary of conclusions from each set of studies, as well as a discussion of future directions for further study.

Figure 1. Structure of the glomerulus. **A.** The glomerular tuft contained within Bowman's capsule is composed of a capillary network fed and drained by an afferent and efferent arteriole, respectively. Plasma filtrate passes through the glomerular filtration barrier into Bowman's space, which drains into the proximal tubule of the nephron. **B.** The glomerular filtration barrier is composed of the fenestrated capillary endothelial cell layer, the glomerular basement membrane, and slit diaphragms formed by the foot processes of podocytes. Mesangial cells are found among the glomerular capillaries. Their extracellular matrix contributes to the glomerular basement membrane. *Adapted from Ganong, 2005* (24)

Figure 1.

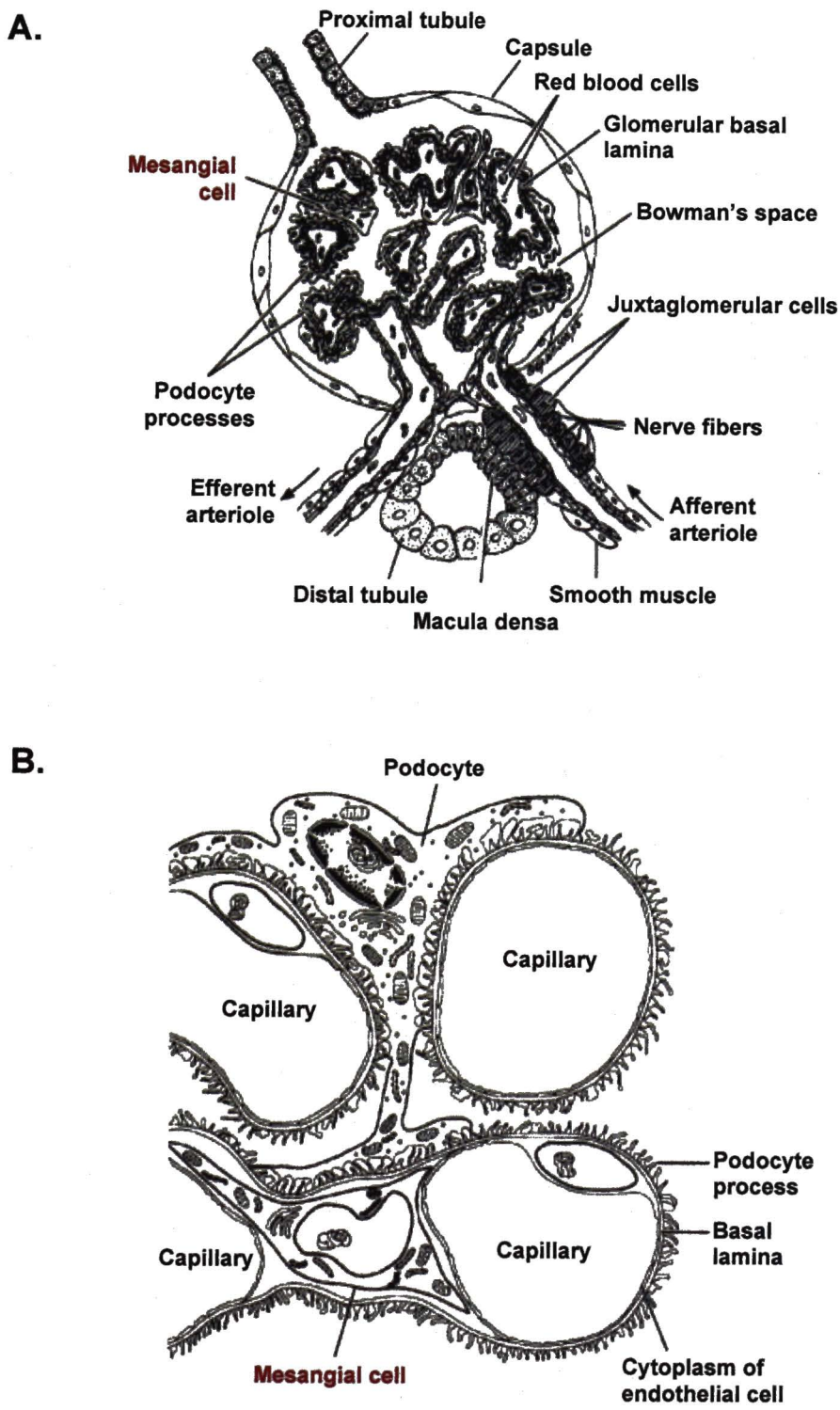

A.
Proximal tubule
Capsule
Red blood cells
Glomerular basal lamina
Mesangial cell
Bowman's space
Juxtaglomerular cells
Podocyte processes
Nerve fibers
Efferent arteriole
Afferent arteriole
Distal tubule
Macula densa
Smooth muscle
B.
Podocyte
Capillary
Capillary
Podocyte process
Basal lamina
Capillary
Capillary
Mesangial cell
Cytoplasm of endothelial cell

Figure 2. Ca^{2+} entry pathways in glomerular mesangial cells. **a.** VOCC activated by membrane depolarization. **b.** ROC activation by DAG resulting from G_q-protein coupled receptor activation, e.g. Ang II. **c.** SOC activation stimulated directly by PKC. **d.** Passive store-depletion by SERCA inhibition also activates SOC (49)

Figure 2.

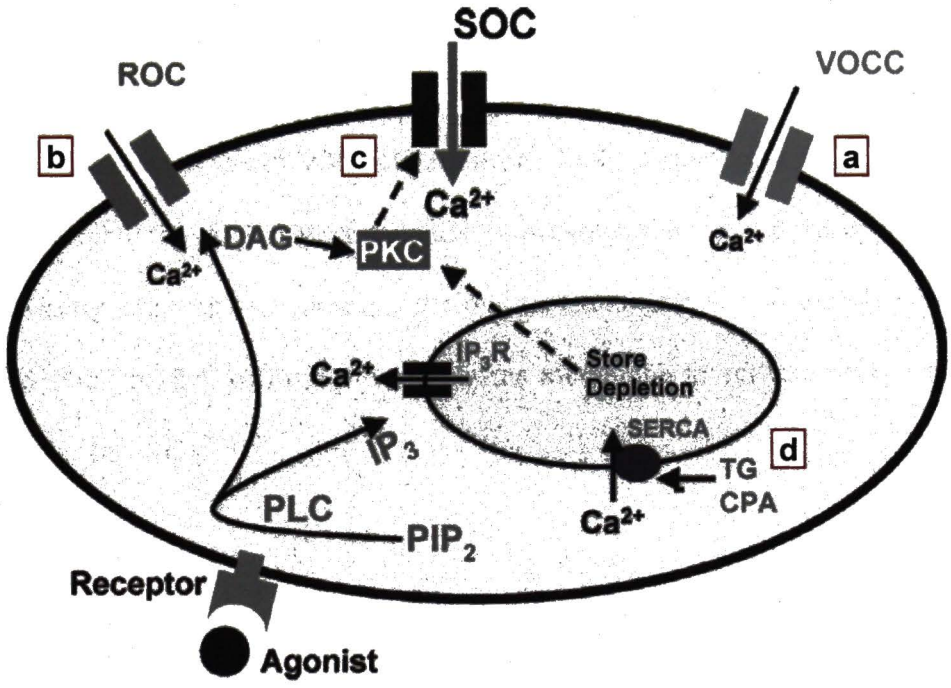

SOC
ROC
VOCC
b
c
a
Ca2+
DAG
PKC
Ca2+
Ca2+
IP3R
Store
Depletion
Ca2+
SERCA
d
IP3
TG
CPA
PLC
Ca2+
PIP2
Receptor
Agonist

Figure 3. Proposed models of SOC activation. **A.** SOC store in vesicles are transported to and inserted into the plasma membrane upon store-depletion. **B.** IP_3 receptors activate SOC by directly coupling to channels at the plasma membrane. **C.** A mobile messenger is released from the ER, diffusing to the plasma membrane to activate SOC. *Adapted from Parekh, 2003* (67).

Figure 3.

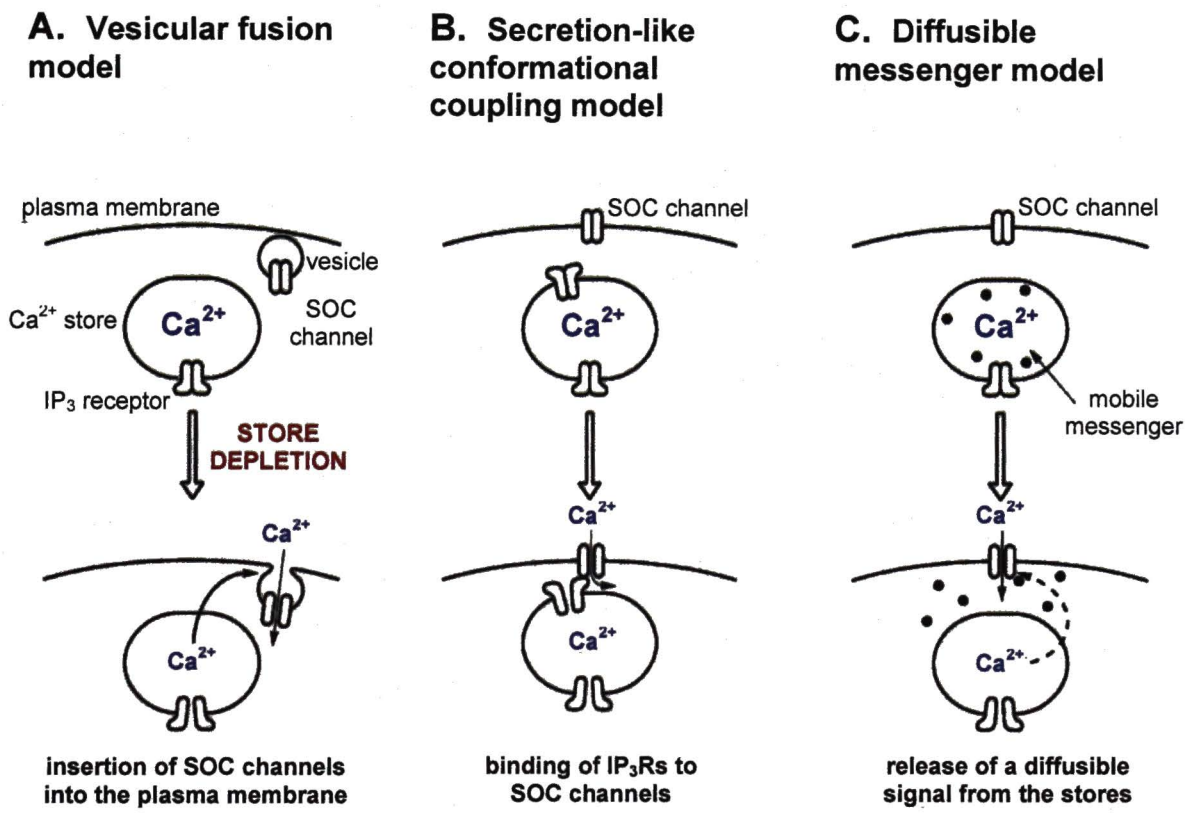

A. Vesicular fusion model
B. Secretion-like conformational coupling model
C. Diffusible messenger model
plasma membrane
vesicle
SOC channel
Ca2+ store
Ca2+
IP3 receptor
STORE DEPLETION
Ca2+
Ca2+
insertion of SOC channels into the plasma membrane
SOC channel
Ca2+
Ca2+
Ca2+
binding of IP3Rs to SOC channels
SOC channel
Ca2+
mobile messenger
Ca2+
Ca2+
release of a diffusible signal from the stores

Figure 4. Structure and relationship of TRPCs. **A.** Predicted transmembrane domains (1-6, yellow) and pore-forming loop (green) of TRP proteins. Three to four ankyrin repeats (purple) are found on the N-terminus; proto-typical 'TRP-box' found (orange) on the C-terminus close to the plasma membrane. **B.** Phylogenetic grouping and structural alignment of mammalian TRPCs compared to *Drosophila* TRP. *Adapted from Venkatchalam,* et al., *2002* (94).

Figure 4.

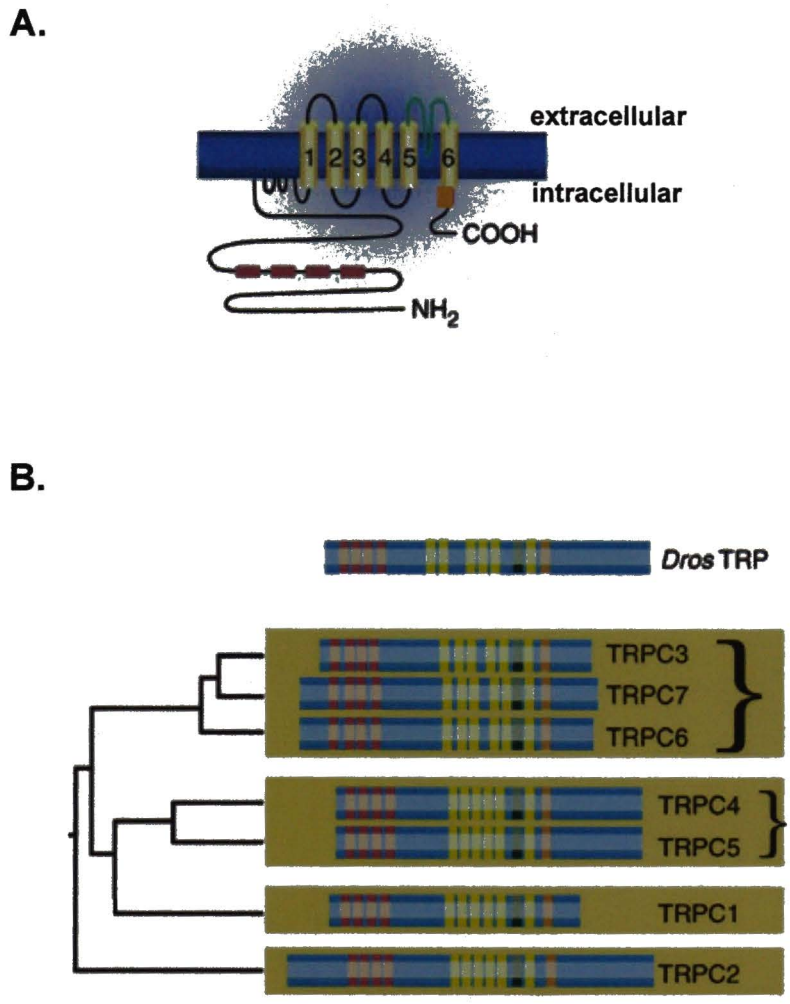
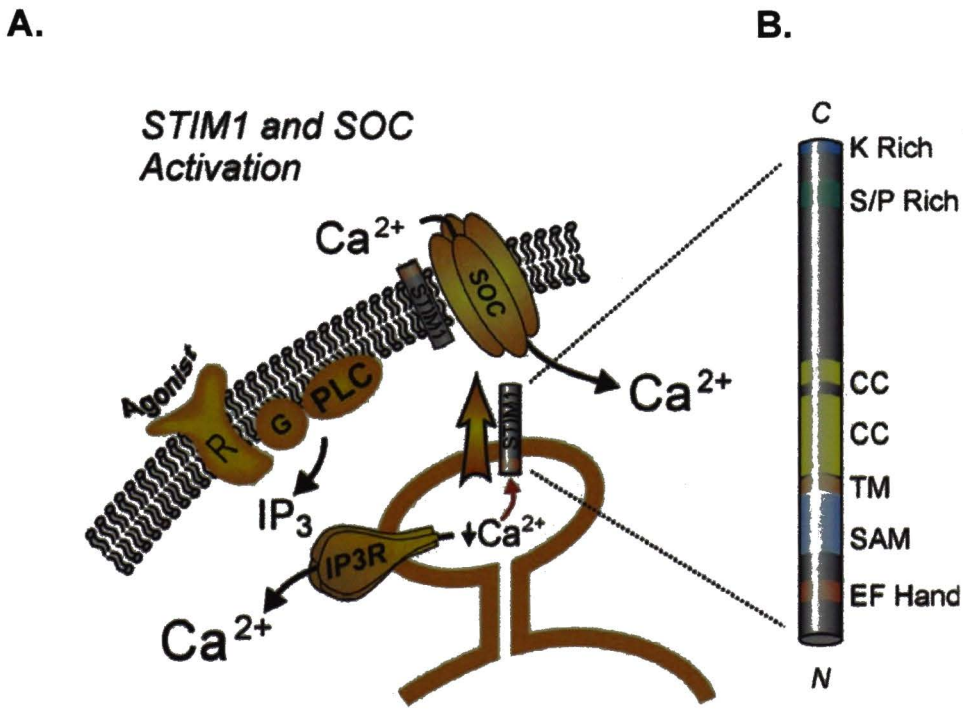

A.
extracellular
1 2 3 4 5 6
intracellular
COOH
NH2
B.
Dros TRP
TRPC3
TRPC7
TRPC6
TRPC4
TRPC5
TRPC1
TRPC2

Figure 5. STIM1 activation of SOC. **A.** Agonist stimulation leading to IP_3-mediated store-depletion activates translocation of STIM1 to regions directly opposed from SOC on in the plasma membrane where it triggers SOCE by an unknown mechanism. **B.** Structure of STIM1, including a luminal EF-hand, a sterile α-motif (SAM), a single transmembrane (TM) domain, multiple coiled-coil domains, a serine/proline-rich (S/P Rich) as well as lysine-rich domain. *From Putney, 2005* (73).

Figure 5.


A.
B.
STIM1 and SOC Activation
Ca2+
SOC
Agonist
R
G
PLC
IP3
IP3R
Ca2+
Ca2+
Ca2+
C
K Rich
S/P Rich
CC
CC
TM
SAM
EF Hand
N

Abbreviations

Ang II: angiotensin II

CIF: Ca^{2+} influx factor

DAG: diacylglycerol

EGF: epidermal growth factor

ER: endoplasmic reticulum

ERM: ezrin/radixin/moesin

ET: endothelin

GFR: glomerular filtration rate

I_{CRAC}: Ca^{2+}-release-activated Ca^{2+} current

IP_3: inositol triphosphate

I_{SOC}: store-operated current

K_V: voltage-gated K^+ channels

MC: mesangial cell

TGF-β_1: transforming growth factor β_1

PGDF: platelet derived growth factor

PKC: protein kinase C

RiVIT: rapid vesicular insertion of TRP

ROC: receptor-operated channel

SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase

SOC: store-operated channel

SOCE: store-operated Ca^{2+} entry

STIM1: stromal interaction molecule 1

TRPC: canonical transient receptor potential

VOCC: voltage-operated Ca^{2+} channel

References

1. **Abboud HE**. Growth factors and the mesangium. *J Am Soc Nephrol* 2: S185-S189, 1992.
2. **Albert AP and Large WA**. Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J Physiol* 544: 113-125, 2002.
3. **Alderton JM, Ahmed SA, Smith LA and Steinhardt RA**. Evidence for a vesicle-mediated maintenance of store-operated calcium channels in a human embryonic kidney cell line. *Cell Calcium* 28: 161-169, 2000.
4. **Alsaad KO and Herzenberg AM**. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update. *J Clin Pathol* 60: 18-26, 2007.
5. **Baba Y, Hayashi K, Fujii Y, Mizushima A, Watarai H, Wakamori M, Numaga T, Mori Y, Iino M, Hikida M and Kurosaki T**. Coupling of STIM1 to store-operated Ca^{2+} entry through its constitutive and inducible movement in the endoplasmic reticulum. *PNAS* 103: 16704-16709, 2006.
6. **Babnigg G, Bowersox SR and Villereal ML**. The Role of pp60c-src in the regulation of calcium entry via store-operated calcium channels. *J Biol Chem* 272: 29434-29437, 1997.
7. **Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T and Harris RC**. Mesangial cell, glomerular and renal vascular responses to endothelin in the rat

kidney. Elucidation of signal transduction pathways. *J Clin Invest* 83: 336-342, 1989.

8. **Bergdahl A, Gomez MF, Dreja K, Xu SZ, Adner M, Beech DJ, Broman J, Hellstrand P and Sward K.** Cholesterol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca^{2+} entry dependent on TRPC1. *Circ Res* 93: 839-847, 2003.
9. **Berridge MJ.** Capacitative calcium entry. *Biochem J* 312: 1-11, 1995.
10. **Berridge M.** Conformational coupling: A physiological calcium entry mechanism. *Science Signaling* 2004: e33, 2004.
11. **Bezzerides VJ, Ramsey IS, Kotecha S, Greka A and Clapham DE.** Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol* 6: 709-720, 2004.
12. **Brazer Sc, Singh BB, Liu X, Swaim W and Ambudkar IS.** Caveolin-1 contributes to assembly of store-operated Ca^{2+} influx channels by regulating plasma membrane localization of TRPC1. *J Biol Chem* 278: 27208-27215, 2003.
13. **Bryant NJ, Govers R and James DE.** Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol* 3: 267-277, 2002.
14. **Cai S, Fatherazi S, Presland R, Belton C, Roberts F, Goodwin P, Schubert M and Izutsu K.** Evidence that TRPC1 contributes to calcium-induced differentiation of human keratinocytes. *Pfl++gers Archiv European Journal of Physiology* 452: 43-52, 2006.

15. **Cioffi DL, Wu S and Stevens T**. On the endothelial cell I_{SOC}. *Cell Calcium* 33: 323-336, 2003.

16. **Clapham DE, Runnels LW and Strubing C**. The trp ion channel family. *Nat Rev Neurosci* 2: 387-396, 2001.

17. **Csutora P, Su Z, Kim HY, Bugrim A, Cunningham KW, Nuccitelli R, Keizer JE, Hanley MR, Blalock JE and Marchase RB**. Calcium influx factor is synthesized by yeast and mammalian cells depleted of organellar calcium stores. *Proceedings of the National Academy of Sciences* 96: 121-126, 1999.

18. **Drenckhahn D, Schnittler H, Nobiling R and Kriz W**. Ultrastructural organization of contractile proteins in rat glomerular mesangial cells. *Am J Pathol* 137: 1343-1351, 1990.

19. **Dziadek MA and Johnstone LS**. Biochemical properties and cellular localisation of STIM proteins. *Cell Calcium* 42: 123-132, 2007.

20. **Engelke M, Friedrich O, Budde P, Schafer C, Niemann U, Zitt C, Jungling E, Rocks O, Luckhoff A and Frey J**. Structural domains required for channel function of the mouse transient receptor potential protein homologue TRP1β. *FEBS Letters* 523: 193-199, 2002.

21. **Facemire CS, Mohler PJ and Arendshorst WJ**. Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation. *Am J Physiol Renal Physiol* 286: F546-F551, 2004.

22. **Fasolato C, Hoth M and Penner R**. A GTP-dependent step in the activation mechanism of capacitative calcium influx. *J Biol Chem* 268: 20737-20740, 1993.

23. **Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M and Rao A**. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441: 179-185, 2006.

24. **Ganong WF**. Review of Medical Physiology, Twenty-Second Edition. 2nd. 2005. United States, McGraw-Hill.

25. **Goel M, Sinkins WG, Zuo CD, Estacion M and Schilling WP**. Identification and localization of TRPC channels in the rat kidney. *Am J Physiol Renal Physiol* 290: F1241-F1252, 2006.

26. **Gomez-Garre D, Ruiz-Ortega M, Ortego M, Largo R, Lopez-Armada MJ, Plaza JJ, Gonzalez E and Egido J**. Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension* 27: 885-892, 1996.

27. **Greka A, Navarro B, Oancea E, Duggan A and Clapham DE**. TRPC5 is a regulator of hippocampal neurite length and growth cone morphology. *Nat Neurosci* 6: 837-845, 2003.

28. **Grigoriev I, Gouveia SM, van der Vaart B, Demmers J, Smyth JT, Honnappa S, Splinter D, Steinmetz MO, Putney JW, Hoogenraad CC and Akhmanova A**. STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. *Curr Biol* 18: 177-182, 2008.

29. **Herman WH, Emancipator SN, Rhoten RL and Simonson MS**. Vascular and glomerular expression of endothelin-1 in normal human kidney. *Am J Physiol Renal Physiol* 275: F8-17, 1998.

30. **Hiraoka-Yoshimoto M, Higashida K, Takeda M, Kawamoto S, Ichikawa I and Hoover RL**. Characterization of myosin heavy and light chains in cultured mesangial cells. *Kidney Int* 40: 1013-1019, 1991.

31. **Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T and Schultz G**. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397: 259-263, 1999.

32. **Hoth M and Penner R**. Calcium release-activated calcium current in rat mast cells. *J Physiol (Lond)* 465: 359-386, 1993.

33. **Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S and Worley PF**. STIM1 carboxyl-terminus activates native SOC, I_{CRAC} and TRPC1 channels. *Nat Cell Biol* 8: 1003-1010, 2007.

34. **Ichikawa I, Miele JF and Brenner BM**. Reversal of renal cortical actions of angiotensin II by verapamil and manganese. *Kidney Int* 16: 137-147, 1979.

35. **Kimura K, Suzuki N, Ohba S, Nagai R, Hiroi J, Mise N, Tojo A, Nagaoka A, Hirata Y, Goto A, Yazaki Y and Omata M**. Hypertensive glomerular damage as revealed by the expression of alpha-smooth muscle actin and non-muscle myosin. *Kidney Int Suppl* 55: S169-S172, 1996.

36. **Kumar B, Dreja K, Shah SS, Cheong A, Xu SZ, Sukumar P, Naylor J, Forte A, Cipollaro M, McHugh D, Kingston PA, Heagerty AM, Munsch CM, Bergdahl A, Hultgardh-Nilsson A, Gomez MF, Porter KE, Hellstrand P and Beech DJ**. Upregulated TRPC1 channel in vascular injury *in vivo* and its role in human neointimal hyperplasia. *Circ Res* 98: 557-563, 2006.

37. **Kunichika N, Yu Y, Remillard CV, Platoshyn O, Zhang S and Yuan JXJ.** Overexpression of TRPC1 enhances pulmonary vasoconstriction induced by capacitative Ca^{2+} entry. *Am J Physiol Lung Cell Mol Physiol* 287: L962-L969, 2004.

38. **Li WP, Tsiokas L, Sansom SC and Ma R.** Epidermal growth factor activates store-operated Ca^{2+} channels through an inositol 1,4,5-trisphosphate-independent pathway in human glomerular mesangial cells. *J Biol Chem* 279: 4570-4577, 2004.

39. **Ling BN.** Regulation of mesangial chloride channels by insulin and glucose: role in diabetic nephropathy. *Clin Exp Pharmacol Physiol* 23: 89-94, 1996.

40. **Ling BN, Matsunaga H, Ma HP and Eaton DC.** Role of growth-factors in mesangial cell ion-channel regulation. *Kidney International* 48: 1158-1166, 1995.

41. **Liou J, Kim ML, Do Heo W, Jones JT, Myers JW, Ferrell J and Meyer T.** STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} Influx. *Current Biology* 15: 1235-1241, 2005.

42. **Liu X, Bandyopadhyay BC, Singh BB, Groschner K and Ambudkar IS.** Molecular analysis of a store-operated and 2-acetyl-sn-glycerol-sensitive non-selective cation channel: Heteromeric assembly of TRPC1-TRPC3. *J Biol Chem* 280: 21600-21606, 2005.

43. **Liu X, Wang W, Singh BB, Lockwich T, Jadlowiec J, O' Connell B, Wellner R, Zhu MX and Ambudkar IS.** Trp1, a candidate protein for the store-operated Ca^{2+} influx mechanism in salivary gland cells. *J Biol Chem* 275: 3403-3411, 2000.

44. **Lockwich T, Singh BB, Liu X and Ambudkar IS**. Stabilization of cortical actin induces internalization of transient receptor potential 3 (Trp3)-associated caveolar Ca^{2+} signaling complex and loss of Ca^{2+} influx without disruption of Trp3-inositol trisphosphate receptor association. *J Biol Chem* 276: 42401-42408, 2001.

45. **Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S and Ambudkar IS**. Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* 275: 11934-11942, 2000.

46. **Ma R, Kudlacek PE and Sansom SC**. Protein kinase Cα participates in activation of store-operated Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol Cell Physiol* 283: C1390-C1398, 2002.

47. **Ma R, Pluznick J, Kudlacek P and Sansom SC**. Protein kinase C activates store-operated Ca^{2+} channels in human glomerular mesangial cells. *J Biol Chem* 276: 25759-25765, 2001.

48. **Ma R and Sansom SC**. Epidermal growth factor activates store-operated calcium channels in human glomerular mesangial cells. *J Am Soc Nephrol* 12: 47-53, 2001.

49. **Ma R, Du J, Sours S and Ding M**. Store-operated Ca^{2+} channel in renal microcirculation and glomeruli. *Experimental Biology and Medicine* 231: 145-153, 2006.

50. **Ma R, Smith S, Child A, Carmines PK and Sansom SC**. Store-operated Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 278: F954-F961, 2000.

51. **Mahieu PR, Foidart JB, Dubois CH, Dechenne CA and Deheneffe J.** Tissue culture of normal rat glomeruli: contractile activity of the cultured mesangial cells. *Invest Cell Pathol* 3: 121-128, 1980.

52. **Mene P, Pugliese G, Pricci F, Di Mario U, Cinotti GA and Pugliese F.** High glucose inhibits cytosolic calcium signaling in cultured rat mesangial cells. *Kidney Int* 43: 585-591, 1993.

53. **Mene P, Simonson MS and Dunn MJ.** Physiology of the mesangial cell. *Physiol Rev* 69: 1347-1424, 1989.

54. **Mene P, Teti A, Pugliese F and Cinotti GA.** Calcium release-activated calcium influx in cultured human mesangial cells. *Kidney Int* 46: 122-128, 1994.

55. **Mene P, Pascale C, Teti A, Bernardini S, Cinotti GA and Pugliese F.** Effects of advanced glycation end products on cytosolic Ca^{2+} signaling of cultured human mesangial cells. *J Am Soc Nephrol* 10: 1478-1486, 1999.

56. **Mene P, Festuccia F, Polci R, Pugliese F and Cinotti GA.** Transmembrane signalling in human monocyte/mesangial cell co-cultures: role of cytosolic Ca^{2+}. *Nephrol Dial Transplant* 17: 42-49, 2002.

57. **Montell C.** The TRP superfamily of cation channels. *Sci STKE* 2005: re3, 2005.

58. **Morel JL, rez-Lepretre N and Mironneau J.** Angiotensin II-activated Ca^{2+} entry-induced release of Ca^{2+} from intracellular stores in rat portal vein myocytes. *Br J Pharmacol* 118: 73-78, 1996.

59. **Mori Y, Wakamori M, Miyakawa T, Hermosura M, Hara Y, Nishida M, Hirose K, Mizushima A, Kurosaki M, Mori E, Gotoh K, Okada T, Fleig A,**

Penner R, Iino M and Kurosaki T. Transient receptor potential 1 regulates capacitative Ca^{2+} entry and Ca^{2+} release from endoplasmic reticulum in B lymphocytes. *J Exp Med* 195: 673-681, 2002.

60. **Nagahama T, Hayashi K, Ozawa Y, Takenaka T and Saruta T**. Role of protein kinase C in angiotensin II-induced constriction of renal microvessels. *Kidney Int* 57: 215-223, 2000.

61. **Nakai K, Ito C, Yumura W, Horita S, Nihei H, Sugino N and Nagai R**. Difference of myosin heavy chain expression between mesangial cells and vascular smooth muscles. *Nippon Jinzo Gakkai Shi* 37: 428-435, 1995.

62. **Nilius B, Owsianik G, Voets T and Peters JA**. Transient receptor potential cation channels in disease. *Physiol Rev* 87: 165-217, 2007.

63. **Nishio M, Tsukahara H, Hiraoka M, Sudo M, Kigoshi S and Muramatsu I**. Calcium channel current in cultured rat mesangial cells. *Mol Pharmacol* 43: 96-99, 1993.

64. **Nutt LK and O'Neil RG**. Effect of elevated glucose on endothelin-induced store-operated and non-store-operated calcium influx in renal mesangial cells. *J Am Soc Nephrol* 11: 1225-1235, 2000.

65. **Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, Imoto K and Mori Y**. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca^{2+}-permeable cation channel that is constitutively activated

and enhanced by stimulation of G protein-coupled receptor. *J Biol Chem* 274: 27359-27370, 1999.

66. **Ong HL, Cheng KT, Liu X, Bandyopadhyay BC, Paria BC, Soboloff J, Pani B, Gwack Y, Srikanth S, Singh BB, Gill D and Ambudkar IS**. Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx: Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem* 282: 9105-9116, 2007.

67. **Parekh AB**. Store-operated Ca^{2+} entry: dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane. *J Physiol (Lond)* 547: 333-348, 2003.

68. **Parekh AB and Putney JW, Jr.** Store-operated calcium channels. *Physiol Rev* 85: 757-810, 2005.

69. **Patterson RL, van Rossum DB and Gill DL**. Store-operated Ca^{2+} entry: Evidence for a secretion-like coupling model. *Cell* 98: 487-499, 1999.

70. **Putney JW**. A model for receptor-regulated calcium entry. *Cell Calcium* 7: 1-12, 1986.

71. **Putney JW, Jr. and McKay RR**. Capacitative calcium entry channels. *Bioessays* 21: 38-46, 1999.

72. **Putney JW, Jr.** New molecular players in capacitative Ca^{2+} entry. *J Cell Sci* 120: 1959-1965, 2007.

73. **Putney JW, Jr.** Capacitative calcium entry: sensing the calcium stores. *J Cell Biol* 169: 381-382, 2005.

74. **Putney J**. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42: 103-110, 2007.

75. **Randriamampita C and Tsien RY**. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* 364: 809-814, 1993.

76. **Rao JN, Platoshyn O, Golovina VA, Liu L, Zou T, Marasa BS, Turner DJ, Yuan JXJ and Wang JY**. TRPC1 functions as a store-operated Ca^{2+} channel in intestinal epithelial cells and regulates early mucosal restitution after wounding. *Am J Physiol Gastrointest Liver Physiol* 290: G782-G792, 2006.

77. **Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G and Stauderman KA**. STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol* 169: 435-445, 2005.

78. **Sakai T and Kriz W**. The structural relationship between mesangial cells and basement membrane of the renal glomerulus. *Anat Embryol (Berl)* 176: 373-386, 1987.

79. **Singh BB, Liu X, Tang J, Zhu MX and Ambudkar IS**. Calmodulin regulates Ca^{2+}-dependent feedback inhibition of store-operated Ca^{2+} influx by interaction with a site in the C terminus of TrpC1. *Molecular Cell* 9: 739-750, 2002.

80. **Smani T, Zakharov SI, Leno E, Csutora P, Trepakova ES and Bolotina VM.** Ca^{2+}-independent phospholipase A2 is a novel determinant of store-operated Ca^{2+} Entry. *J Biol Chem* 278: 11909-11915, 2003.

81. **Sorokin A and Kohan DE.** Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285: F579-F589, 2003.

82. **Stockand JD and Sansom SC.** Glomerular mesangial cells: Electrophysiology and regulation of contraction. *Physiol Rev* 78: 723-744, 1998.

83. **Takeda K, Meyer-Lehnert H, Kim JK and Schrier RW.** Effect of angiotensin II on Ca^{2+} kinetics and contraction in cultured rat glomerular mesangial cells. *Am J Physiol Renal Physiol* 254: F254-F266, 1988.

84. **Takenaka T, Suzuki H, Okada H, Inoue T, Kanno Y, Ozawa Y, Hayashi K and Saruta T.** Transient receptor potential channels in rat renal microcirculation: actions of angiotensin II. *Kidney Int* 62: 558-565, 2002.

85. **Thomas D and Hanley MR.** Evaluation of calcium influx factors from stimulated jurkat T-lymphocytes by microinjection into Xenopus Oocytes. *J Biol Chem* 270: 6429-6432, 1995.

86. **Thyagarajan B, Poteser M, Romanin C, Kahr H, Zhu MX and Groschner K.** Expression of Trp3 determines sensitivity of capacitative Ca^{2+} entry to nitric oxide and mitochondrial Ca^{2+} handling. Evidence for a role of Trp3 as a subunit of capacitative Ca^{2+} entry channels. *J Biol Chem* 276: 48149-48158, 2001.

87. **Trepakova ES, Csutora P, Hunton DL, Marchase RB, Cohen RA and Bolotina VM**. Calcium influx factor directly activates store-operated cation channels in vascular smooth muscle cells. *J Biol Chem* 275: 26158-26163, 2000.

88. **Trepakova ES, Gericke M, Hirakawa Y, Weisbrod RM, Cohen RA and Bolotina VM**. Properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. *J Biol Chem* 276: 7782-7790, 2001.

89. **Tryggvason K and Wartiovaara J**. How does the kidney filter plasma? *Physiology* 20: 96-101, 2005.

90. **Vaca L and Kunze DL**. Depletion of intracellular Ca^{2+} stores activates a Ca^{2+}-selective channel in vascular endothelium. *Am J Physiol Cell Physiol* 267: C920-C925, 1994.

91. **Vazquez G, de Boland AR and Boland RL**. Involvement of calmodulin in 1alpha ,25-Dihydroxyvitamin D3 stimulation of store-operated Ca^{2+} influx in skeletal muscle cells. *J Biol Chem* 275: 16134-16138, 2000.

92. **Vazquez G, Lievremont JP, Bird J and Putney JW, Jr.** Human Trp3 forms both inositol trisphosphate receptor-dependent and receptor-independent store-operated cation channels in DT40 avian B lymphocytes. *PNAS* 98: 11777-11782, 2001.

93. **Vazquez G, Wedel BJ, Aziz O, Trebak M and Putney J**. The mammalian TRPC cation channels. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1742: 21-36, 2004.

94. **Venkatachalam K, van Rossum DB, Patterson RL, Ma HT and Gill DL**. The cellular and molecular basis of store-operated calcium entry. *Nat Cell Biol* 4: E263-E272, 2002.

95. **Venkatachalam K, Zheng F and Gill DL**. Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *J Biol Chem* 278: 29031-29040, 2003.

96. **Wu X, Zagranichnaya TK, Gurda GT, Eves EM and Villereal ML**. A TRPC1/TRPC3-mediated increase in store-operated calcium entry is required for differentiation of H19-7 hippocampal neuronal cells. *J Biol Chem* 279: 43392-43402, 2004.

97. **Yamamoto T, Hayashi K, Matsuda H, Kubota E, Tanaka H, Ogasawara Y, Nakamoto H, Suzuki H, Saruta T and Kajiya F**. In vivo visualization of angiotensin II- and tubuloglomerular feedback-mediated renal vasoconstriction. *Kidney Int* 60: 364-369, 2001.

98. **Yao Y, Ferrer-Montiel AV, Montal M and Tsien RY**. Activation of store-operated Ca^{2+} current in Xenopus Oocytes requires SNAP-25 but not a diffusible messenger. *Cell* 98: 475-485, 1999.

99. **Yu YM, Lermioglu F and Hassid A**. Modulation of Ca^{2+} by agents affecting voltage-sensitive Ca^{2+} channels in mesangial cells. *Am J Physiol Renal Physiol* 257: F1094-F1099, 1989.

100. **Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S and Worley PF**. Homer binds

TRPC family channels and is required for gating of TRPC1 by IP3 receptors. *Cell* 114: 777-789, 2003.

101. **Yuan JP, Zeng W, Huang GN, Worley PF and Muallem S.** STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* 9: 636-645, 2007.

CHAPTER II

EXPRESSION OF CANONICAL TRANSIENT RECEPTOR POTENTIAL (TRPC) PROTEINS IN HUMAN GLOMERULAR MESANGIAL CELLS

Sherry Sours,[1] Juan Du,[1,2] Shaoyou Chu,[3] Min Ding,[1] Xin J. Zhou,[4] and Rong Ma[1]

[1]Department of Integrative Physiology and [3]Department of Cell Biology and Genetics, University of North Texas Health Science Center at Fort Worth, Fort Worth; [4]Department of Pathology, Division of Renal Pathology, UT Southwestern Medical Center at Dallas, Dallas, Texas; and [2]Department of Physiology, Anhui Medical University, Hefei, People's Republic of China.

American Journal of Physiology, Renal Physiology, 290: F1507-F1515, 2006; doi:10.1152/ajprenal.00268.2005.

Abstract

Mesangial cells are located within glomerular capillary loops and contribute to the physiological regulation of glomerular hemodynamics. The function of mesangial cells is controlled by a variety of ion channels in the plasma membrane, including nonselective cation channels, receptor-operated Ca^{2+} channels, and recently identified store-operated Ca^{2+} channels. Although the significance of these channels has been widely acknowledged, their molecular identities are still unknown. Recently, the members of the canonical transient receptor potential (TRPC) protein family have been demonstrated to behave as cation channels. The present study was performed to identify the isoforms of endogenous TRPC proteins in human mesangial cells (HMCs) and their interactions. Western blotting showed that TRPC1, 3, 4, and 6 were expressed in cultured HMCs. Consistently, immunofluorescent confocal microscopy revealed specific stainings for TRPC1, 3, 4, and 6 with predominant intracellular localization. However, TRPC5 and 7 were not detectable at protein level by either Western blotting or immunofluorescent staining. The expression of TRPC1, 3, 4, and 6 was also observed in rat and human glomeruli using fluorescent immunohistochemistry. Furthermore, coimmunoprecipitation experiments and immunofluorescent double staining displayed that TRPC1 had physical interaction with TRPC4 and 6, while no interactions were detected among other isoforms of TRPCs. Ca^{2+} fluorescent ratiometry measurement showed that store-operated Ca^{2+} entry in HMCs was significantly reduced by knocking down TRPC1, but enhanced by overexpressing TRPC1. These results suggest that HMCs specifically express isoforms of

TRPC1, 3, 4, and 6 proteins. These isoforms of TRPCs might selectively assemble to form functional complexes.

Keywords: store-operated Ca^{2+} entry; Ca^{2+} channel; Ca^{2+} signaling; glomeruli

Introduction

Mesangial cells (MCs) reside in the glomerular tuft, strategically positioned for the regulation of glomerular hemodynamics (26, 35). Like vascular smooth muscle cells, MCs contract in response to ANG II and relax in response to nitric oxide (25, 35). Altered responsiveness of MCs to the vasoactive hormones is one of the major causes leading to certain severe renal diseases, such as diabetic nephropathy. Modulation of mesangial cell ion transport plays an integral role in mediating many physiological and pathological responses, including mesangial cell contraction, mesangial cell growth, and mesangial matrix accumulation. It has been documented that binding of both vasoactive hormones and growth factors to mesangial cell receptors promotes an increase in mesangial cell cytosolic Ca^{2+} (26, 27, 30, 35). This process involves both the release of intracellular Ca^{2+} stores and extracellular Ca^{2+} entry. The latter occurs via classic, voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+} channels, and recently identified store-operated Ca^{2+} channels in the mesangial cell plasma membrane (22).

Although a variety of channels have been described in various mesangial cultures using patch-clamp techniques (22), the majority of channels involved in Ca^{2+} influx via the plasma membrane have not been defined at the molecular level. These channels include nonselective cation channels, receptor-operated Ca^{2+} channels, and store-operated Ca^{2+} channels. Recently, mammalian canonical TRP (transient receptor potential) channels (TRPCs) have been proposed as Ca^{2+}-permeable cation channels that are activated in response to stimulation of G protein-coupled receptors or receptor tyrosine kinases (1, 12, 14, 32). Belonging to the TRP superfamily, TRPC family includes seven

related members, designated TRPC1–7 (29). TRPC2 is a pseudogene in humans (47). The remaining six appear to fall into two groups based on structural and functional similarities: TRPC1, -4, and -5, and TRPC3, -6, and -7 (13). TRPCs have been discovered in a variety of cell types, including vascular smooth muscle (8, 9, 34, 43). In addition, the seven TRPC proteins can assemble to form homomers or heteromers (6, 10, 13, 36, 37). The loss-of- and gain-of-function experiments have provided convincing evidence that various TRPC proteins might function as receptor-operated or store-operated Ca^{2+}-permeable channels or nonselective cation channels in a wide range of cell lines, primary cultures or native tissues (21, 24, 32, 40, 41, 46). However, with respect to the function of a particular subtype of TRPC protein, the data are contradictory among investigators partially because expression of TRPC isoforms is tissue and cell type specific (8, 9, 34). This can be even more sophisticated by the fact that different assembly of TRPC isoforms expresses differential channel behavior and gating mechanisms, and therefore, might have different physiological significance (1, 20, 28, 37, 42). Thus identifying subtypes of endogenous TRPC proteins and the rules governing subunit assembly of individual TRPCs in a particular type of cells should be an essential step to unravel molecular entity of receptor-operated, store-operated Ca^{2+} channels, or nonselective cation channels. Because this information is extremely devoid in human glomerular mesangial cells (HMCs), important cells relevant to physiological function and the pathophysiological process in kidneys, we have, in the present study, employed Western blotting, RT-PCR, confocal microscopy, immunohistochemistry, and coimmunoprecipitation to systematically assess subtypes of TRPC proteins present in

HMCs and the physical interaction among them. Our data show that TRPC1, 3, 4, and 6 are expressed in HMCs at the protein level, whereas TRPC5 and 7 are only detectable at the messenger level. TRPC1 might have physical interaction with TRPC4 and 6 in this type of cell.

Materials and Methods

Preparation of cultures of HMCs. HMCs used in this study were purchased from Cambrex (East Ruthersord, NJ). The procedures and methods for culturing HMC were described previously (16). Briefly, HMCs were cultured in DMEM (GIBCO, Carlsbad, CA) supplemented with 25 mM HEPES, 4 mM glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum. Only subpassages less than 11 generations of cells were used in the present study.

Transient transfection. Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) were used to transiently transfect *trpc1–7* cDNA plasmids or *trpc*1 or 4 or 6 RNAi constructs into H293T cells (in 10-cm plates) following instruction provided by the manufactor. The cells were lysed 24–48 h after transfection.

Immunoprecipitation and immunoblots. When cell monolayers were 80% confluent, the cells were washed twice with PBS and then lysed in 1 ml of 1% Triton X-100 buffer (per 10-ml plate) containing (in mM) 150 NaCl, 10 Tris·HCl (pH 7.5), 1 EGTA, 0.2 sodium orthovanadate, 0.2 phenylmethylsulfonyl fluoride, 0.5% NP-40, aprotinin (1 μg/ml), pepstatin (1 μg/ml), and proteinase inhibitor cocktail (Roche Applied

Science, Indianapolis, IN). The cell lysates were centrifuged at 6,000 *g* for 15 min at 4°C. For coimmunoprecipitation experiments, the cell lysates were incubated for 2 h with specific TRPC antibody and complexes were captured for 1 h with 30 μl slurry of protein G or A (Amersham Biosciences, Piscataway, NJ) in 50 mM Tris·HCl. Immunocomplexes were then washed five times in lysis buffer. The cell lysates (without precipitation, for regular Western blotting) or immunoprecipitates (for coimmunoprecipitation) were fractionated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated primary TRPC antibodies. Bound antibodies will be visualized with Super Signal West Femto Luminol/Enhancer Solution (Pierce Biotechnology, Rockford, IL).

TRPC antibodies and plasmids. TRPC1 mouse monoclonal and rabbit polyclonal antibodies were obtained from Dr. L. Tsiokas's laboratory (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The specificity and efficiency of the antibodies have been described previously (23, 31). TRPC3–7 goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRPC3 and 6 rabbit polyclonal antibodies were from Alomone Labs. *trpc* Plasmids were obtained from following sources. Human TRPC1-pGEX-3X and TRPC3-pcDNA3 were obtained from C. Montell (Johns Hopkins Medical School, Baltimore, MD). Bovine TRPC4-pcDNA3, mTRPC5-pcDNA3, and mTRPC6-pcDNA3 were obtained from L. Birnbaumer (National Institutes of Health). mTRPC7-pCIneo was obtained from Y. Mori (Okazaki National Research Institute). Trpc1-RNAi construct (trpc1-pSHAG1–2219) was provided by Dr. L.

Tsiokas (University of Oklahoma Health Sciences Center), and trpc4- and 6-RNAi construct was obtained from Dr. M. Villereal (University of Chicago, Chicago, IL).

Fluorescent immunocytochemistry. HMC cells were plated on 22 × 22.1-mm coverslips, fixed with iced acetone/methanol, and incubated with a TRPC antibody or two TRPC antibodies (for double staining) in PBS plus 2% heat-inactivated goat or fetal bovine serum and 0.2% Triton X-100 for 1 h. Three washes with PBS containing 2% goat or fetal bovine serum and 0.2% Triton X-100 were followed by blocking at 4°C overnight in blocking buffer containing 2% goat or fetal bovine serum and 0.2% Triton X-100. The cells were then incubated with secondary antibodies (goat anti-rabbit conjugated with Alexa Fluor 488 or goat anti-mouse conjugated with Alexa Fluor 568 or donkey anti-goat conjugated with Alexa Fluor 488, Molecular Probes, Eugene, OR) for 1 h. Cells were washed three times with PBS and processed for confocal microscopy using a Leica Confocal Laser Scanning microscope.

Fluorescent immunohistochemistry. Adult male Wistar-Kyoto rats (weighing 200–250 g) were kept under environmentally controlled conditions (12:12-h light-dark cycle, 20–22°C) with food and water available ad libitum until used. All animals were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. Kidneys were perfused with physiological saline solution via a catheter inserted into abdominal aorta followed by 4% paraformaldehyde and then excised from the animals. The kidneys were fixed in 2% paraformaldehyde in K^+-free PBS over 2 h at 4°C, immersed in 30% sucrose overnight at 4°C, and cryosectioned at 20 μm thickness (Cryostat 2800 Frigocut-E, Leica

Instruments). The sections were washed with K^+-free PBS and treated with blocking buffer containing 50 mM NH_4Cl, 2% BSA, 0.05% saponin in K^+-free PBS for 20 min at room temperature for permeabilization. The sections were then incubated overnight at 4°C in blocking buffer containing mouse monoclonal anti-TRPC1, rabbit polyclonal anti-TRPC3 or 6, or goat polyclonal anti-TRPC4 antibody. To label glomerular mesangial cells, we also incubated the sections with rabbit polyclonal or mouse monoclonal anti-desmin antibody (depending on the host of TRPC antibodies) overnight at 4°C. The concentrations for all primary antibodies were 2–5 μg/ml. The sections were rinsed and incubated for 30 min at room temperature with Alexa Fluor 488 goat anti-rabbit IgG or donkey anti-mouse IgG, or Alexa Fluor 568 goat anti-mouse IgG or donkey anti-goat IgG (Molecular Probes), depending on primary antibodies. The concentrations of the secondary antibodies were 2 μg/ml. In control slides, equal amounts of rabbit IgG, mouse IgG, or goat IgG were used instead of the primary antibodies. All stainings were visualized under confocal laser-scanning microscopy (Zeiss LSM410). Normal human kidney tissues were obtained from archival nephrectomy specimens. Paraffin sections of each specimen were stained with hematoxylin and eosin and were examined to confirm the preservation of histological structures and to validate the normality. For immunostaining, the sections were cut at 5 μm and a standard protocol of xylene and graded ethanol was employed to deparaffinize and rehydrate. TRPC protein stainings were detected using the same protocol as described above for rat kidney sections.

Ratiometric Ca^{2+} measurements. Cells were harvested in PBS containing 0.5 mM EDTA, washed with PSS, and loaded with 2 μM indo-1/AM in PSS containing 0.05%

Pluronic F-127 (Molecular Probes) for 40 min at room temperature. After the 40-min incubation, cells were washed three times with a nominally Ca^{2+}-free solution. About 2 × 106 cells were resuspended in 2 ml of Ca^{2+}-free solution. Cells in Ca^{2+}-free solution were first incubated with 1 μM TG for 30 min to deplete the internal Ca^{2+} stores and then Ca^{2+} entry was determined by Ca^{2+} readdition (10 mM $CaCl_2$). Ratiometric measurements representing free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were obtained by a PTI QuantaMaster spectrofluorometer equipped with an excitation monochromator set at 350 nm and two emission monochromators set at 405 and 485 nm.

Results

Expression of TRPC proteins. We employed regular Western blotting to detect subtypes of endogenous TRPC proteins expressed in HMCs. With specific TRPC antibodies, we were able to identify immunoblots probed with anti-TRPC1, 3, 4, and 6 antibodies in the lysates from HMCs (Fig. 1). The sizes of the bands (80–90 kDa) were a little lower than predicted. However, they were located at the same level as their corresponding positive controls (lysates from TRPC-transfected H293T cells). To further confirm the specificity of the imunoblots, we also Western-blotted lysates from H293T or HMCs cells transfected with trpc1- or 4-RNAi constructs for knocking down corresponding TRPC (Fig. 1, *A* and *C*) or lysates from HMCs with preadsorbing corresponding primary antibodies (Fig. 1, *B* and *E*). As shown in Fig. 1, the immunoblots were either completely blocked or significantly reduced by the specific gene silencing or antigen competition. As reported by many investigators (3, 8, 31), immunoblotting

endogenous TRPC proteins in native tissues is very difficult, presumably because of immunogenicity of TRPC antibodies or inherent properties of TRPC proteins. In the present study, we had to use a Femto chemiluminescent substrate to obtain clear TRPC bands. In contrast to TRPC1, 3, 4, and 6, TRPC5 and 7 were not detectable in HMCs (Fig. 1) even though a ~90-kDa band was clearly shown in the lysates from *trpc5*- and *trpc7*-transfected H293T cells. The specific TRPC5 and 7 immunoblots were also unable to be detected in the lysates of HMCs using the antibodies from W. P. Schilling (Case Western Reserve University School of Medicine, Cleveland, OH; data not shown).

Expression of TRPC proteins was also detected with fluorescence from immunocomplexes of specific TRPC protein antibodies and their Fluor-conjugated secondary antibodies using confocal laser-scanning microscopy. In agreement with the results from Western blotting, immunofluorescence stainings specific for TRPC1, 3, 4, and 6, but not TRPC5 and 7, were observed (Fig. 2). All of the expressed TRPCs displayed ubiquitous subcellular distribution, showing predominant intracellular localization with a pattern consistent with the endoplasmic reticulum and/or vesicles. In addition, TRPC1 and TRPC4 stainings were also clearly seen in the region of the plasma membrane while TRPC6 staining appears mostly localized to the nucleus.

In vivo *expression of TRPCs in rat and human glomeruli.* Because of the inaccessibility of mesangial cells in vivo, their function and cell biology have been mostly studied after several generations of growth in a culture environment. However, culture condition could change the phenotypes of mesangial cells from those in in vivo environment (15, 22). Therefore, the results obtained from cultured HMCs might not

necessarily represent physiological presence of TRPCs in this type of cells. In particular, a recent study reported that expression of TRPCs in arterial smooth muscle might be associated with organ culture per se (4). Thus immunohistochemistry was performed in rat kidney sections and isotypes of TRPCs identified in cultured HMCs (TRPC1, 3, 4, and 6) were detected with specific anti-TRPC antibodies. Glomeruli were easily distinguished by their characteristic circular morphological aspect bordered by peripheral lumen. Glomerular mesangial cells were identified with positive staining with antibody against desmin. Consistent with the data described in cultured cells, stainings specific for TRPC1, 3, 4, and 6 were detected in desmin-labeled cells (Fig. 3, *A* and *B*). The specificity of the stainings was confirmed by failure in detecting stainings in the samples treated with control immunoglobulins (Fig. 3*A*). Not surprisingly, TRPC stainings were also found in the regions inside glomeruli, which were not stained by desmin antibody, suggesting that TRPCs also reside in other types of glomerular cells.

Expression of TRPC proteins in glomeruli was also detected in human kidney tissue sections by immunohistochemistry. Although there is no specific marker for mesangial cells in *in vivo* human glomeruli, the diffuse pattern of TRPC stainings indicates that TRPC1, 3, 4, and 6 are strongly and widely expressed in human glomerular cells, most likely including mesangial cells (Fig. 3*C*). In addition, TRPC stainings were also found in renal tubular epithelia. These results are consistent with the findings from recent studies which demonstrated expression of TRPC proteins in glomerular podocytes and renal tubule epithelial cells (11, 33).

Heteromeric interaction between endogenous TRPCs in HMCs. It has been reported that both exogenously expressed and endogenous TRPC proteins form multimeric protein assembly giving rise to biophysically and functionally discernible channel entities (1, 10, 13, 20). Thus we examined possible heteromerization between the natively present TRPCs in HMCs using coimmunoprecipitation. As shown in Fig. 4, when anti-TRPC1 antibody was used to pull down TRPC1, TRPC4 and 6, but not TRPC3, were coimmunoprecipitated (Fig. 4*A*). However, neither IP of TRPC4 pull down TRPC3 or TRPC6 (Fig. 4*B*) nor IP of TRPC6 pull down TRPC3 (Fig. 4*C*). These data suggest a selective interaction of TRPC1 with TRPC4 and TRPC1 with TRPC6 in HMCs.

The selective interaction between TRPC1 and TRPC4 and 6 was verified by immunofluorescent double staining. Similar to Fig. 2, stainings for TRPC1, 3, 4, and 6 were seen in multiple fixed HMCs (Fig. 5). Overlaying TRPC1 signal (red) with TRPC3 or 4 or 6 (green) shows clear colocalization (yellow) between TRPC1 and TRPC4 and 6, but not TRPC3 (Fig. 5).

Mediation of store-operated Ca^{2+} entry by TRPC1 in HMCs. We selected TRPC1 as a representative of TRPC channel proteins to detect their physiological function in mesangial cells because TRPC1 is the founding member of TRPC family and is highly expressed in HMCs. Emerging evidence demonstrated that specific TRPC proteins might be candidates of store-operated Ca^{2+} channel (21, 32). Therefore, we tested the role of TRPC1 in store-operated Ca^{2+} entry using ratiometric measurement of $[Ca^{2+}]_i$ in response to Ca^{2+} readmission, a typical protocol of testing store-operated Ca^{2+} entry. Internal Ca^{2+} stores were depleted by incubating HMCs with 1 μM thapsigargin for 30 min in the

nominally Ca^{2+}-free solution. As shown in Fig. 6*A*, re-addition of Ca^{2+} into the bathing solution induced a remarkable rise of $[Ca^{2+}]_i$ in control HMCs. However, this response was significantly attenuated by knocking down TRPC1 and enhanced by overexpressing TRPC1 (Fig. 6, *A* and *B*), suggesting an essential role of TRPC1 in mediating store-operated Ca^{2+} entry in HMCs.

Discussion

In the present study, we employed multiple approaches and found that in HMCs, *1*) TRPC1, 3, 4, and 6, but not TRPC5 and 7, were expressed at protein level; *2*) TRPC1 physically interacted with TRPC4 and 6; *3*) the proteins of TRPC1, 3, 4, and 6 were also found in rat and human glomeruli; and *4*) TRPC1 participated in store-operated Ca^{2+} entry.

It has been documented that TRPC proteins exist almost ubiquitously in mammalian tissues. However, the expression of TRPC isoforms is tissue and cell type specific and may also be species specific (8, 9, 45). For instance, high levels of TRPC1, 3, and 5 mRNA were found in human cerebellum (34). Human parotid gland ductal cells endogenously expressed TRPC1, 3, and 4, but not TRPC5 or 6 (20). However, TRPC1, 3, 4, 5, and 6 mRNA and protein were detected in rat renal resistance vessels, whereas TRPC2 and 7 mRNA were not expressed (8). The data in the present study revealed that human-originated mesangial cells expressed the proteins of TRPC1, 3, 4, and 6. These results were further supported by immunohistochemistry data from rat and human kidney sections. Incapability of identifying TRPC5 and 7 proteins indicates that either TRPC5

and 7 is deficient in HMCs because of deficiency in posttranscriptional mechanism or the amounts of TRPC5 and 7 proteins are below the detectable level. Our results are not completely in agreement with those from a recent study by Wang et al. (45) in which TRPC1 and TRPC4 mRNA were the only two TRPC messengers identified in mouse glomerular mesangial cells. This discrepancy might be due to species difference (human vs. mouse). Indeed, rat glomeruli, in which glomerular mesangial cells are located, express TRPC1, 3, 5, and 6 protein, but not TRPC4 and 7 (8). The species-dependent TRPC expression is also seen in other tissues. For example, TRPC3 and 6 are not detectable in adult rat brain (37) but highly expressed in human central nervous system (34).

Fluorescence resonance energy transfer measurements and immunoprecipitation experiments demonstrated that the seven TRPC proteins (TRPC1–7) can assemble to form heteromers (10, 13, 36, 37). The heteromeric interactions between the TRPC proteins are proposed to occur between members of two groups of TRPCs: TRPC1/TRPC4/TRPC5 and TRPC3/ TRPC6/TRPC7. Consistent with those studies, we found that TRPC1 was associated with TRPC4 in HMCs. In addition to this known interaction between TRPCs, in the present study we also found that TRPC1 physically interacted with TRPC6. Exceptions to the TRPC protein interaction governed by the principals described by Hofmann et al. (13) have been reported by several groups. For instance, that TRPC1 and TRPC3 co-assemble to form a heteromeric complex has been reported in exogenously expressed HEK293 cells and in native human parotid gland ductal cells (19, 20). The novel heteromeric associations between endogenous TRPCs

were also described by Strübing et al. (37) in embryonic brain. The novel interaction between TRPC1 and TRPC6 found in the present study suggests that this heteromeric TRPC protein complex might underlie a channel mechanism specific for HMCs. It is true that diversity of TRPC function and regulation in different types of tissues or cells is derived from diverse assembly of TRPC subunits (1, 19, 20, 36, 37). The tissue- and cell type-dependent expression profile of TRPCs (9, 34) also fits the diverse properties of store-operated or receptor-operated channels found in a variety of type of cells given that TRPCs are potential molecular candidates of these channels. Different assemblies of TRPC isoforms found in the present study (TRPC1 with TRPC4, and TRPC1 with TRPC6) might underlie differential molecular entities of different types of ion channels (nonselective cation channel, receptor-operated Ca^{2+} channel, or store-operated Ca^{2+} channel) present in HMCs. Another interesting finding in this study, which contradicted the results from Hofmann et al. (13), is that no interaction between TRPC3 and TRPC6 was detected even though the two isoforms of TRPCs are expressed in HMCs. We have used different anti-TRPC3 and TRPC6 antibodies (goat IgG and rabbit IgG) from different sources (Santa Cruz Biotechnology and Alomone Lab) and the interaction between TRPC3 and TRPC6 was never observed in coimmunoprecipitation or reverse coimmunoprecipitation. We speculate that the TRPC3- TRPC6 interaction might not exist in HMCs and each of the TRPCs might have another partner for its specific function.

As founding members of the TRP superfamily, TRPCs take part in a wide range of physiological functions, including nerve growth (5, 17, 44), vascular tone (7, 14, 46), permeability of vascular endothelium (39), cell proliferation (38, 49), and

mechanosensation (2). TRPC proteins might also be associated with development of certain diseases, such as pulmonary hypertension (18, 48). Glomerular mesangial cells have important physiological and pathophysiological relevance, and the function of mesangial cells is controlled by a variety of ion channels, including nonselective cation channel, receptor-operated and store-operated Ca^{2+} channels. However, the molecular entities of these channels are still unknown. Because expressed or endogenous individual TRPCs or TRPC complexes with differential assembly behave as nonselective cation channels or receptor-operated Ca^{2+} channels or store-operated Ca^{2+} channels depending on tissue and cell type, we speculate that the existing TRPCs found in the present study might constitute a functional entity of the three types of important channels in HMCs. This speculation was supported by the findings that TRPC1 and TRPC4 stainings were clearly seen in the region of the plasma membrane (Figs. 2 and 5), implying that the two subtypes of TRPCs might be important components of cation channels in the cell membrane of HMCs. Our functional data provide further support for the notion by showing mediation of store-operated Ca^{2+} entry via TRPC1 protein in HMCs (Fig. 6). Furthermore, immunocytochemistry revealed that TRPC6 is mostly localized to the nucleus of HMCs (Figs. 2 and 5). This unique localization of TRPC6 might imply an important role of this TRPC isoform in regulating mesangial proliferation and contractile function by modulating particular processes of gene transfer and transcription. Apparently, more functional and mechanistic studies are required to explore the physiological function of the TRPC proteins and their regulation under normal and disease states.

In conclusion, our results suggest that HMCs specifically express isoforms of TRPC1, 3, 4, and 6 proteins. These isoforms of TRPCs might selectively assemble together to form functional complexes, which might underlie diverse channel mechanisms found in mesangial cells.

Acknowledgements

We thank Dr. L. Tsiokas (University of Oklahoma Health Sciences Center, Oklahoma City, OK) for generously providing the anti-TRPC1 antibody and human *trpc1*-RNAi construct, Dr. W. Schilling (MetroHealth Campus of Case Western Reserve University, Clevelend, OH) for providing the anti-TRPC3, anti-TRPC5, and -TRPC7 antibodies (rabbit IgG), and Dr. M. Villereal (University of Chicago, Chicago, IL) for providing human *trpc4*- and *trpc6*-RNAi constructs.

Grants

This study was supported by a Scientist Development Grant from American Heart Association National Center (R. Ma, 0430026N).

Figure 1. Western blotting. Transient receptor potential channel (TRPC)1, 3, 4, and 6 were detected in native human mesangial cells (HMCs). However, TRPC5 and 7 were not detectable. *A*: H293T *trpc1*(-) indicates lysates from H293T cells transfected with human *trpc1* expression plasmids, served as a positive control. H293T *trpc1*(-) represents lysates from H293T cells cotransfected with human *trpc1* expression plasmids and human *trpc1*-RNAi constructs, serving as a negative control. *B*, *left*: H293T *trpc3*(-) indicates lysates from H293T cells transfected with human *trpc3* expression plasmids, serving as a positive control. *B*, *right*: anti-TRPC 3 antibody was preadsorbed by incubating the antibody with its blocking peptide for 1 h at room temperature, serving as a negative control. *C*, *left*: TRPC4 immunoblots in HMCs treated with human *trpc4*-RNAi constructs [*trpc4*(-)] or empty vectors (Con). *C*, *right*: TRPC4 immunoblots in H293T cells transfected with bovine *trpc4* expression plasmids [H293T-*trpc4*(-) and in HMCs]. HMC-*trpc4*(-) and H293T-*trpc4*(-) served as negative and positive controls for TRPC4, respectively. *D*: H293T *trpc5*(-) indicates lysates from H293T cells transfected with mouse *trpc5* expression plasmids, serving as a positive control. H293T con represents lysates from H293T cells transfected with empty vectors, serving as a negative control. *E*: HMC *trpc6(-)* indicates lysates from HMC cells transfected with human *trpc6*-RNAi constructs, serving as a negative control. H293T *trpc6(-)* indicates lysates from H293T cells transfected with mouse *trpc6* expression plasmids, serving as positive control for TRPC6. *F*, *left*: H293T *trpc*7(-) represent lysates from H293T cells tranfected with mouse *trpc7* expression plasmids, serving as a positive control. *F*, *right*: anti-TRPC7 antibody was preadsorbed by preincubating the antibody with its blocking peptide for 1 h

at room temperature, serving as negative control. Antibodies used: anti-TRPC1, monoclonal mouse IgG, 1:1,000 dilution; anti-TRPC3–7, polyclonal goat IgG, 1:200 dilution. Immunoblots were visualized with ECL reagents at Femto level. Actin was used as loading control.

Figure 1.

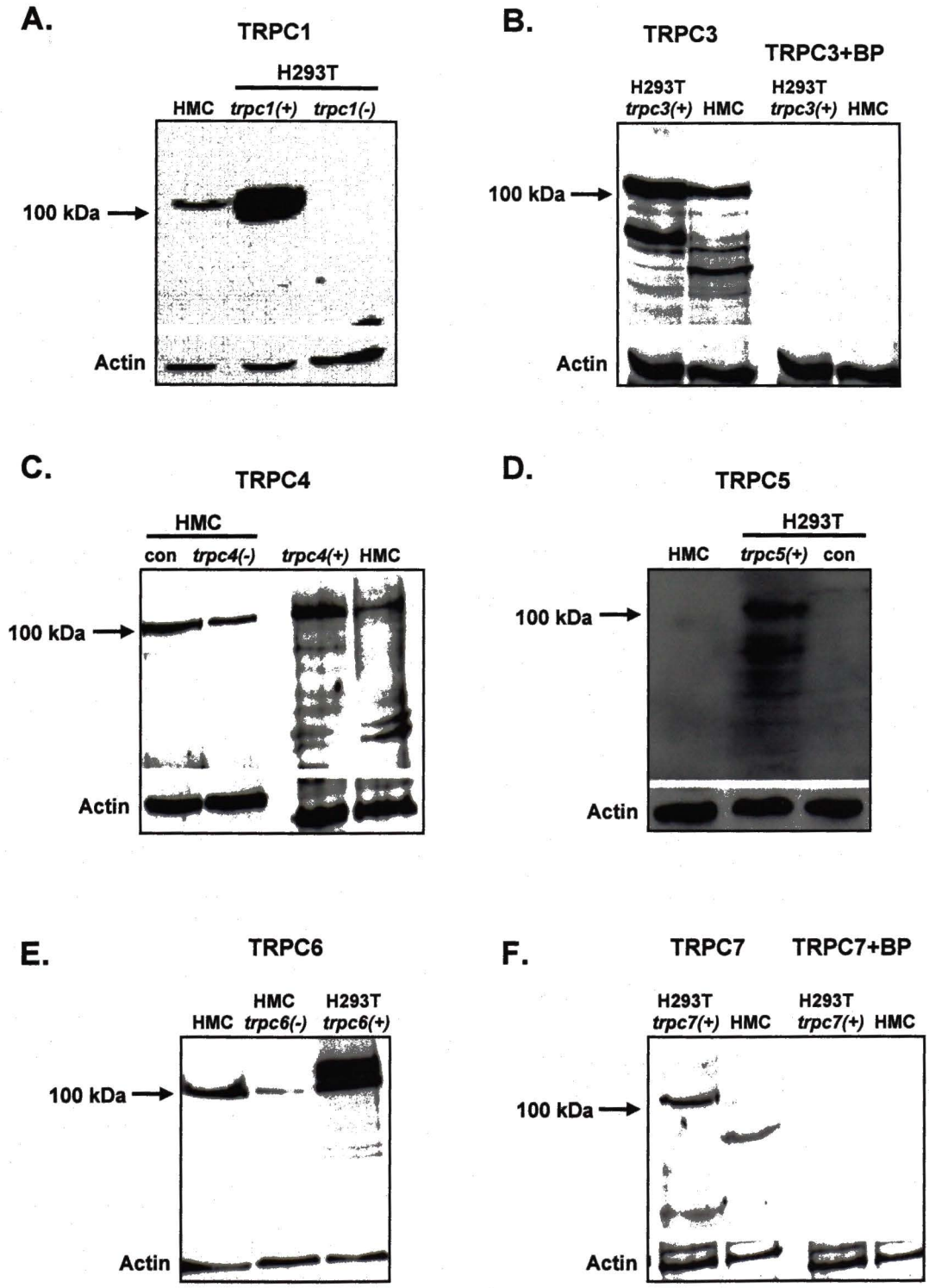

A.
TRPC1
H293T
HMC
trpc1(+)
trpc1(-)
100 kDa
Actin
B.
TRPC3
TRPC3+BP
H293T
trpc3(+)
HMC
H293T
trpc3(+)
HMC
100 kDa
Actin
C.
TRPC4
HMC
con
trpc4(-)
trpc4(+)
HMC
100 kDa
Actin
D.
TRPC5
H293T
HMC
trpc5(+)
con
100 kDa
Actin
E.
TRPC6
HMC
H293T
HMC
trpc6(-)
trpc6(+)
100 kDa
Actin
F.
TRPC7
TRPC7+BP
H293T
trpc7(+)
HMC
H293T
trpc7(+)
HMC
100 kDa
Actin

Figure 2. Confocal microscopy, showing immunofluorescent staining of TRPCs in HMCs. TRPC1 was stained with mouse monoclonal antibody (1:500) followed by Alexa Fluor-568-conjugated secondary antibody (1:1,000). TRPC3–7 were stained with goat polyclonal antibodies (1:100) followed by Alexa Fluor-488-conjugated secondary antibody (1:1,000). Mouse and goat IgG were negative controls for TRPC1 and TRPC3–7 stainings, respectively. Magnification: ×40.

Figure 2.

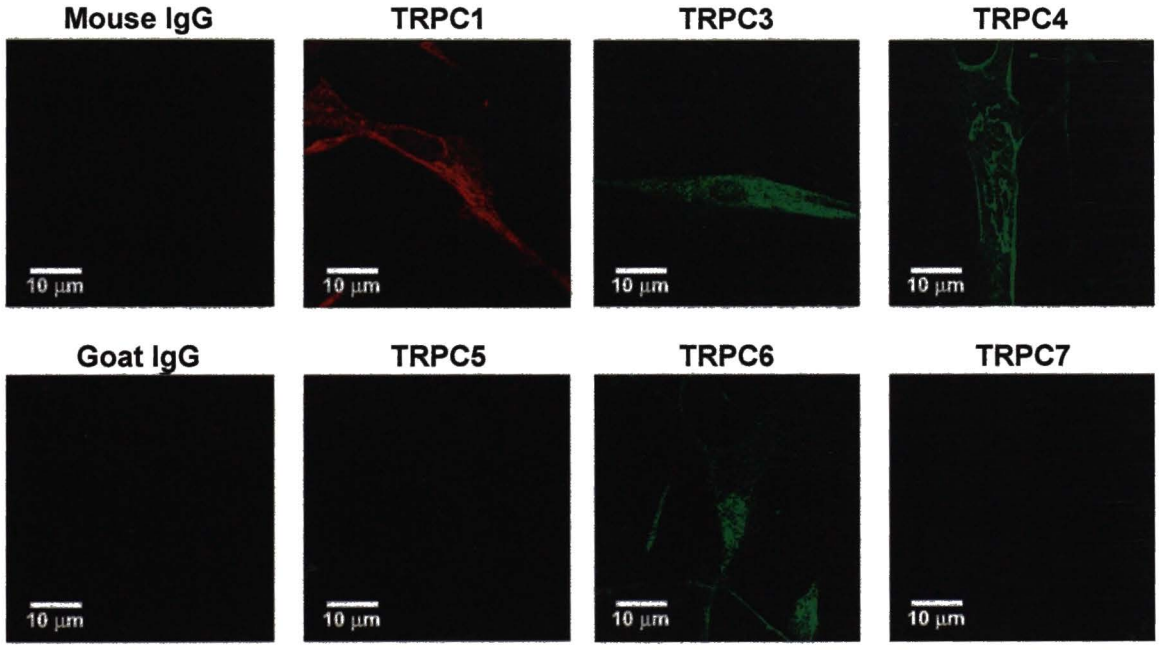


Figure 3. Immunohistochemistry showing expression of TRPC proteins in rat (*A* and *B*) and human (*C*) kidney sections. *A* and *B*: glomerular mesangial cells were labeled with desmin. *A*: IgG were stained with red fluorescence while desmin (probed with anti-desmin rabbit IgG) was stained with green fluorescence. *B*: TRPC4 and 6 (probed with anti-TRPC4 and 6 goat IgG, respectively) were stained with green fluorescence and desmin (probed with anti-desmin mouse IgG) were stained with red fluorescence. *C*: TRPC1, 3, 4, and 6 (probed with corresponding anti-TRPC goat IgG) were stained with green fluorescence in human glomeruli. Rabbit IgG was used as a negative control for TRPC stainings. The brightfield image shows normal morphology of glomerulus treated with rabbit IgG (negative control). The bars inside imagings indicate 50 μm.

Figure 3.

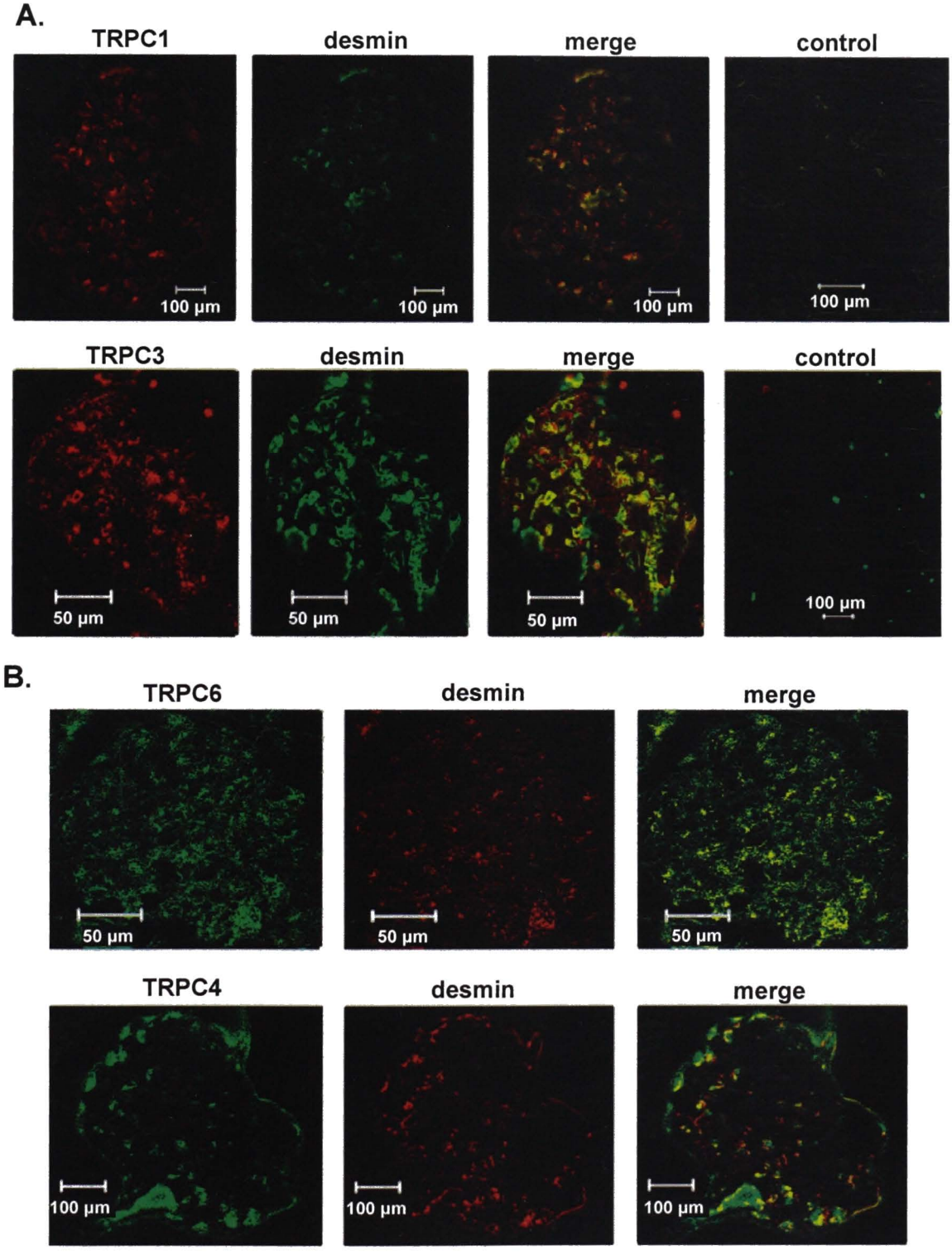

A.
TRPC1
desmin
merge
control
100 µm
100 µm
100 µm
100 µm
TRPC3
desmin
merge
control
50 µm
50 µm
50 µm
100 µm
B.
TRPC6
desmin
merge
50 µm
50 µm
50 µm
TRPC4
desmin
merge
100 µm
100 µm
100 µm

Figure 3. (continued)

C.

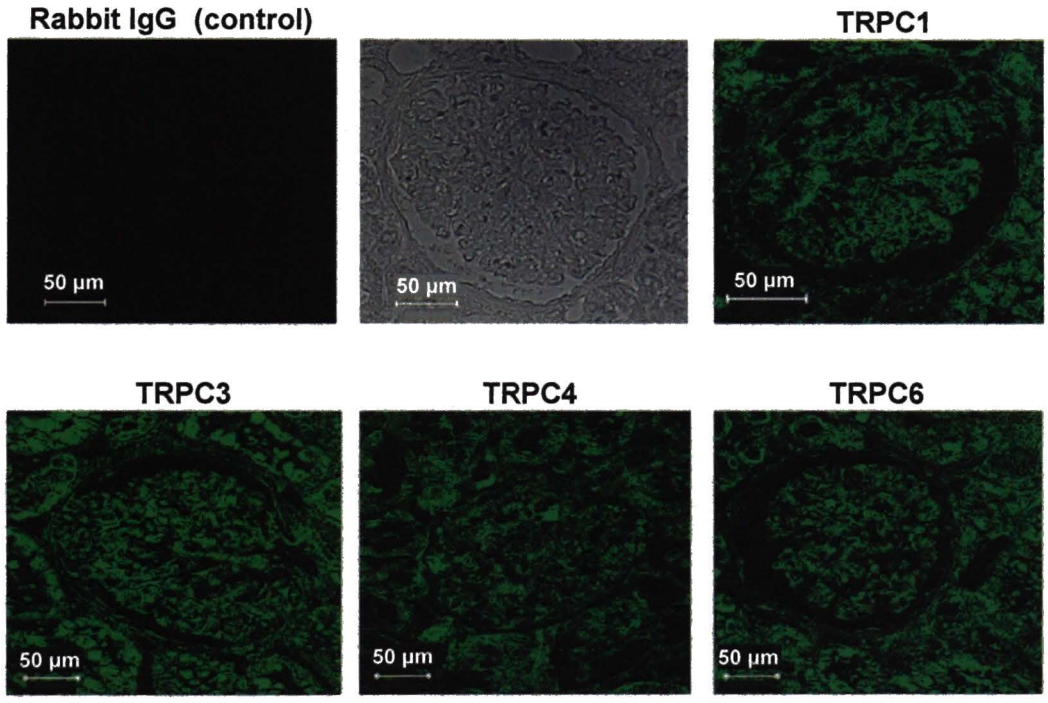


Figure 4. Coimmunoprecipitation, showing interaction between the isoforms of TRPCs. *A*: immunoprecipitating TRPC1 with mouse monoclonal TRPC1 antibody and immunoblotting TRPC3, 4, or 6 with goat polyclonal antibodies. Input indicates cell lysates (without TRPC1 pull down). *B*: immunoblotting TRPC3 or TRPC6 with rabbit polyclonal antibodies in cell lysates (Input) or TRPC4 immunoprecipitates. *C*: immunoblotting TRPC3 with rabbit polyclonal antibody in cell lysates (Input) or TRPC6 immunoprecipitates. IP, immunoprecipitation; IB, immunoblotting. All inputs shown here were 1/20 of the proteins used for IP.

Figure 4.

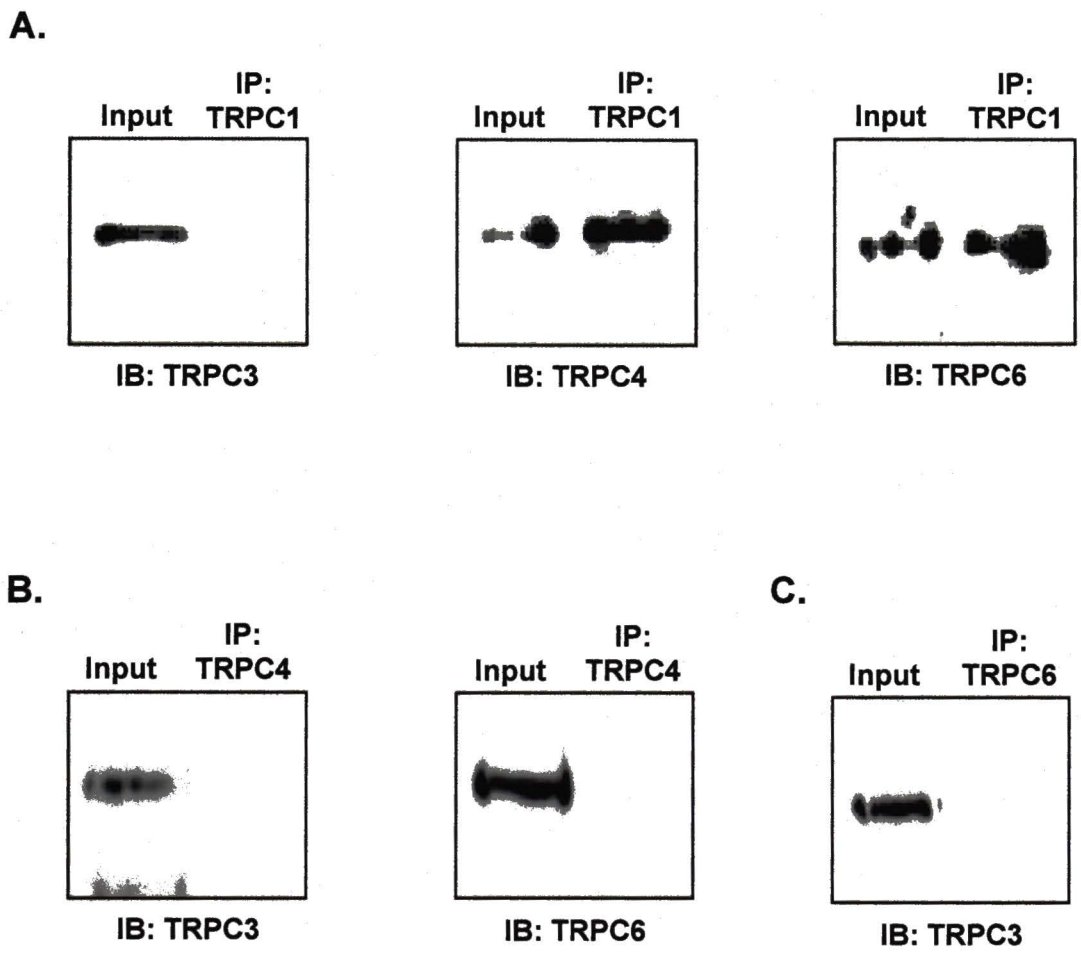

A.
Input
IP: TRPC1
IB: TRPC3
Input
IP: TRPC1
IB: TRPC4
Input
IP: TRPC1
IB: TRPC6
B.
Input
IP: TRPC4
IB: TRPC3
Input
IP: TRPC4
IB: TRPC6
C.
Input
IP: TRPC6
IB: TRPC3

Figure 5. Selective colocalization of TRPC1 with TRPC4 and 6 in HMCs. TRPC1 (red signals) was probed with anti-TRPC 1 mouse monoclonal antibody (1F1, 1:500) followed by Alexa Fluor-568-conjugated goat anti-mouse secondary antibody (1:1,000) while TRPC3, 4, and 6 (green signals) were stained with corresponding rabbit polyclonal antibodies (1:100) followed by Alexa Fluor-488-conjugated goat anti-rabbit secondary antibody (1:1,000). Colocalization of TRPCs was indicated by yellow signals from overlaying red and green signals (*right* and *bottom*). The bars inside imagings indicate 50 μm.

Figure 5.

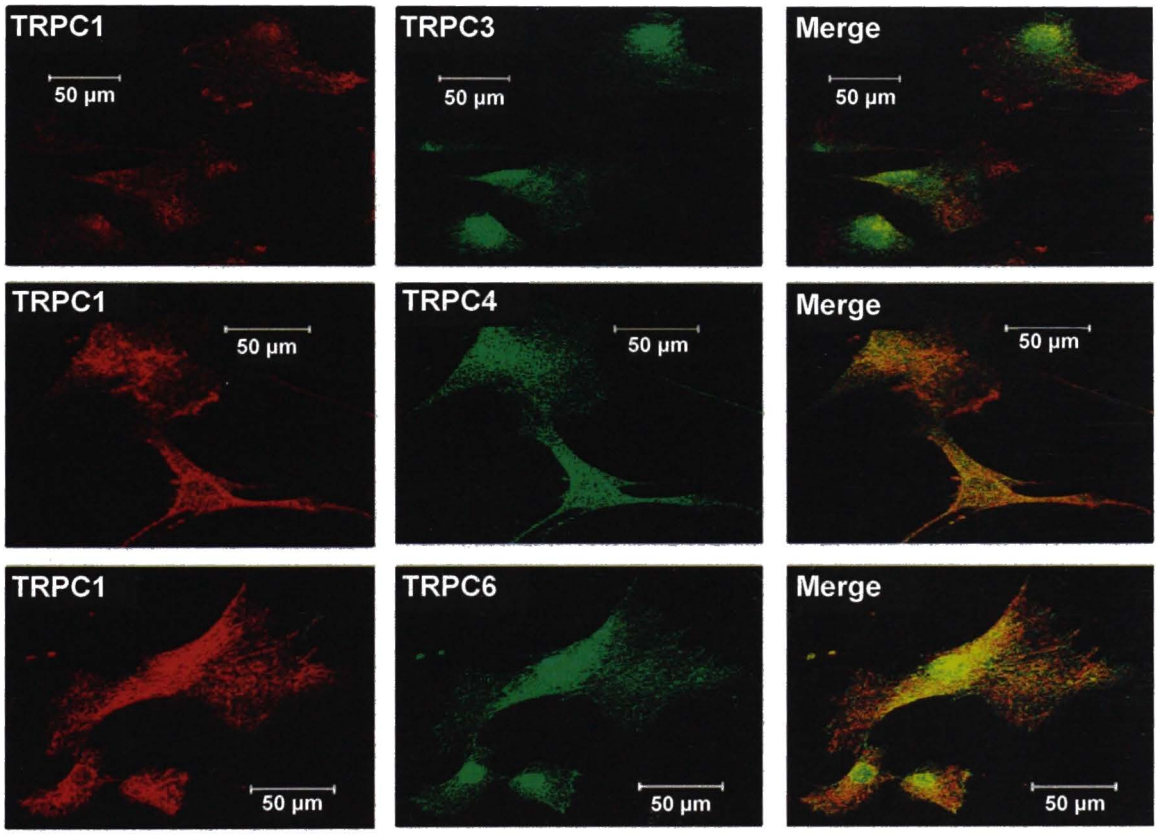
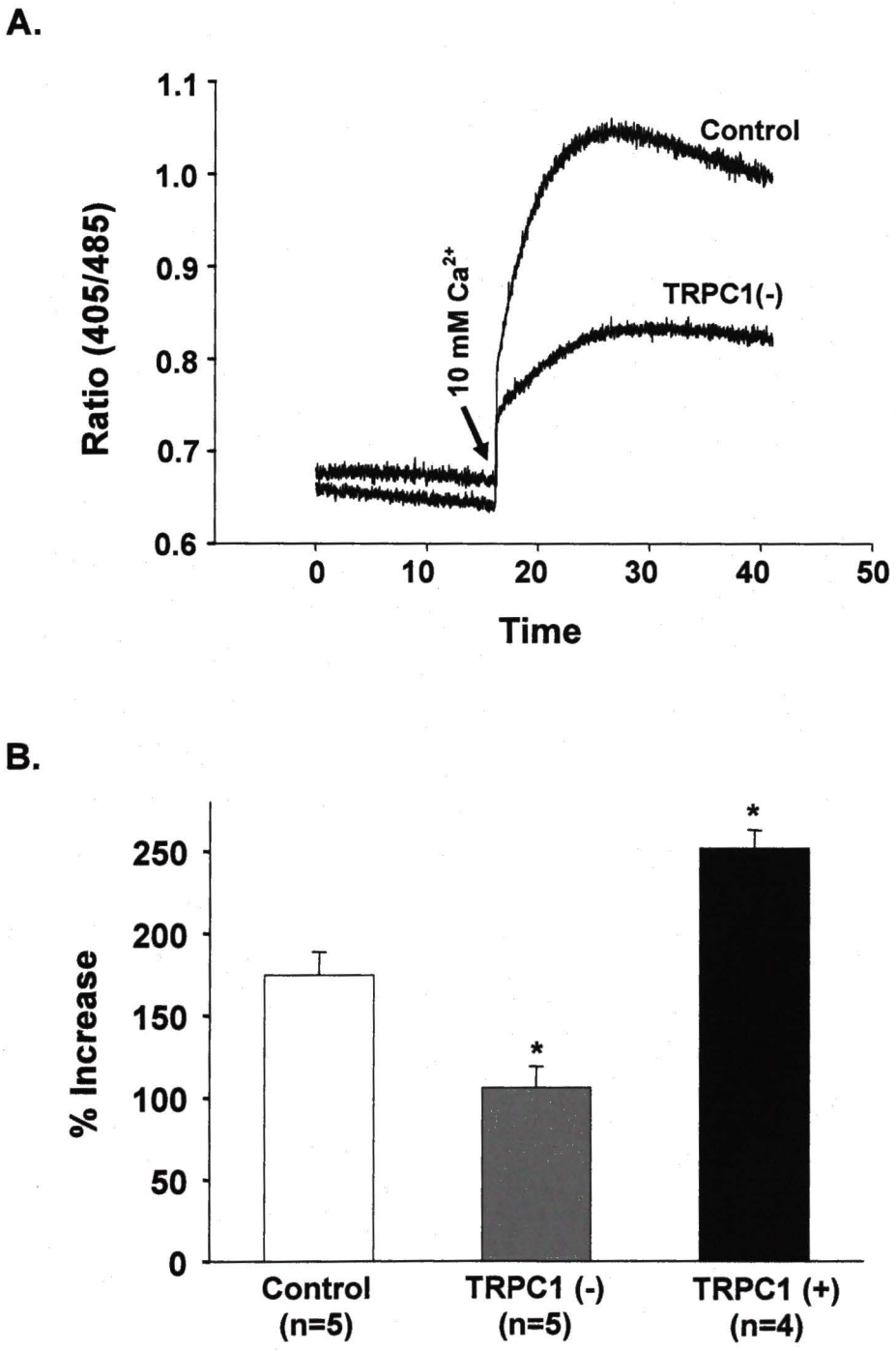

TRPC1
50 µm
TRPC3
50 µm
Merge
50 µm
TRPC1
50 µm
TRPC4
50 µm
Merge
50 µm
TRPC1
50 µm
TRPC6
50 µm
Merge
50 µm

Figure 6. Suppression of store-operated Ca^{2+} entry by inactivating TRPC1 in HMCs. *A*: representative experiment, showing the effect of Ca^{2+} addition (10 mM $CaCl_2$, added as indicated) on intracellular [Ca^{2+}] (shown as 405/485 fluorescence ratio of indo-1/AM) in trpc1-RNAi construct transfected [TRPC(-)] and untransfected (Control) HMCs incubated in a nominally Ca^{2+}-free solution with 1 μM thapsigargin for 30 min. *B*: summary bar graph from experiment shown in *A*. TRPC1(-) indicates HMCs transfected with *trpc4* cDNA. *Significant difference from control; *n* in each group indicates the sample size.

Figure 6.


A.
1.1
1.0
0.9
0.8
0.7
0.6
Ratio (405/485)
Control
TRPC1(-)
10 mM Ca2+
0
10
20
30
40
50
Time
B.
250
200
150
100
50
0
% Increase
*
*
Control
(n=5)
TRPC1 (-)
(n=5)
TRPC1 (+)
(n=4)

Abbreviations

ANG II: angiotensin II

HMC: human mesangial cell

IP: immunoprecipitationMC: mesangial cell

TG: thapsigargin

TRP: transient receptor potential

TRPC: canonical transient receptor protein

References

1. **Bandyopadhyay BC, Swaim WD, Liu X, Redman RS, Patterson RL, and Ambudkar IS.** Apical localization of a functional TRPC3/TRPC6 Ca^{2+}-signaling complex in polarized epithelial cells. *J Biol Chem* 280: 12908–12916, 2005.
2. **Barritt G and Rychkov G.** TRPs as mechanosensitive channels. *Nat Cell Biol* 7: 105–107, 2005.
3. **Beech DJ, Xu SZ, McHugh D, and Flemming R.** TRPC1 store-operated cationic channel subunit. *Cell Calcium* 33: 433–440, 2003.
4. **Bergdahl A, Gomez MF, Wihlborg AK, Erlinge D, Eyjolfson A, Xu SZ, Beech DJ, Dreja K, and Hellstrand P.** Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca^{2+} entry. *Am J Physiol Cell Physiol* 288: C872–C880, 2005.
5. **Bezzerides VJ, Ramsey IS, Kotecha S, Greka A, and Clapham DE.** Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol* 6: 709–720, 2004.
6. **Birnbaumer L, Zhu X, Jiang M, Boulay G, Peyton M, Vannier B, Brown D, Platano D, Sadeghi H, Stefani E, and Birnbaumer M.** On the molecular basis and regulation of cellular capacitative calcium entry: roles for trp proteins. *Proc Natl Acad Sci USA* 93: 15195–15202, 1996.
7. **Dietrich A, Schnitzler MM, Kalwa H, Storch U, and Gudermann T.** Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedebergs Arch Pharmacol* 371: 257–265, 2005.

8. **Facemire CS, Mohler PJ, and Arendshorst WJ.** Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation. *Am J Physiol Renal Physiol* 286: F546–F551, 2004.

9. **Garcia RL and Schilling WP.** Differential expression of mammalian TRP homologues across tissues and cell lines. *Biochem Biophys Res Commun* 239: 279–283, 1997.

10. **Goel M, Sinkins WG, and Schilling WP.** Selective association of TRPC channel subunits in rat brain synaptosomes. *J Biol Chem* 277: 48303–48310, 2002.

11. **Goel M, Sinkins WG, Zuo CD, Estacion M, and Schilling WP.** Identification and localization of TRPC channels in rat kidney. *Am J Physiol Renal Physiol* 290: F1241–F1252, 2006.

12. **Hardie RC.** Regulation of TRP channels via lipid second messengers. *Annu Rev Physiol* 65: 735–759, 2003.

13. **Hofmann T, Schaefer M, Schultz G, and Gudermann T.** Subunit composition of mammalian transient receptor potential channels in living cells. *Proc Natl Acad Sci USA* 99: 7461–7466, 2002.

14. **Inoue R, Okada T, Onoue H, Hara Y, Shimizu S, Naitoh S, Ito Y, and Mori Y.** The transient receptor potential protein homologue TRP6 is the essential component of vascular α_1-adrenoceptor-activated Ca^{2+}-permeable cation channel. *Circ Res* 88: 325–332, 2001.

15. **Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG, and Alpers C.** The activated mesangial cell: a glomerular "myofibroblast"? *J Am SOC Nephrol* 2, *Suppl* 10: S190–S197, 1992.

16. **Li WP, Tsiokas L, Sansom SC, and Ma R.** Epidermal growth factor activates store-operated Ca^{2+} channels through an IP3 independent pathway in human glomerular mesangial cells. *J Biol Chem* 279: 4570–4577, 2004.

17. **Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, and Yuan XB.** Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. *Nature* 434: 894–898, 2005.

18. **Lin MJ, Leung GPH, Zhang WM, Yang XR, Yip KP, Tse CM, and Sham JSK.** Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. *Circ Res* 95: 496–505, 2004.

19. **Lintschinger B, Balzer-Geldsetzer M, Baskaran T, Graier W, Romanin C, Zhu MX, and Groschner K.** Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+}-sensitive cation channels. *J Biol Chem* 275: 27799–27805, 2000.

20. **Liu XB, Bandyopadhyay BC, Singh BB, Groschner K, and Ambudkar IS.** Molecular analysis of a store-operated and OAG sensitive nonselective cation channel: heteromeric assembly of TRPC1-TRPC3. *J Biol Chem* 280: 21600–21606, 2005.

21. **Liu XB, Singh BB, and Ambudkar IS.** TRPC1 is required for functional store-operated Ca^{2+} channels. *J Biol Chem* 278: 11337–11343, 2003.

22. **Ma R, Pluznick JL, and Sansom SC.** Ion channels in mesangial cells: function, malfunction, or fiction. *Physiology* 20: 102–111, 2005.

23. **Ma R, Rundle D, Jacks J, Koch M, Downs T, and Tsiokas L.** Inhibitor of myogenic family: a novel suppressor of store-operated Ca^{2+} currents through an interaction with TRPC1. *J Biol Chem* 278: 52763–52772, 2003.

24. **Maroto R, Raso A, Wood TG, Kurosky A, Martina B, and Hamill OP.** TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nat Cell Biol* 7: 179–185, 2005.

25. **Marrero MB, Schieffer B, ma HP, Berstein KE, and Ling BN.** ANG II-induced tyrosine phosphorylation stimulates phospholipase C-γ a and C1-channels in mesangial cells. *Am J Physiol Cell Physiol* 270: C1834–C1842, 1996.

26. **Menè P, Simonson MS, and Dunn MJ. Physiology of mesangial cell.** *Physiol Rev* 69: 1347–1424, 1989.

27. **Menè P, Teti A, Pugliese F, and Cinotti GA.** Calcium release-activated calcium influx in cultured human mesangial cells. *Kidney Int* 46: 122–128, 1994.

28. **Moneer Z, Pino I, Taylor EJA, Broad LM, Liu Y, Tovey SC, Staali L, and Taylor CW.** Different phospholipase C-coupled receptors differentially regulate capacitative and noncapacitative Ca^{2+} entry in A7r5 cells. *Biochem J* 389: 821–829, 2005.

29. **Montell C, Birnbaumer L, Flockerzi V, Bindels R, Bruford EA, Caterina MJ, Clapham DE, Harteneck C, Heller S, Julius D, Kojima I, Mori Y, Penner R, Prawitt D, Scharenberg AM, Schultz G, Shimizu N, and Zhu MX.** A unified nomenclature for the superfamily of TRP cation channels. *Mol Cell* 9: 229–231, 2002.

30. **Nutt LK and O'Neil RG.** Effect of elevated glucose on endothelin induced store-operated and nonstore-operated calcium influx in renal mesangial cells. *J Am Soc Nephrol* 11: 1225–1235, 2000.

31. **Ong HL, Chen J, Chataway T, Brereton H, Zhang L, Downs T, Tsiokas L, and Barritt G.** Specific detection of the endogenous transient receptor potential (TRP)-1 protein in liver and airway smooth muscle cells using immunoprecipation and Western-blot analysis. *Biochem J* 364: 641–648, 2002.

32. **Parekh AB and Putney JW.** Store-operated calcium channels. *Physiol Rev* 85: 757–810, 2005.

33. **Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE, and Pollak MR.** TRPC6 is a glomerular slit diapharam-associated channel required for normal renal function. *Nat Genet* 37: 739–744, 2005.

34. **Riccio A, Medhurst AD, Mattei C, Kelsell RE, Calver AR, Randall AD, Benham CD, and Pangalos MN.** mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Mol Brain Res* 109: 95–104, 2002.

35. **Stockand JD and Sansom SC.** Glomerular mesangial cells: electrophysiology and regulation of contraction. *Physiol Rev* 78: 723–744, 1998.

36. **Strübing C, Krapivinsky G, Krapivinsky L, and Clapham DE.** TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* 29: 645–655, 2001.

37. **Strübing C, Krapivinsky G, Krapivinsky L, and Clapham DE.** Formation of novel TRPC channels by complex subunit interactions in embryonic brain. *J Biol Chem* 278: 39014–39019, 2003.

38. **Sweeney M, Yu Y, Platoshyn O, Zhang S, mcDaniel SS, and Yuan JCJ.** Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. *Am J Physiol Lung Cell Mol Physiol* 283: L144–L155, 2002.

39. **Tiruppathi C, Freichel M, Vogel SM, Paria BC, Mehta D, Flockerzi V, and Malik AB.** Impairment of store-operated Ca^{2+} entry in TRPC4(-/-) mice interferes with increase in lung microvascular permeability. *Circ Res* 91: 70–76, 2002.

40. **Trebak M, Bird GStJ, Mckay RR, and Putney JW.** Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. *J Biol Chem* 277: 21617–21623, 2002.

41. **Trebak M, St. J. Bird G, Mckay RR, Birnbaumer L, and Putney JW.** Signaling mechanism for receptor-activated canonical transient receptor potential 3 (TRPC3) channels. *J Biol Chem* 278: 16244–16252, 2003.

42. **Vazquez G, Wedel BJ, Trebak M, Bird G, and Putney JW.** Expression level of the canonical transient receptor potential 3 (TRPC3) channel determines its mechanism of activation. *J Biol Chem* 278: 21649–21654, 2003.

43. **Walker RL, Hume JR, and Horowitz B.** Differential expression and alternative splicing of TRP channel genes in smooth muscles. *Am J Physiol Cell Physiol* 280: C1184–C1192, 2001.

44. **Wang GX and Poo MM.** Requirement of TRPC channels in netrin-1-induced chemotropic turning of nerve growth cones. *Nature* 434: 898–904, 2005.

45. **Wang X, Pluznick JL, Wei P, Padanilam BJ, and Sansom SC.** TRPC4 forms store-operated Ca^{2+} channels in mouse mesangial cells. *Am J Physiol Cell Physiol* 287: C357–C364, 2004.

46. **Welsh DG, Morielli AD, Nelson MT, and Brayden JE.** Transient receptor potential channels regulate myogenic tone of resistance arteries. *Circ Res* 90: 248–250, 2002.

47. **Wissenbach U, Schroth G, Philipp S, and Flockerzi V.** Structure and mRNA expression of a bovine trp homologue related to mammalian trp2 transcripts. *FEBS Lett* 429: 61–66, 1998.

48. **Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigno DD, Thistlethwaite PA, Rubin LJ, and Yuan JXJ.** Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci USA* 101: 13861–13866, 2004.

49. **Yu Y, Sweeney M, Zhang S, Platoshyn O, Landsberg J, Rothman A, and Yuan JXJ.** PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. *Am J Physiol Cell Physiol* 284: C316–C330, 2003.

CHAPTER III

CANONICAL TRANSIENT RECEPTOR POTENTIAL 1 CHANNEL IS INVOLVED IN CONTRACTILE FUNCTION OF GLOMERULAR MESANGIAL CELLS

Juan Du*[†§], Sherry Sours-Brothers*[§], Rashadd Coleman[‡], Min Ding*,

Sarabeth Graham*, De-Hu Kong[†], and Rong Ma*.

*Department of Integrative Physiology, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas; [†]Department of Physiology, Anhui Medical University, Hefei, People's Republic of China; and [‡]Jackson State University, Jackson, Mississippi.

Journal of the American Society of Nephrology, 18: 1437–1445, 2007.

doi: 10.1681/ASN.2006091067.

[§] *These authors contributed equally to this work.*

Abstract

Contractility of mesangial cells (MC) is tightly controlled by $[Ca^{2+}]_i$. Ca^{2+} influx across the plasma membrane constitutes a major component of mesangial responses to vasoconstrictors. Canonical transient receptor potential 1 (TRPC1) is a Ca^{2+}-permeable cation channel in a variety of cell types. This study was performed to investigate whether TRPC1 takes part in vasoconstrictor-induced mesangial contraction by mediating Ca^{2+} entry. It was found that angiotensin II (AngII) evoked remarkable contraction of the cultured MC. Downregulation of TRPC1 using RNA interference significantly attenuated the contractile response. Infusion of AngII or endothelin-1 in rats caused a decrease in GFR. The GFR decline was significantly reduced by infusion of TRPC1 antibody that targets an extracellular domain in the pore region of TRPC1 channel. However, the treatment of TRPC1 antibody did not affect the AngII-induced vasopressing effect. Electrophysiologic experiments revealed that functional or biologic inhibition of TRPC1 significantly depressed AngII-induced channel activation. Fura-2 fluorescence-indicated that Ca^{2+} entry in response to AngII stimulation was also dramatically inhibited by TRPC1 antibody and TRPC1-specific RNA interference. These results suggest that TRPC1 plays an important role in controlling contractile function of MC. Mediation of Ca^{2+} entry might be the underlying mechanism for the TRPC1-associated MC contraction.

Introduction

Glomerular mesangial cells (MC) are located within glomerular capillary loops and contribute to the physiologic regulation of glomerular hemodynamics (1). Altered responsiveness of MC to hormones is one of the major causes that lead to various renal diseases. Ca^{2+} influx across the plasma membrane is a major component of MC responses to vasoconstrictors (1). Several types of Ca^{2+}-conductive channels in the plasma membrane are involved in the physiologic processes. These channels include voltage-operated Ca^{2+} channel (VOCC), receptor-operated channel (ROC), and recently found store-operated channel (SOC) (1,2). In contrast to the widely known VOCC, the molecular identity, physiologic significance, and regulatory mechanism of ROC and SOC in the glomerular contractile cells remain unknown.

Recently, the channel proteins from a new family, canonical transient receptor potential (TRPC), were found in a variety of cells (3). TRPC family includes seven related members, designated as TRPC1 through 7 (3). Pharmacologic and electrophysiologic studies in conjunction with molecular biologic tools and Ca^{2+} imagings have demonstrated that TRPC channel activity is tightly linked to the signaling cascade of G protein–coupled receptor or receptor tyrosine kinase (4,5), supporting the current hypothesis that TRPC proteins are potential candidates for ROC and SOC. TRPC proteins have been identified in glomeruli and glomerular MC (6–8). Our previous work also demonstrated that human MC selectively express TRPC1, 3, 4, and 6 (9). However, the function, regulation, and physiologic relevance of these glomerular TRPC are unexplored at large extent. In this study, we focused on TRPC1 and investigated its

contribution to mesangial contraction *in vitro* and *in vivo*. Our results indicate that TRPC1 is an important component mediating contractile responses of MC. The TRPC1-involved mesangial contraction is attributed to TRPC1-associated Ca^{2+} influx.

Materials and Methods

Animals. Two- to 3-mo-old male Sprague-Dawley rats were used in this study. All rats were purchased from Harlan (Indianapolis, IN). Care and use of all animals in this study were in strict agreement with the guidelines set forth by the University of North Texas Health Science Center.

Measurement of GFR and Renal Blood Flow. GFR and renal blood flow (RBF) were estimated by measurement of inulin and para-aminohippurate (PAH) plasma clearances as described by Pluznick *et al.* (10) and by Waugh and Beall (11), respectively. All rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and the left jugular vein was cannulated with PE-10 tubing for infusion of fluids and chemicals. The bladder was exposed and implanted with PE-50 tubing for urine collection. The right and left carotid arteries were cannulated with PE-10 tubing and a Fiber Optic Blood Pressure Sensor (WPI, Sarasota, FL) for collecting blood samples and monitoring arterial BP, respectively. BP was measured by Fiber Optic Measurement System (WPI) and analyzed by MP100 system with AcqKnowledge 3.8 software (BIOPAC System, Goleta, CA). Physiological saline solution (PSS) that contained 10 mg/ml FITC-inulin or PAH was infused at a rate of 1 ml/h per 100 g body wt. After a 1-h equilibration period, a blood sample (approximately 100 μl) was taken and urine was

collected during the next 30-min period. Then, angiotensin II (AngII)-containing inulin or PAH saline solution was infused into the rats and the 30-min urine sample was collected again. At the end of the period, a larger plasma sample was taken. Urinary volume was determined gravimetrically. GFR and RBF were calculated on the basis of urinary volume, urine and plasma inulin, or PAH concentrations. FITC-inulin was measured using a fluorescence microplate reader with excitation at 485 nm and emission at 538 nm in a Spectrophotometer (Victor3–1420 Multilable Counter; Perkin Elmer, Wellesley, MA), whereas PAH concentration was evaluated by measurement of absorbance in a Microplate Spectrophotometer (Spectramax 340 PC; Molecular Devices Corp., Sunnyvale, CA) at 450 nm. The concentrations of electrolytes in blood and urine were measured by GEM Premier 3000 (Instrumentation Laboratory, Lexington, MA), and osmolalities of the samples were measured by the Advanced Micro-Osmometer (Model 3MO Plus' Advanced Instruments, Norwood, MA).

Cell Culture and Transient Transfection. Human MC were purchased from Cambrex Co. (East Rutherford, NJ). MC were subcultured to no more than 10 generations by standard methods (12). All plasmids were transiently transfected into MC using LipofectAmine and Plus reagent (Invitrogen-BRL, Carlsbad, CA) following the protocols provided by the manufacturer.

MC Contractility Assay. AngII-induced MC contraction was measured by changes in planar surface area. The cover slip on which the MCs grew was mounted to a perfusion chamber, and MCs were visualized under an inverted fluorescence microscope (Nikon TE-2000S, upgraded, Nikon, Melville, NY). The perfusates were heated through

an inline heater, and the temperature was controlled by an Automatic Temperature Controller (TC- 324B; WPI). Changes in the mesangial planar surface area in response to AngII were observed at 37°C with 95% O_2 and 5% CO_2 gassing. Using a digital camera, images of the same cells were captured serially every 5 min for 30 min. The perimeters of individual cells with clearly defined borders were outlined, and the planar surface areas were calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

Patch-Clamp Procedure. The conventional cell-attached voltage clamp was used as described in our previous study (12). Single-channel analysis was made with a Warner PC-505B amplifier (Warner Instrument Corp., Hamden, CT) and pClamp 9.2 (Axon Instrument, Foster City, CA). The extracellular solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, and 10 glucose. The pipette solution contained (in mM) 135 NaCl, 5 KCl, 1 $CaCl_2$, 3 EGTA, and 10 HEPES. The calculated free Ca^{2+} concentration in the pipette solution was <10 nM (MTK software). At the time of the experiment, the pipette solution was supplemented with 100 μM niflumic acid, 10 mM TEA, and 100 nM iberiotoxin to block Ca^{2+}-activated Cl^- channels and K^+ channels, respectively. For exclusion of the influence of fluid flow on channel activity on AngII infusion, the bathing solution continuously flowed throughout the experiments. The flow rate was adjusted by gravity and controlled by a multiple channel perfusion system (ValveLink8, Automate Scientific, San Francisco, CA). Channel activity was calculated as Open probability. Clampfit 9.2 software (Axon Instrument, Foster City, CA) was used to analyze single channel currents.

Fluorescence Measurement of $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ in MC using fura-2 were performed using dual excitation wavelength fluorescence microscopy. MC, grown on a coverslip (22 × 22 mm), were loaded with fura-2 by incubation for approximately 50 min at room temperature in the dark in PSS that contained 2 μM acetoxymethyl ester of fura-2 (fura-2/AM), 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 (Molecular Probes, Eugene, OR) followed by washing three times. The cells were then incubated with fura-2 free PSS for an additional approximately 20 min. The coverslip was then placed in a perfusion chamber (Model RC-2OH; Warner) mounted on the stage of a Nikon Diaphot inverted microscope. Fura-2 fluorescence was monitored by a ratio technique (excitation at 340 and 380 nm, emission at 510 nm) using Metafluor software (Universal Imaging, West Chester, PA). Bath $[Ca^{2+}]$ was reduced to <10 nM during the experiments by addition of EGTA according to the Ca^{2+} concentration program by MTK Software. $[Ca^{2+}]_i$ was calculated using the formula described by Grynkiewicz *et al.* (13). Calibrations were performed *in vivo* at the end of each experiment, and conditions of high $[Ca^{2+}]$i were achieved by addition of 4 μM ionomycin, whereas conditions of low $[Ca^{2+}]_i$ were obtained by addition of 10 mM EGTA.

Materials. TRPC1 antibody and all chemicals were purchased from Sigma-Aldrich (Sigma, St. Louis, MO). pSHAG, pSHAG-trpc1/RNA interference (RNAi) plasmids, and HA-tagged TRPC1 expression plasmids were obtained from Dr. L. Tsiokas (University of Oklahoma Health Sciences Center, Oklahoma City, OK).

Statistical Analyses. Data are reported as means ± SEM. The one-way ANOVA plus Student-Newman-Keuls test, unpaired *t* test, and paired *t* test were used to analyze

the differences among multiple groups, between two groups, and before and after treatment in the same group, respectively. $P < 0.05$ was considered statistically significant.

Results

TRPC1 Contributed to AngII-Induced Contraction of MC. Our previous study (9) demonstrated that TRPC1 is present in human and rat MC. To determine whether TRPC1 was involved in contractile function of MC, we assessed AngII-induced contraction in cultured MC with and without knockdown of TRPC1. TRPC1 knockdown was achieved by transient transfection of short hairpin RNAi constructs (pSHAG-trpc1/RNAi) that specifically silenced human trpc1 gene. As shown in Figure 1C, TRPC1 protein was remarkably overexpressed in HEK293 cells that were transiently transfected with human TRPC1 expression plasmids. The exogenous TRPC1 protein was significantly reduced by co-transfection of the cells with RNAi constructs specific for human TRPC1 gene (pSHAG-trpc1/RNAi). These results were consistent with our previous report (9), suggesting successful knockdown of TRPC1 protein by this tool. This construct was used for subsequent experiments in this study.

AngII-induced MC contraction was measured by changes in planar surface area of the cell. This contractility assay was carried out in transiently green fluorescence protein (GFP) +pSHAG–transfected (control) and GFP+pSHAG-*trpc1*/RNAi–transfected (knocking down TRPC1) human MC. Positively transfected cells were identified with green fluorescence. As shown in Figure 1, A and B, with AngII treatment for 10 min, the

surface area of control MC decreased by 37.2 ± 4.0%. However, the contractile response was significantly attenuated in MC with TRPC1 knockdown (20.1 ± 3.0%). These results suggest that TRPC1 participated in the contractile function of MC.

TRPC1 Regulated Glomerular Filtration in Rats. Because the major function of glomerular MC is to regulate glomerular filtration by regulating effective filtration surface area, we reasoned that if TRPC1 protein contributes to the contractile function of MC, then inhibition of mesangial TRPC1 function should increase GFR or compromise mesangial contraction–induced GFR decline, accordingly. Therefore, inulin clearance was measured to evaluate rats' GFR in the presence or absence of AngII with or without TRPC1 inhibition. In agreement with other studies (14,15), infusion of AngII (1.7 ng/min per 100 g body wt) significantly reduced GFR from 2.7 ± 0.6 of basal level to 1.4 ± 0.3 ml/min per 100 g body wt (Figure 2A, normal), representing 42 ± 10% decrease (Figure 2B, normal). Inclusion of a TRPC1 antibody (polyclonal rabbit IgG, 300 ng/ml), which is directed to an extracellular epitope (predicted pore region) of TRPC1, into perfusate did not alter the basal GFR. However, the AngII-induced decline of GFR was dramatically attenuated (from 2.3 ± 0.5 to 2.0 ± 0.7 ml/min per 100 g body wt, representing 8.9 ± 6% decrease; Figure 2, A and B, TRPC1 Ab). This inhibitory effect was specific because the same amount of rabbit IgG did not affect the AngII-induced reduction of GFR (2.0 ± 0.3 to 1.3 ± 0.3 ml/min per 100 g body wt, representing 36 ± 8.6% decrease; Figure 2, A and B, Rb IgG). To determine whether the inhibitory effect of TRPC1 antibody is specific for AngII or general for vasoconstrictors, we assessed the effect of TRPC1 antibody on endothelin-1 (ET-1)-induced decrease in GFR in rats using the same experimental

procedures. ET-1 has been known as one of the most potent agonists that initiates contraction of MC (1). We found that ET-1 resulted in a comparable decline of GFR (approximately 49% from baseline) to that induced by AngII in the rats that were treated with nonimmune IgG. However, similar to the AngII-treated rats, infusion of immune active TRPC1 antibody significantly attenuated the ET-1–induced GFR decrease. These results suggest that TRPC1 channel protein might be a convergent pathway contributing to MC contraction in response to a broad range of vasoconstrictors (Figure 2C).

To determine further whether the inhibition of AngII-induced GFR decline by TRPC1 antibody was owing to changes in renal hemodynamics, we monitored arterial BP throughout the experiments. RBF in response to AngII was also evaluated in rats with and without TRPC1 antibody treatment. As shown in Figure 3A, the baseline mean arterial pressure (MAP) greatly increased in all rats (approximately 150 mmHg) because the baroreceptor reflex had been removed after bilateral catheterization of carotid arteries. Consistent with other studies (16,17), infusion of AngII increased MAP and decreased RBF (Figure 3). Thus, renal vascular resistance (ratio of MAP to RBF) increased in response to AngII. However, these changes in renal hemodynamics were not significantly affected by TRPC1 antibody, because the AngII-induced vasopressing effect and reduction of RBF were comparable among normal, rabbit IgG–treated, and TRPC1 antibody–treated rats (Figure 3). We also measured concentrations of electrolytes and osmolalities of blood and urine in normal, rabbit IgG–, and TRPC1 antibody–treated rats. As summarized in Table 1, all of these values were within physiologic ranges, and no significant difference was observed among the three groups. These results suggest that

the suppression of AngII-induced GFR decrease by TRPC1 antibody was most likely attributed to intraglomerular factors (*i.e.*, change in mesangial contractility).

TRPC1 Channels Mediated AngII-Stimulated Membrane Currents. Mesangial tone is controlled by intracellular Ca^{2+} homeostasis that is regulated by Ca^{2+} intrusion and extrusion across the plasma membrane. It has been known that several types of cation channels, including ROC and SOC, are involved in vasoconstrictor-induced Ca^{2+} responses in MC (1,2). TRPC1 has been considered a Ca^{2+}-permeable cation channel linked to G protein–coupled receptor-phospholipase C pathway for activation (18). Therefore, we speculated that TRPC1 regulated mesangial contraction by mediating agonist-stimulated ionic currents. Cell-attached patch clamp was used to measure membrane currents in response to AngII. Figure 4A shows the current-voltage relation curve (I-V curve) before application of AngII. Because the resting membrane potentials of the patched MC were unknown, the membrane potentials in the analysis were expressed as negative pipette potentials (-Vp). In the range from -80 to 0 mV of command potentials (-Vp), the calculated single channel conductance was 17.2 pS. The extrapolated linear I-V relation was reversed at -Vp of 54 mV. Considering that intracellular K^+ concentration in MC is approximately 120 mM and the resting membrane potential of MC is estimated at approximately -45 mV (19,20), the reversal membrane potential for the observed channels is estimated to be approximately 9 mV. These biophysical features are consistent with those of TRPC1 channel (21,22).

We then investigated the responses of the membrane currents to AngII and the contribution of TRPC1 to the responses. In the untreated group, AngII increased channel

activity by approximately 2.2-fold (Figure 4, B and C). However, inclusion of TRPC1 antibody (200 ng/ml) into the pipette significantly depressed the AngII-induced channel activation. Similar to *in vivo* experiments, the inhibitory effect of TRPC1 antibody seemed to be specific because the same concentration of rabbit IgG did not significantly affect the AngII-induced currents (approximately 2.5-fold increase; Figures 4C and 5A).

We further assessed the AngII-induced channel response in TRPC1-knocked down MC. As shown in Figure 5A, transient transfection of pSHAG-trpc1/RNAi constructs into human MC significantly inhibited AngII-stimulated channel activity compared with control transfected cells (pSHAG empty vector). The inhibition of TRPC1 channel either by its antibody or by RNAi also significantly reduced the basal activities of the AngII-responsive channels (Figure 5B). These results suggest that TRPC1 contributes not only to vasoconstrictor-induced mesangial contraction but also to the basal tone of MC.

TRPC1 Contributed to AngII-Induced Ca^{2+}. Entry in MC An increase in $[Ca^{2+}]_i$ is a downstream event of AngII-induced channel responses and a key factor to trigger mesangial contraction. Contribution to elevation of $[Ca^{2+}]_i$ might be an underlying mechanism for TRPC1-associated MC contraction. To test this speculation, we assessed fura-2 fluorescence-indicated $[Ca^{2+}]_i$ in response to AngII in cultured MC with and without TRPC1 inhibition. Consistent with our previous studies (23,24), AngII evoked a rapid and striking cytosolic Ca^{2+} transient followed by a steady state of lower elevation of $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+} extracellular solution. Removal of extracellular Ca^{2+} immediately reduced the $[Ca^{2+}]_i$ to a level lower than baseline. Re-addition of Ca^{2+}

resulted in an increase in $[Ca^{2+}]_i$ that is attributed to Ca^{2+} influx (12) (Figure 6A, untreated). Bath application of rabbit IgG or TRPC1 antibody (200 ng/ml) did not alter the profile of the AngII-induced Ca^{2+} responses. However, the AngII-stimulated Ca^{2+} entry in response to Ca^{2+} re-addition was significantly inhibited by TRPC1 antibody but not by rabbit IgG treatment (Figure 6, A and C). The inhibition of Ca^{2+} influx by TRPC1 antibody was not due to changes in AngII-induced Ca^{2+} release from sarcoplasmic reticulum, because the initial Ca^{2+} transients were comparable among untreated, rabbit IgG–treated, and TRPC1 antibody–treated MC (Figure 6A). Normalization of Ca^{2+} entry to Ca^{2+} release revealed a significantly lower ratio in TRPC1-treated group than that in the other two groups (Figure 6D). Similarly, inhibition of TRPC1 by transfection of pSHAG-trpc1/RNAi constructs into MC also significantly suppressed AngII-stimulated Ca^{2+} entry without affecting Ca^{2+} release from sarcoplasmic reticulum (Figure 6, B through D). It should be mentioned that nifedipine (1 μM) was present in the bath during re-addition of Ca^{2+}; therefore, involvement of VOCC in the Ca^{2+} entry can be excluded or neglected.

Discussion

MCs contribute to the physiologic regulation of glomerular hemodynamics by responding to locally produced or circulating vasoactive peptides (1). Like smooth muscle cells, the contractility of MC is tightly controlled by $[Ca^{2+}]_i$. Ca^{2+} influx across the plasma membrane constitutes a major component of the responses of MC to vasoconstrictors. The results from this study suggest that TRPC1 channel protein is an

indispensable contributor to contractile function of MC. This conclusion is based on two lines of findings from both *in vitro* and *in vivo* experiments. In cultured human MC, AngII- or ET-1–induced mesangial contraction was significantly attenuated by downregulation of TRPC1 protein. In rats, infusion of TRPC1 antibody significantly reduced the AngII-induced GFR decline. In addition to mesangial tone, glomerular capillary pressure and RBF are the other two major determinants of glomerular filtration and might also contribute to the AngII-induced GFR decline. The involvement of these factors could be indicated by increases in MAP and decreases in RBF during infusion of AngII (Figure 3). However, inhibition of AngII-induced vasopressing effect might not be the underlying mechanism for TRPC1 antibody treatment because the changes in resistance of renal vasculature (ratio of MAP to RBF) were comparable between control and antibody-treated rats. The TRPC1 antibody that was used in this study is directed to an extracellular epitope that is located at the predicted pore region of TRPC1 and therefore might act as a channel blocker. Indeed, using specific TRPC antibodies selectively to antagonize function of individual TRPC has become a powerful tool for studying specific TRPC functions (25,26). The same TRPC1 antibody has been reported to depress significantly TRPC1 channel-mediated currents in platelets, neurons, and endothelial cells (27–29).

In addition, we repeated the *in vivo* experiments using another TRPC1 antibody (T1E3; from Dr. David Beech, University of Leeds, Leeds, UK) that also targets on the outer vestibule of TRPC1 channel (25) in four rats. Like the commercial TRPC1 antibody, the T1E3 antibody attenuated the AngII-induced GFR decline at an equivalent degree

(data not shown). Therefore, we reasoned that the infused TRPC1 antibody filtered out from glomerular capillaries and then bound to the extracellular domains of TRPC1 channel protein located on the plasma membrane of MC and further blocked the AngII-stimulated Ca^{2+} entry through TRPC1 channel. As a support for this inference, glomerular deposit of an Ig from systemic circulation and further reaction to its antigen protein on the surface of MC has been well established in a disease model of glomerulonephritis (30).

TRPC1 has been known to function as a Ca^{2+}-permeable cation channel linked to the G protein–coupled signaling pathway for activation (4,5). However, whether the channel is activated by IP_3-induced internal Ca^{2+} store depletion (SOC) or by a second messenger between the receptor activation and Ca^{2+} store depletion (ROC) is debatable (31,32). Recently, stromal interaction molecule 1 (STIM1) was proposed to be a key activator of SOC by functioning as a Ca^{2+} sensor of internal stores (33,34). It is interesting that STIM1 was found to interact with endogenous or transfected TRPC1, and this interaction is required for SOC activation (35,36), strongly suggesting a store-operated mechanism of TRPC1. We are not able to identify the nature of the TRPC1 channel that mediates the AngII-stimulated ionic responses (SOC or ROC) from this study. We recently found that TRPC1 was also involved in thapsigargin-induced Ca^{2+} influx, a classical mechanism of store-operated Ca^{2+} entry, and STIM1 was strongly expressed in MC (data not shown), indicating that TRPC1 might be an important subunit of SOC in the glomerular contractile cells. Apparently, further study is required to define the precise mechanism of TRPC1 in AngII-evoked channel responses.

It has been documented that TRPC proteins can assemble one another as well as with auxiliary proteins to form channel complexes (3,37). The heteromultimerization and auxiliary proteins play important roles in determining the biophysical properties and activation mechanism of the TRPC channels (37). We have demonstrated that TRPC1 selectively interacts with TRPC4 and 6 in human MC (9). It is conceivable that in MC, the channel that mediates AngII-stimulated currents might be a complex that contains multiple subtypes of TRPC proteins and unknown auxiliary proteins. TRPC1 might be only one component of the channel complex.

Conclusion

The findings from the *in vitro* and *in vivo* studies provide evidence that TRPC1 participates in contractile function of MC by mediating vasoconstrictor-stimulated Ca^{2+} responses. Because contractility of MC has important physiologic significance in regulation of glomerular function and an impairment of mesangial tone is involved in progression of various renal diseases, such as diabetic hyperfiltration at an early stage of diabetic nephropathy, the data from this study have important physiologic and clinical relevance.

Acknowledgments

This study was supported by a Scientist Development Grant from the American Heart Association (R.M.) and a Young Investigator Grant from National Kidney

Foundation (R.M.). We thank Dr. David J. Beech (School of Biomedical Sciences, University of Leeds, Leeds, UK) for providing the anti-TRPC1 antibody (T1E3).

Table 1. Electrolyte concentrations and osmolality of plasma and urine.

Groups	B_{Na} (mM)	B_K (mM)	B_{Ca} (mM)	B_{Osm} (mM)	U_{Na} (mM)	U_{Ca} (mM)	U_{Osm} (mM)
Untreated	135.9 ± 1.2	5.8 ± 0.6	0.9 ± 0.1	298.0 ± 5.5	146.5 ± 8.4	0.6 ± 0.2	999.3 ± 9.7
Rb IgG	135.4 ± 1.3	4.7 ± 0.2	0.7 ± 0.03	280.1 ± 4.4	155.5 ± 14.2	0.5 ± 0.1	967.8 ± 152.9
TRCP1 Ab	135.1 ± 0.7	5.1 ± 0.3	0.7 ± 0.06	280.8 ± 4.3	168.8 ± 14.6	0.6 ± 0.2	905.4 ± 127.7

B_{Na}, B_K, B_{Ca}, and B_{Osm} represent plasma concentrations of Na^+, K^+, and Ca^{2+} and plasma osmolality, respectively. U_{Na}, U_{Ca}, and U_{Osm} represent urine concentrations of Na^+ and Ca^{2+} and urine osmolality, respectively. Rb IgG, rabbit IgG. TRPC1 Ab, canonical transient receptor potential 1 antibody.

Figure 1. Changes in the planar surface area of mesangial cells (MC) with angiotensin II (AngII) stimulation. (A) Representative morphology of MC that were used in contraction assays in green fluorescence protein (GFP)+pSHAG (pSHAG)- and GFP+pSHAG-trpc1/RNA interference (pSHAG-trpc1/RNAi)-transfected cells before and 10 min after AngII treatment. Arrows indicate positively transfected cells. The changes in size of the transfected cells in response to AngII are illustrated on the right (Overlap), by overlapping the images of the same cell before and after AngII treatment using Photoshop software. The cells before AngII treatment were colored as red and after treatment as green. The right panels are enlarged regions indicated by dashed rectangles in the left and middle panels. (B) Bar graph showing the AngII-induced decreases in the surface area of MC with and without canonical transient receptor potential 1 (TRPC1) knockdown, calculated as [(the surface area of MC after AngII - the original surface area of the cells)/the original surface area of the cells] × 100%. "n" indicates the number of cells counted in each group. *Significant difference between the indicated groups. (C) Western blot in HEK293 cells, showing TRPC1 protein expression in untransfected HEK293 cells (lane 1) and HEK293 cells with transient transfection of HA-tagged human TRPC1 expression plasmids (lane 2) or human TRPC1 expression plasmids plus RNAi constructs for human TRPC1 (lane 3). Actin was used as a loading control. Magnification, × 250.

Figure 1.

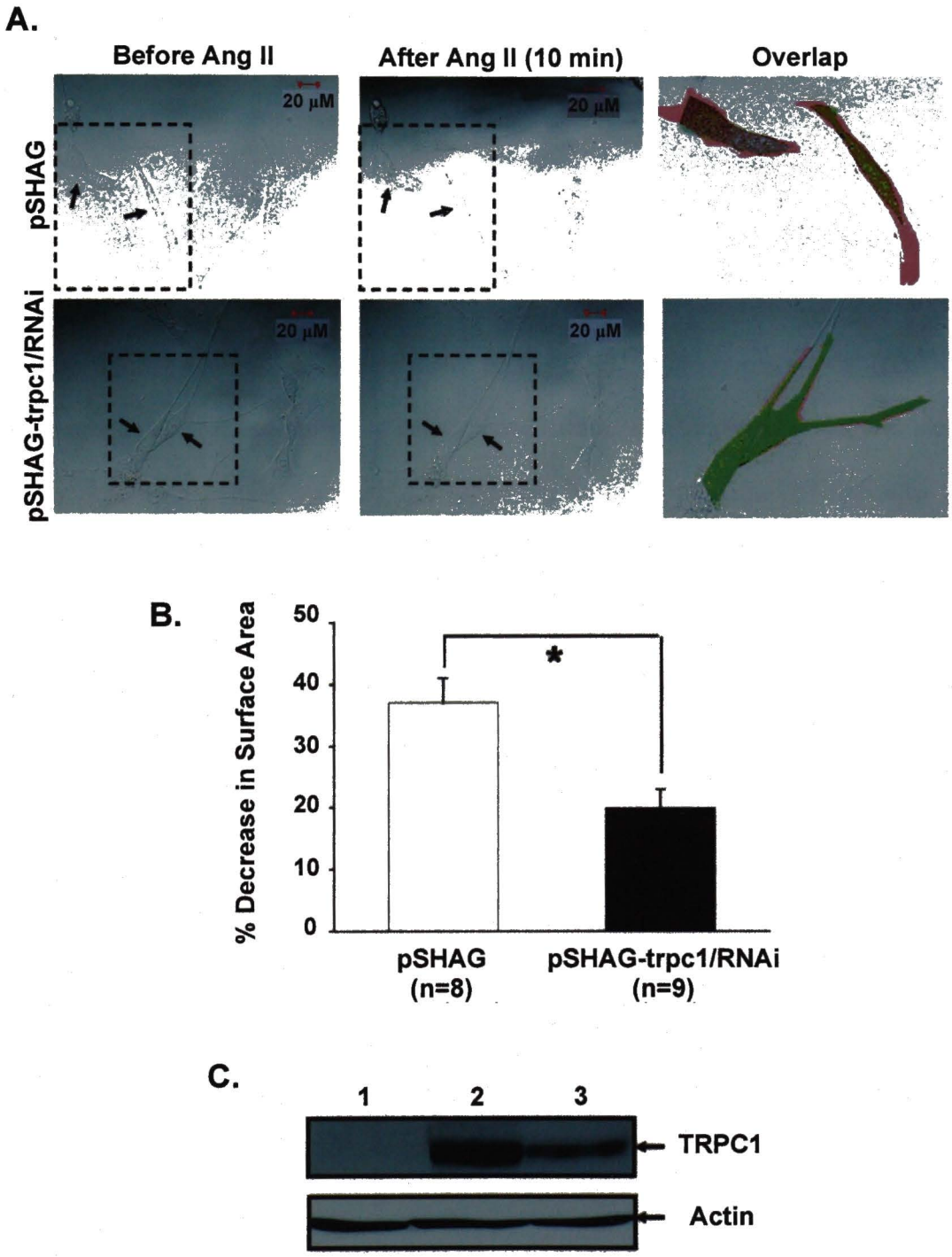

A.
Before Ang II
After Ang II (10 min)
Overlap
pSHAG
pSHAG-trpc1/RNAi
20 μM
B.
% Decrease in Surface Area
50
40
30
20
10
0
*
pSHAG
(n=8)
pSHAG-trpc1/RNAi
(n=9)
C.
1
2
3
TRPC1
Actin

Figure 2. GFR before and during infusion of AngII (1.7 ng/min per 100 g body wt) or endothelin-1 (ET-1; 1.3 ng/min per 100 g body wt) with and without (Untreated) TRPC1 antibody (TRPC1 Ab; 300 ng/ml) or rabbit IgG (Rb IgG; 300 ng/ml) in rats. (A) GFR was calculated on the basis of urine output, concentrations of inulin in urine, and blood samples that were collected during corresponding infusion periods. "n" indicates the number of rats in each group. (B) Percentage of decrease in GFR induced by AngII in each group shown in A, calculated by [(GFR before AngII – GFR during infusion of AngII)/GFR before AngII] × 100%. (C) Percentage decrease in GFR in response to ET-1. *Significant difference between the indicated groups.

Figure 2.

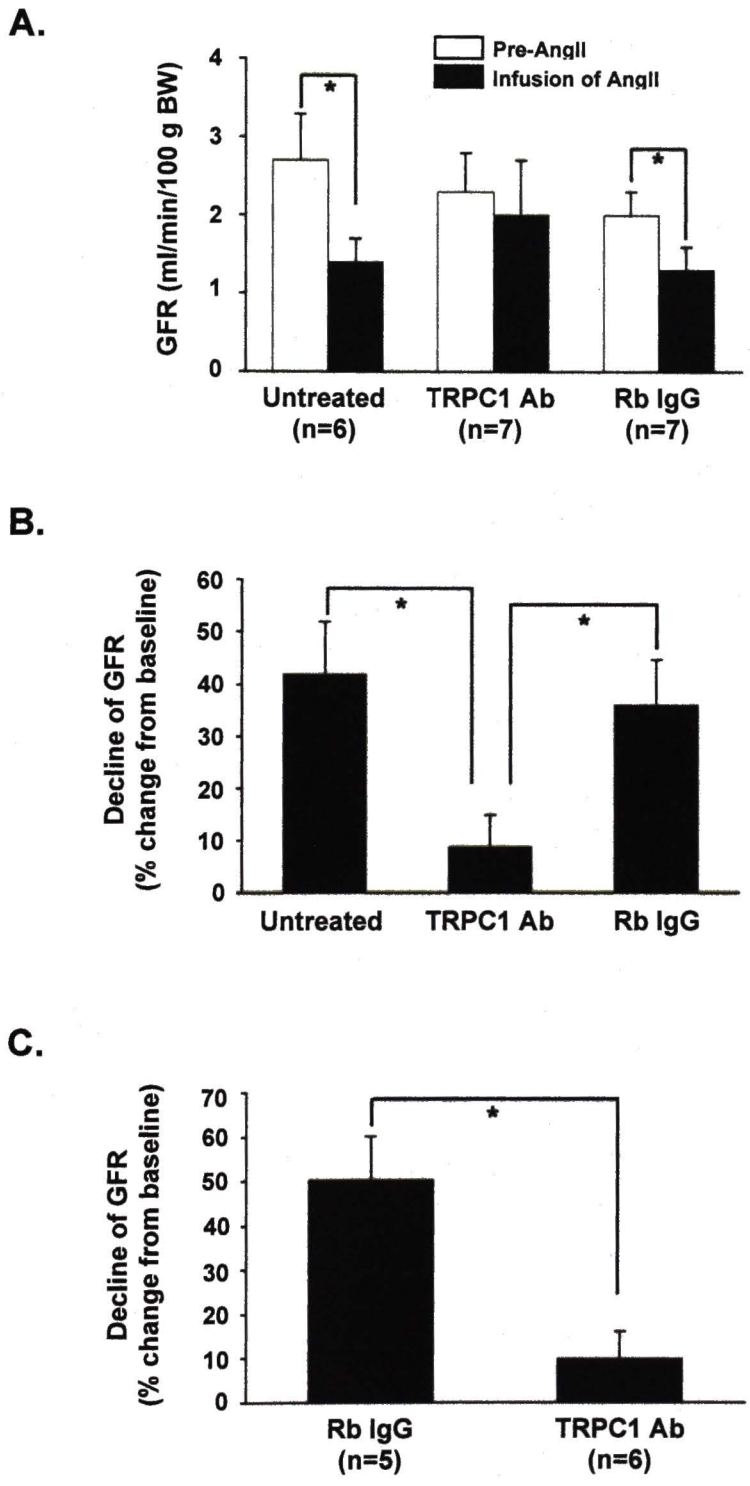
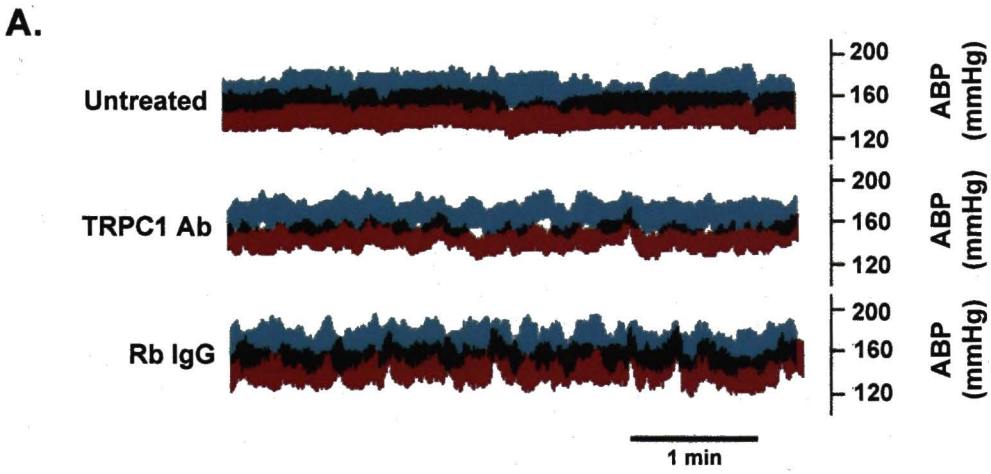
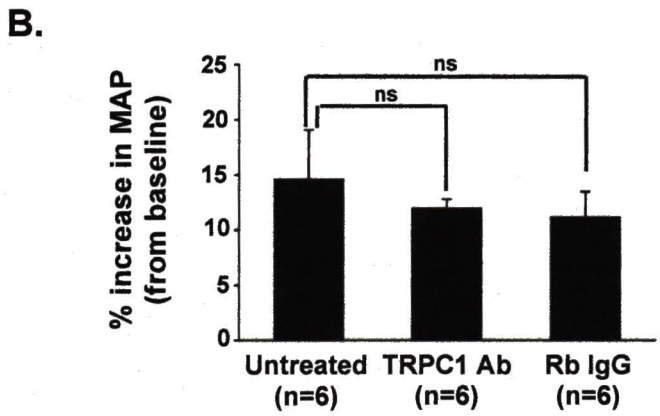

A.
Pre-AngII
Infusion of AngII
GFR (ml/min/100 g BW)
0
1
2
3
4
*
*
Untreated
(n=6)
TRPC1 Ab
(n=7)
Rb IgG
(n=7)
B.
Decline of GFR
(% change from baseline)
0
10
20
30
40
50
60
*
*
Untreated
TRPC1 Ab
Rb IgG
C.
Decline of GFR
(% change from baseline)
0
10
20
30
40
50
60
70
*
Rb IgG
(n=5)
TRPC1 Ab
(n=6)

Figure 3. Mean arterial BP (MAP) and renal blood flow (RBF) before and during infusion of AngII (1.7 ng/min per 100 g body wt) with and without TRPC1 Ab or Rb IgG in rats. (A) Original traces of arterial BP (ABP) before (red traces) and during infusion of AngII (blue traces) with or without (Untreated) Rb IgG or TRPC1 Ab. The traces for AngII were sampled approximately 10 min after onset of AngII infusion. (B) Summary data from experiments described in A, showing AngII-induced increases in MAP in the three groups, expressed as percentages: [(MAP during infusion of AngII – basal MAP)/basal MAP] × 100%. (C) Para-aminohippurate (PAH) clearance-estimated RBF in response to AngII with or without concomitant infusion of TRPC1 Ab or Rb IgG, expressed as percentages: [(RBF during infusion of AngII – basal RBF)/basal RBF] × 100%. In both B and C, "n" indicates the number of rats in each group.

Figure 3.


A.
Untreated
TRPC1 Ab
Rb IgG
200
160
120
ABP (mmHg)
1 min


B.
% increase in MAP (from baseline)
25
20
15
10
5
0
ns
ns
Untreated (n=6)
TRPC1 Ab (n=6)
Rb IgG (n=6)

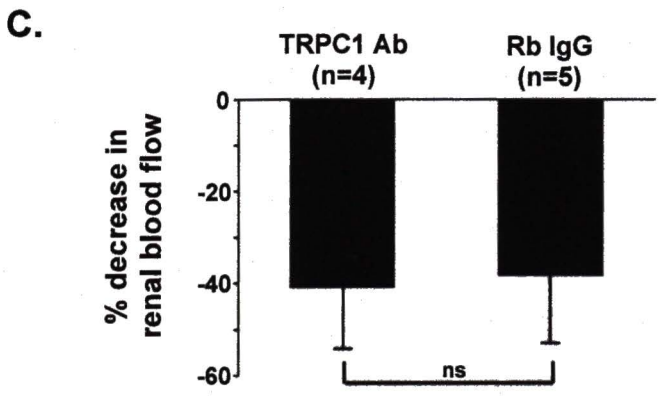

C.
TRPC1 Ab (n=4)
Rb IgG (n=5)
% decrease in renal blood flow
0
-20
-40
-60
ns

Figure 4. Single-channel currents in response to 1 μM AngII stimulation in MC with and without (Untreated) Rb IgG (200 ng/ml) or TRPC1 Ab (200 ng/ml). (A) Current-voltage (I-V) relation curves, showing development of single-channel currents with changes in membrane potentials in untreated MC. Membrane potentials are expressed as negative pipette potentials ($-V_P$). The calculated single-channel conductance was 17.2 pS. The reversal potential was estimated as 54 mV ($-V_P$). (B) Open probability (NP_O) of single channels before and after application of AngII in untreated MC. Holding potential was 80 mV (pipette). "n" indicates the number of cells analyzed. *Significant difference before and after application of AngII. (C) Representative traces, showing channel activity before (Before AngII) and after (AngII) AngII stimulation in untreated, Rb IgG–treated, and TRPC1 Ab–treated MC, respectively. Arrows indicate closed state of channels. Downward deflects indicate inward currents. Holding potential was 80 mV (pipette). Bottom trace on the right (inside a dashed rectangle) is the time-expanded portion of the trace indicated by a small dashed rectangle (indicated by a dashed arrow).

Figure 4.

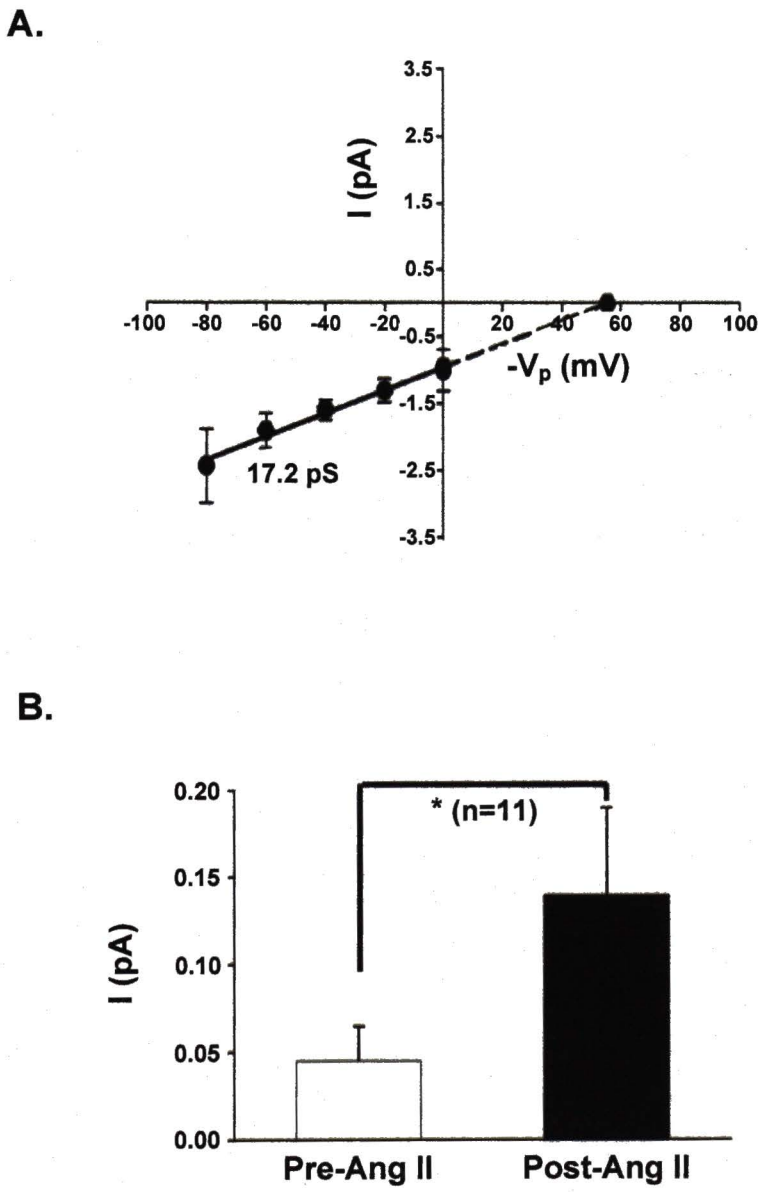

A.
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
I (pA)
-100
-80
-60
-40
-20
20
40
60
80
100
-Vp (mV)
17.2 pS
B.
0.20
0.15
0.10
0.05
0.00
I (pA)
* (n=11)
Pre-Ang II
Post-Ang II

Figure 4. (continued)

C.

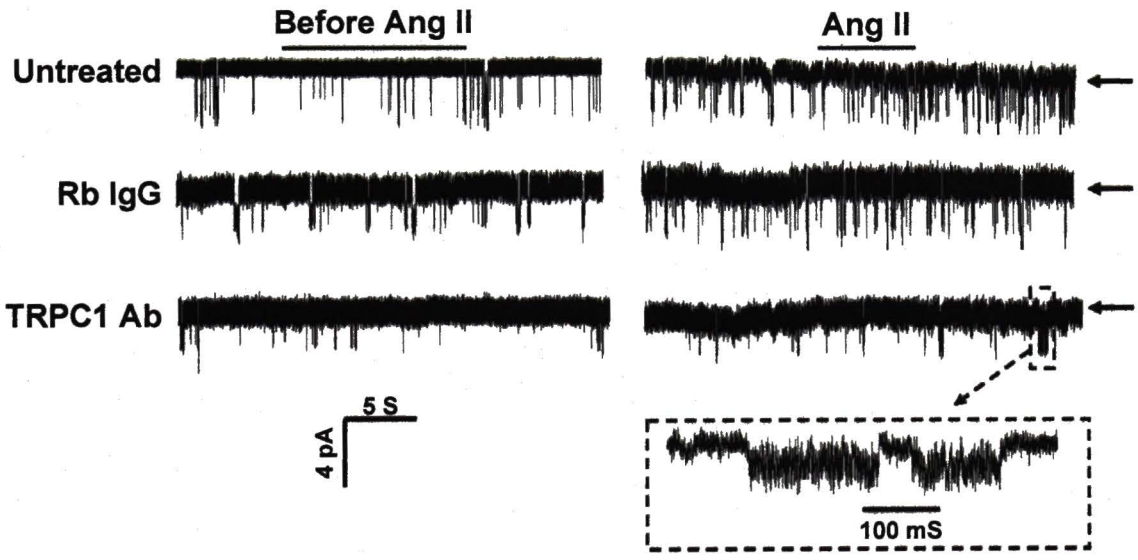


Figure 5. (A) Percent changes in channel activity (NP_O) induced by AngII stimulation in Rb IgG–or TRPC1 Ab–treated MC and in GFP+pSHAG- (pSHAG) or GFP+pSHAG-trpc1/RNAi- (pSHAG-trpc1/RNAi) transiently transfected MC. (B) Basal activities of the observed channels in MC of Rb IgG, TRPC1 Ab, pSHAG, and pSHAG-trpc1/RNAi groups. In both A and B, "n" indicates the number of cells analyzed in corresponding groups. *Significant difference between the indicated groups.

Figure 5.

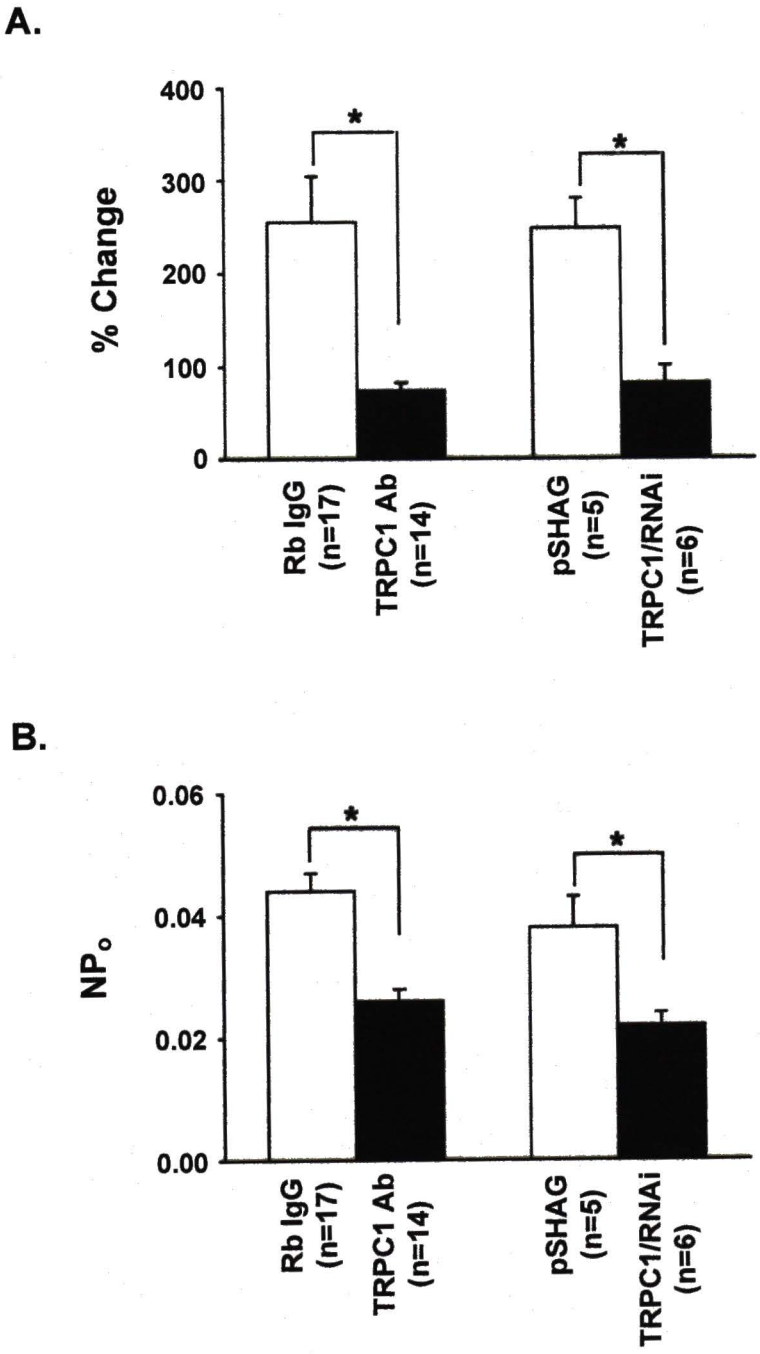

A.
400
300
200
100
0
% Change
*
*
Rb IgG (n=17)
TRPC1 Ab (n=14)
pSHAG (n=5)
TRPC1/RNAi (n=6)
B.
0.06
0.04
0.02
0.00
NPo
*
*
Rb IgG (n=17)
TRPC1 Ab (n=14)
pSHAG (n=5)
TRPC1/RNAi (n=6)

Figure 6. Fura-2 fluorescence ratiometry measurement of $[Ca^{2+}]_i$ in response to AngII stimulation. (A and B) Representative experiments, showing AngII-stimulated Ca^{2+} responses in untreated, Rb IgG–treated, and TRPC1 Ab–treated MC (A) and in yellow fluorescent protein (YFP) +pSHAG- (control) and YFP+pSHAG-trpc1/RNAi-transiently transfected MC (B). Application of AngII (1 μM) is indicated by the middle horizontal bars at the top of A and B. For exclusion of the involvement of voltage-operated Ca^{2+} channel in the AngII-induced Ca^{2+} responses, 1 μM nifedipine was included into the extracellular solution as indicated. "$[Ca^{2+}]_B$" represents Ca^{2+} concentration in the bathing solution, indicated by the lower horizontal bars at the top of the panels. (C) Summary data, showing the difference in the AngII-induced Ca^{2+} entry in response to Ca^{2+} re-addition in MC of all groups described in A and B. (D) Ratios of AngII-stimulated Ca^{2+} influx to Ca^{2+} release in MC of the five groups described in A and B. In both C and D, the numbers at the bottom of bars indicate the number of cells analyzed in corresponding groups. *Significant difference between the indicated groups.

Figure 6.

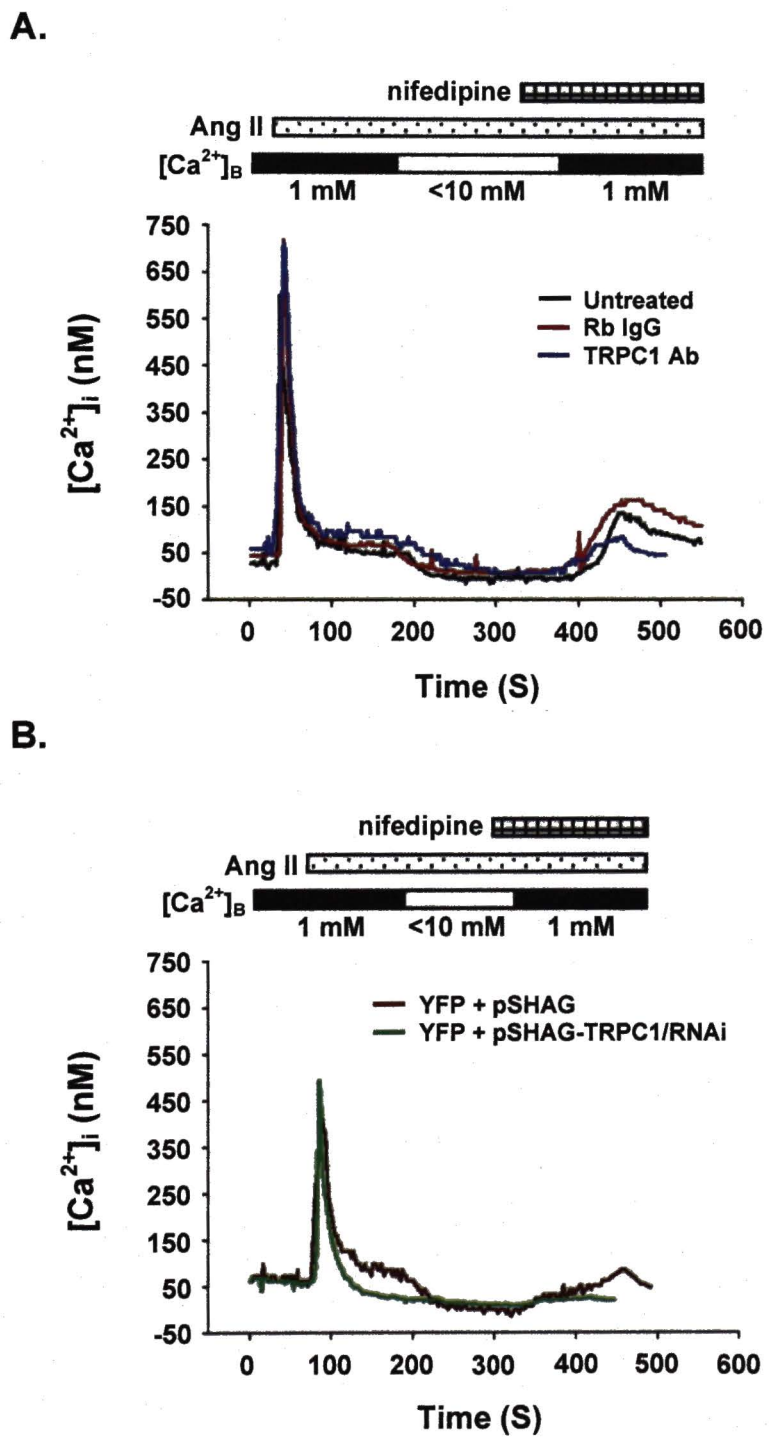
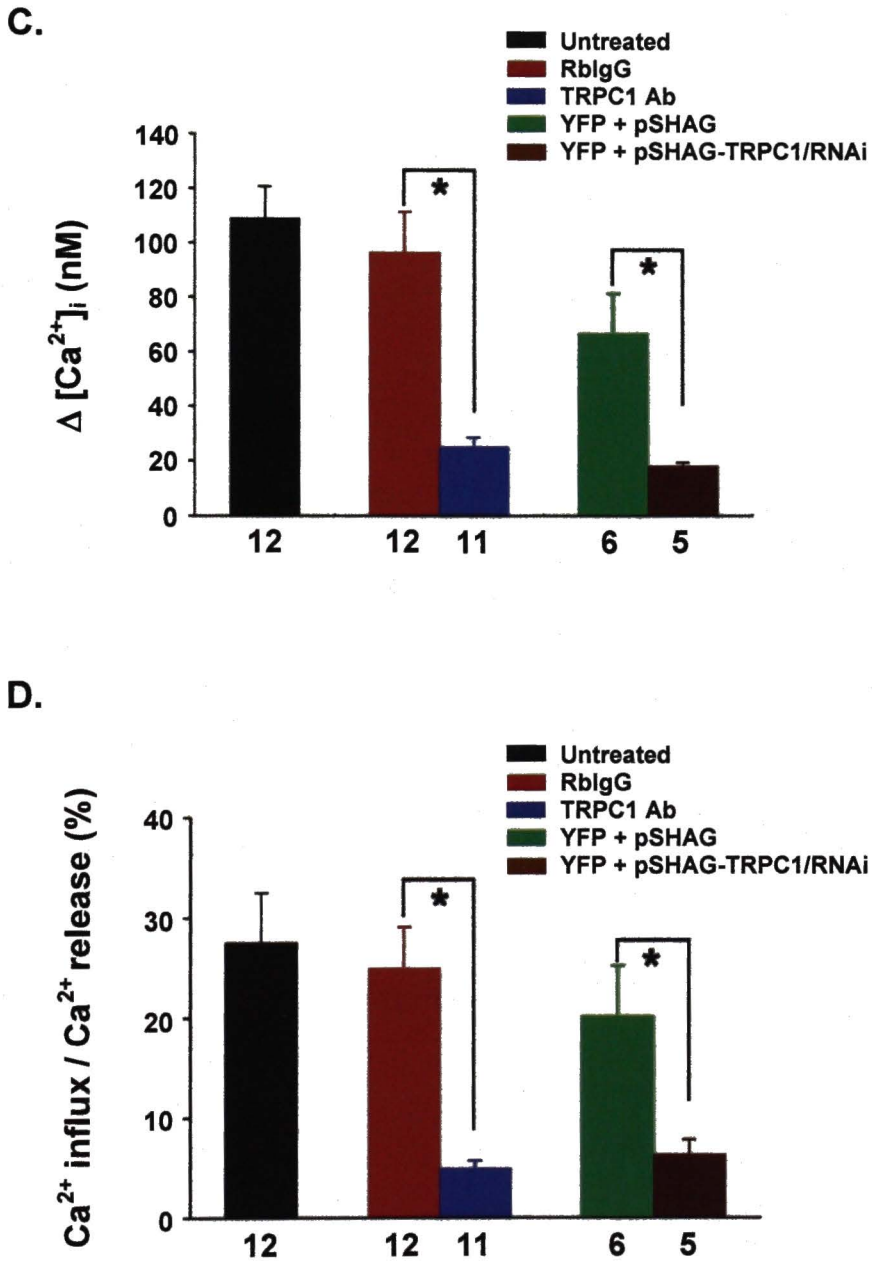

A.
nifedipine
Ang II
[Ca2+]B
1 mM
<10 mM
1 mM
750
650
550
450
350
250
150
50
-50
[Ca2+]i (nM)
Untreated
Rb IgG
TRPC1 Ab
0
100
200
300
400
500
600
Time (S)
B.
nifedipine
Ang II
[Ca2+]B
1 mM
<10 mM
1 mM
750
650
550
450
350
250
150
50
-50
[Ca2+]i (nM)
YFP + pSHAG
YFP + pSHAG-TRPC1/RNAi
0
100
200
300
400
500
600
Time (S)

Figure 6. (continued)



Abbreviations:

Ang II: angiotensin II

BP: blood pressure

ET-1: endothelin-1

FITC: fluorescein isothiocyanate

GFR: glomerular filtration rate

MAP: mean arterial pressure

MC: mesangial cell

PAH: para-aminohippurate

PSS: physiological saline solution

RBF: renal blood flow

RNAi: RNA interference

ROC: receptor-operated channel

SOC: store-operated channel

STIM1: stromal interaction molecule 1

TRPC: canonical transient receptor channel

VOCC: voltage-operated Ca^{2+} channel

References

1. Stockand JD, Sansom SC: Glomerular mesangial cells: Electrophysiology and regulation of contraction. *Physiol Rev* 78: 723–744, 1998
2. Ma R, Pluznick JL, Sansom SC: Ion channels in mesangial cells: Function, malfunction, or fiction. *Physiology* 20: 102–111, 2005
3. Ramsey IS, Delling M, Clapham DE: An introduction to TRP channels. *Annu Rev Physiol* 68: 18.1–18.29, 2006
4. Soboloff J, Spassova M, Xu W, He LP, Cuesta N, Gill DL: Role of endogenous TRPC6 channels in Ca2_ signal generation in A7r5 smooth muscle cells. *J Biol Chem* 280: 39786–39794, 2005
5. Odell AF, Scott JL, Van Helden DF: Epidermal growth factor induces tyrosine phosphorylation, membrane insertion, and activation of transient receptor potential channel 4. *J Biol Chem* 280: 37974–37987, 2005
6. Goel M, Sinkins WG, Zuo CD, Schilling WP: Identification and localization of TRPC channels in rat kidney. *Am J Physiol Renal Physiol* 290: F1241–F1252, 2006
7. Wang X, Pluznick JL, Wei P, Padanilam BJ, Sansom SC: TRPC4 forms store-operated Ca^{2+} channels in mouse mesangial cells. *Am J Physiol Cell Physiol* 287: C357–C364, 2004
8. Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE, Pollak MR: TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* 37: 739–744, 2005

9. Sours S, Du J, Chu S, Zhou JX, Ding M, Ma R: Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 290: F1507–F1515, 2006

10. Pluznick JL, Wei P, Carmines PC, Sansom SC: Renal fluid and electrolyte handling in BKCa-beta1 -/- mice. *Am J Physiol Renal Physiol* 284: F1274–F1279, 2003

11. Waugh WH, Beall PT: Simplified measurement of p-aminohippurate and other arylamines in plasma and urine. *Kidney Int* 5: 429–436, 1974

12. Ma R, Smith S, Child A, Carmines PK, Sansom SC: Storeoperated Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol* 278: F954–F961, 2000

13. Grynkiewicz G, Poenie MT: A new generation of Ca^{2+}-indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985

14. Novak J, Reckelhoff J, Bumgarner L, Cockrell K, Kassab S, Granger JP: Reduced sensitivity of the renal circulation to angiotensin II in pregnant rats. *Hypertension* 30: 580, 1997

15. Kerstens MN, van der Kleli FG, Boonstra AH, Sluiter WJ, van der Moles JC, Navis G, Dullaart RP: Angiotensin administration stimulates renal 11beta-hydroxysteroid dehydrogenase activity in healthy men. *Kidney Int* 65: 2065–2070, 2004

16. Perlstein TS, Gumieniak O, Hopkins PN, Murphey LJ, Brown NJ, Williams GH, Hollenberg NK, Fisher ND: Uric acid and the state of the intrarenal renin-angiotensin system in humans. *Kidney Int* 66: 1465–1470, 2004

17. Haque MZ, Majid DS: Assessment of renal functional phenotype in mice lacking gp91PHOX subunit of NAD(P)H oxidase. *Hypertension* 43: 335–340, 2004

18. Ambudkar IS: Ca2_ signaling microdomains: Platforms for the assembly and regulation of TRPC channels. *TiPS* 27: 25–32, 2006

19. Kasner SE, Ganz MB: Regulation of intracellular potassium in mesangial cells: A fluorescence analysis using the dye PBFI. *Am J Physiol Renal Fluid Electrolyte Physiol* 262: F462–F467, 1992

20. Matsunaga H, Yamashita N, Miyajima Y, Okuda T, Chang H, Ogata E, Kurokawa K: Ion channel activities of cultured rat mesangial cells. *Am J Physiol* 261: F808–F814, 1991

21. Vazquez G, Wedel BJ, Aziz O, Trebak M, Putney JW Jr: The mammalian TRPC cation channels. *Biochim Biophys Acta* 1742: 21–36, 2004

22. Clapham DE, Runnels LW, Strubing C: The TRP ion channel family. *Nat Rev Neurosci* 2: 387–396, 2001

23. Hall D, Carmines PK, Sansom SC: Dihydropyridine-sensitive Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 278: F97–F103, 2000

24. Li WP, Tsiokas L, Sansom SC, Ma R: Epidermal growth factor activates store-operated Ca^{2+} channels through an IP_3 independent pathway in human glomerular mesangial cells. *J Biol Chem* 279: 4570–4577, 2004

25. Xu SZ, Beech DJ: trpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. *Circ Res* 88: 84–87, 2001

26. Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S, Worley PF: Homer binds TRPC family

channels and is required for gating of TRPC1 by IP3 receptors. *Cell* 114: 777–789, 2003

27. Rosado JA, Brownlow SL, Sage SO: Endogenously expressed TRP1 is involved in store-mediated Ca^{2+} entry by conformational coupling in human platelets. *J Biol Chem* 277: 42157–42163, 2002

28. Kim SJ, Kim YS, Yuan JP, Petralla RS, Worley PF, Linden DJ: Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. *Nature* 426: 285–291, 2003

29. Ahmmed GU, Mehta D, Vogel S, Holinstat M, Paria BC, Tiruppathi C, Malik AB: Protein kinase Calpha phosphorylates transient receptor potential channel-1 (TRPC1) and regulates store operated Ca^{2+} entry. Role in signaling increased endothelial permeability. *J Biol Chem* 279: 20941–20949, 2004

30. Bagchus WM, Hoedemaeker PJ, Rozing J, Bakker WW: Glomerulonephritis induced by monoclonal anti-Thy 1.1 antibodies. *Lab Invest* 55: 680–687, 1986

31. Strubing C, Krapivinsky G, Krapivinsky L, Clapham DE: TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* 29: 645–655, 2001

32. Liu X, Singh BB, Ambudkar IS: TRPC1 is required for functional store-operated Ca^{2+} channels. *J Biol Chem* 278:11337–11343, 2003

33. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD: STIM1 is a Ca^{2+}_ sensor that activates CRAC channels and migrates from the $Ca^{2+}Ca^{2+}$ store to the plasma membrane. *Nature* 437: 902–905, 2005

34. Spassova MA, Soboloff J, He L, Xu W, Dziadek MA, Gill DL: STIM1 has a plasma membrane role in the activation of store-operated Ca^{2+} channels. *Proc Natl Acad Sci USA* 103: 4040–4045, 2006

35. Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF: STIM1 carboxyl-terminus activates native SOC, ICRAC and TRPC1 channels. *Nat Cell Biol* 8: 1003–1010, 2006

36. Lopez J, Salido GM, Pariente JA, Rosado JA: Interaction of STIM1 with endogenously expressed hTRPC1 upon depletion of intracellular Ca^{2+} stores. *J Biol Chem* 281: 28254–28264, 2006

37. Kiselyov K, Kim JY, Zeng W, Muallem S: Protein-protein interaction and function TRPC channels. *Eur J Physiol* 451:116–124, 2005

CHAPTER IV

STORE-OPERATED CA^{2+} ENTRY THROUGH TRPC1/TRPC4 COMPLEXES INTERACTING WITH STIM1 PROTEIN IN GLOMERULAR MESANGIAL CELLS

Sherry Sours-Brothers, Min Ding, Joseph Oberdorfer, Sarabeth Graham and Rong Ma

Department of Integrative Physiology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX 76107

Submitted manuscript under revision

Abstract

Although Orai1 protein was recently identified as the component of CRAC channels in hemopoietic cells, store-operated channels in other cell types might have different molecular entity. Also, the activation mechanism of store-operated channels still remains unclear in general. In the present study, we tested the hypothesis that TRPC1 and TRPC4 proteins were functional subunits of store-operated channels in glomerular mesangial cells and STIM1, a known Ca^{2+} sensor in the endoplasmic reticulum, was required for the channel activation through interaction with the TRPC proteins. Cell-attached patch clamp and fura-2 fluorescence measurements showed that single knockdown of either TRPC1 or TRPC4 significantly attenuated, but did not abolish thapsigargin-induced channel activation and Ca^{2+} entry. However, double knockdown of both TRPCs resulted in a significantly greater inhibition. Reciprocal co-immunoprecipitation indicated a physical interaction between the two TRPC isoforms and this interaction was enhanced by thapsigargin treatment. Confocal microscopy and biotinylation assays suggested that store depletion by thapsigargin triggered the membrane translocation of TRPC1, but not that of TRPC4. Furthermore, we found that knockdown of STIM1 using RNAi significantly reduced the thapsigargin-stimulated membrane currents. Co-immunoprecipitation showed that STIM1 interacted with TRPC4, but not TRPC1. Taken together, we concluded that in glomerular mesangial cells, store depletion triggers membrane recruitment of cytosolic TRPC1 and heteromultimerization with TRPC4 in the plasma membrane. The TRPC1/TRPC4 complexes constitute the

functional subunits of store-operated channels and are activated through interaction between STIM1 and TRPC4 in the channel complexes.

Keywords: TRPC1, TRPC4, STIM1, store-operated Ca^{2+} channel, store-operated Ca^{2+} entry, glomerular mesangial cell

Introduction

Store-operated Ca^{2+} entry (SOCE) is a ubiquitous signaling mechanism in non-excitable and excitable cells that is responsible for the regulation of diverse cellular functions ranging from cell proliferation and gene expression to cell contraction and secretion (28). Physiologically, this Ca^{2+} entry mechanism is triggered by activation of phospholipase C by G-protein-coupled receptors or receptor tyrosine kinases that induces Ca^{2+} release from the endoplasmic reticulum (ER), which then activates store-operated Ca^{2+} channels (SOC) in the plasma membrane. Despite intensive studies in the last two decades, the molecular identity of the SOC and the cellular mechanisms underlying the coupling of store depletion and Ca^{2+} entry remain elusive. This case is even more complicated by the fact that SOC behaves differently in different types of cells, implying that the protein components or regulatory mechanisms of SOC might be cell type specific. For instance, the SOC of lymphocytes, known as Ca^{2+} release-activated Ca^{2+} channels (CRAC), are distinguished from other SOCs by their extremely high selectivity for Ca^{2+} and extremely low conductance (15; 19). However, these properties are not displayed in excitable cells (1; 2; 26).

Members of the canonical transient receptor potential (TRPC) protein family have been proposed as candidates for SOC (27; 28). These include studies on TRPC1 (16; 25), TRPC3 (17), TRPC4 (39), TRPC5 (29), TRPC6 (9259}, and TRPC7 (32). TRPCs are thought to exist as tetramers and in many cases different members within TRPC subgroups may exist as functional heteromultimers (11; 14; 27). For example, SOCE is mediated by TRPC1 complex forming with TRPC3 in human parotid gland ductal cells

and hippocampal neuronal cells, or with TRPC4 in endothelial cells (6; 23; 40). This variability of subunit complex composition in different tissues may confer specificity of channel function. Recently, a major breakthrough in the field of SOC is the finding that Orai1 protein is the component, most likely the pore-forming subunit of CRAC channel, the prototypic SOC (9; 30; 36; 37; 41; 43). However, whether the *Orai* genes account for all SOCs existing in different cell types and whether the Orai 1 protein requires binding partners to form native SOCs remain unknown.

Recently, the stromal interaction molecule 1 (STIM1) has been identified as a Ca^{2+} sensor in the ER, activating SOC at the plasma membrane upon store-depletion (22; 33). There is evidence that STIM1 diffusely distributed in the ER membrane translocates to the regions of the ER directly apposed to the plasma membrane upon store depletion, where it triggers SOCE (3; 18; 21). Thus, SOCs can be considered channels that are regulated by STIM1 and require the store depletion-mediated clustering of STIM1.

Our laboratory has previously demonstrated that TRPC1, -3, -4, and -6 were selectively expressed in human glomerular mesangial cells (MCs) and that TRPC1 associated with TRPC4 and -6 (34). Further study demonstrated that TRPC1 mediated Ang II-induced Ca^{2+} signaling and contraction in these vascular smooth muscle-like contractile cells (7). However, the nature of the TRPC1 channel activation (store-operated or non store-operated) and the requirement for binding partners have not been defined. In the present study, we showed evidence that TRPC1 interacted with TRPC4 to form channel complexes mediating SOCE in MCs. STIM1 was required for activation of the SOC, probably via interaction with TRPC4 protein.

Materials and Methods

Mesangial Cell culture. Human MCs used in this study were purchased from Cambrex Company (East Ruthersord, NJ). The procedures and methods for culturing MCs have been described previously (7; 34). Subpassages of less than ten generations of cells were used.

Transient transfection. Transient transfection was carried out using Lipofectamine and Plus reagents or LTX according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). MCs were transiently transfected with TRPC1, TRPC4 or STIM1 RNAi constructs or EYFP-tagged TRPC1 expression plasmids. Cells were used 24-48 hours after transfection. Knockdown of gene expression with RNAi constructs was confirmed in HEK293 cells co-transfected with corresponding overexpression plasmids.

Stable transfection. Stable transfection was performed to simultaneously knockdown TRPC1 and TRPC4 in MCs. Double knockdown of the two proteins was carried out by co-expression of TRPC1 and TRPC4 RNAi constructs containing blasticidin and hygromycin selectable markers. The antibiotics were added to cell culture medium 48 hours after transfection. Cells surviving after at least one week were used for functional experiments.

Immunoprecipitation and immunoblotting. Cell monolayers were collected with PBS then lysed in 1% Triton X-100 buffer containing (in mM) 150 NaCl, 10 Tris-HCl (pH 7.5), 1 EGTA, 0.2 sodium orthovanadate, 0.2 phenylmethylsulfonyl fluoride, 0.5% NP-40, aprotinin (1 μg/ml), pepstatin (1 μg/ml), and proteinase inhibitor cocktail (Roche

Applied Science, Indianapolis, IN) followed by centrifugation at 6,000 g for 15 min at 4°C. For coimmunoprecipitation (co-IP) experiments, the cell lysates were incubated for 2 h with the primary antibody indicated, followed by immunoprecipitation (IP) for 1 h with 30-μl slurry of protein G or A (Amersham Biosciences, Piscataway, NJ) in 50 mM Tris-HCl. Immunocomplexes were then washed five times in lysis buffer. The cell lysates (without precipitation, for inputs) and immunoprecipitates (for co-IP) were undertaken into regular Western blotting.

For immunoblotting experiments, all samples were fractionated by 10% SDS-PAGE, transferred to PVDF membranes, and probed with the indicated primary antibodies. Bound antibodies were visualized with Super Signal West Femto Luminol/Enhancer Solution (Pierce Biotechnology, Rockford, IL).

Fluorescent immunocytochemistry. MCs were plated on 22×22-1 mm coverslips, fixed with iced acetone/methanol, and incubated with TRPC1 antibody in PBS plus 2% heat inactivated goat serum and 0.2% Triton X-100 for 1 h. Three washes with PBS containing 2% goat serum and 0.2% Triton X-100 were followed by blocking at 4°C overnight in blocking buffer containing 2% goat serum and 0.2% Triton X-100. The cells were then incubated with secondary antibodies (goat anti-mouse conjugated with Alexa Fluor 568, Molecular Probe) for 1 h. Cells were washed 3 times with PBS and processed for confocal microscopy using a Leica Confocal Laser Scanning microscope.

Biotinylation assays. When cell monolayers were 80% confluent, the cells were washed twice with PBS and then scraped in 1 ml PBS (per 10 cm plate). The cell suspension was centrifuged at 6,000 g for 15 min at 4OC and the pellet was resuspended

with 1 ml PBS and biotinylated with 0.05 mg/ml of sulfo-NHS-SS-biotin (Pierce) for 30 min at room temperature. After centrifugation at 6,000 g for 15 min at 4°C, the pellet was washed 3 times with PBS and then lysed in 1 ml of lysis buffer. The cell lysates were then centrifuged at 21,000 g for 15 min at 4°C. 300 µl of lysates were saved for immunoblotting. 700 µl of supernatants were mixed with 300 µl of lysis buffer (total volume: 1 ml) and then incubated with 30 µl of slurry of immobilized streptavidin beads (Pierce) for 1 h with shaking. The beads were spun down and washed 2 times with lysis buffer. The biotinylated samples were then analyzed by Western blot.

Patch clamp procedure. Conventional cell-attached voltage clamp was carried out as described previously (7; 12). Single channel analysis was made with a Warner PC-505B amplifier (Warner Instrument Corp., Hamden, CT) and pClamp 9.2 (Axon Instrument, Foster City, CA). The extracellular solution contained (mM): 135 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, and 10 glucose. The pipette solution contained (mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 3 EGTA, 10 HEPES. The calculated free Ca^{2+} concentration in the pipette solution was <10 nM (MTK software). At the time of each experiment, the pipette solution was supplemented with 100 µM niflumic acid, 10 mM TEA, and 100 nM iberiotoxin to block Ca^{2+}-activated Cl^- channels and K^+ channels, respectively. Recordings were made at a holding potential of 80 mV (pipette).

Ratiometric Ca^{2+} imaging. $[Ca^{2+}]_i$ was measured in MCs using dual excitation wavelength fluorescence microscopy with fura-2 as described previously (7; 12). Briefly, MCs grown on coverslips (22×22 mm), were loaded with fura-2 by incubation for ~45 min at room temperature in the dark in physiological saline solution (PSS) containing 2

μM acetoxymethyl ester of fura-2 (fura-2/AM), 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 (Molecular Probes, Eugene, OR). After washout, the coverslip was then mounted to a perfusion chamber (Warner, Model RC-2OH) and placed on the stage of a Nikon Diaphot inverted microscope. Fura-2 fluorescence was monitored by a ratio technique (excitation at 340 and 380 nm, emission at 510 nm) using Metafluor software (Universal Imaging, West Chester, PA). Bath $[Ca^{2+}]$ was reduced to less than 10 nM during the experiments by addition of EGTA according to the Ca^{2+} concentration program by MTK Software. $[Ca^{2+}]_i$ was calculated using the formula described by Grynkiewicz *et al* (Grynkiewicz *et al.*, 1985). Calibrations were performed in vivo at the end of each experiment, and conditions of high $[Ca^{2+}]_i$ were achieved by adding 4 μM ionomycin, whereas conditions of low $[Ca^{2+}]_i$ were obtained by adding 10 mM EGTA.

Antibodies, plasmids and chemicals. TRPC1 mouse monoclonal antibody (1F1), TRPC1-pEYFP-N1 and HA-TRPC1 overexpression plasmids, and TRPC1-RNAi construct (shRNA-TRPC1) were obtain from Dr. Leonidas Tsiokas (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Rabbit anti-TRPC4 antibody was obtained from Sigma-Aldrich (St. Louis, MO) and GOK/STIM1 mouse monoclonal antibody was obtained from BD Biosciences Pharmingen (San Jose, CA). Bovine TRPC4-pcDNA3 was obtained from L. Birnbaumer (National Institutes of Health). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or as indicated.

Statistical Analysis. Data are reported as means ± SE. The one-way ANOVA plus Student-Newman-Keuls test, Student unpaired t-test, and Student paired t-test were used to analyze the differences among multiple groups, between two groups, and before and

after treatment in the same group, respectively. $P<0.05$ was considered statistically significant.

Results

TRPC1 and TRPC4 contribute to SOC activity. TRPC1 and TRPC4 have been previously identified in MCs *in vitro* as well as *in vivo* (34). In the current study, cell-attached patch clamp was employed to examine whether the two TRPC subtypes mediated SOCE in cultured MCs. Thapsigargin (TG) was used to activate SOC by passive depletion of internal Ca^{2+} stores. Consistent with our previous report (26), MCs have spontaneous, but relatively low channel activity under resting state. After stimulation with 1 μM TG, single channel currents increased strikingly (Fig. 1A). The TG-induced channel activation usually onset within 1 min and reached peak about 3 min after treatment. In some cases, multiple levels of the channel opening could be seen in one patch. Because the pipette solution contained niflumic acid, TEA, and iberiotoxin, the recorded channel currents were unlikely mediated by Cl^- or K^+ channels, both expressed in MCs (24). The calculated NP_O from 8 cells showed that TG treatment significantly raised channel activity (0.024 ± 0.003 vs. 0.11 ± 0.05, before TG vs. TG).

Our previous study has demonstrated that the TG-stimulated single channel currents in MCs were mediated by SOC (26). To further verify the store-operated nature of the currents, we examined the blocking effect of MRS1845 and La^{3+} on the TG-induced response. MRS1845 at 10 μM has been reported to selectively inhibit SOC (13). La^{3+} is another reliable and selective SOC inhibitor at concentrations in the low

micromolar (1-5 μM) (5; 26; 35). As shown in Fig. 1 A and B, inclusion of 10 μM MRS1845 into the pipette nearly abolished the TG-evoked channel activation. In agreement with our previous report (26), 2 μM La^{3+} not only blocked TG-induced response completely, but significantly inhibited basal activity as well.

We then determined if TRPC1 and TRPC4 proteins were components of SOC in MCs by examining the effect of knocking down the individual TRPCs on SOC activity. Knockdown of TRPC1 and TRPC4 was accomplished by transient transfection with RNAi plasmids that produced short hairpin RNAs specifically targeting on human *trpc1* (designated as shRNA-TRPC1) or *trpc4* (designated as shRNA-TRPC4). GFP expression plasmids were co-transfected with the RNAi constructs or empty vectors (Mock control) at 10 fold less for identification of successfully-transfected cells. Western blot showed that these RNAi constructs successfully and efficiently reduced the expression levels of corresponding TRPCs (Fig. 2A). As shown in Fig. 2B, either knockdown of TRPC1 or TRPC4 significantly, but not completely inhibited the channel activation induced by TG. These results indicated that both TRPC1 and TRPC4 are contributors to SOC in MCs. However, the native SOCs in MCs were not solely made of either of the single TRPC proteins.

TRPC1 and TRPC4 form channel complexes mediating SOCE. Our previous study has demonstrated a physical interaction between natively expressed TRPC1 and TRPC4 in MCs (34). The patch clamp experiments described above showed that individual TRPC1 or TRPC4 contributed partial, but not entire TG-evoked channel activity. Thus, we speculated that the functional SOCs in MCs might be formed by

protein complexes of TRPC1 and TRPC4. If this is true, then simultaneous knockdown of TRPC1 and TRPC4 should result in an additive inhibition on the channel function. Fluorescent dual-excitation wavelength imaging of fura-2 was used to measure changes in $[Ca^{2+}]_i$. A standard Ca^{2+}-readmission protocol was adopted to evaluate SOCE (26). In agreement with our previous report (26), under 1 mM Ca^{2+} extracellular solution, application of 1 μM TG evoked a rapid and striking cytosolic Ca^{2+} transient followed by a sustained elevation of $[Ca^{2+}]_i$. Removal of Ca^{2+} from the extracellular solution resulted in a decrease in $[Ca^{2+}]_i$ and then readmission of Ca^{2+} into the Ca^{2+} free bath resulted in a marked rise in $[Ca^{2+}]_i$ (Fig. 2C), an indicator of SOCE (26). Consistent with the patch clamp data (Fig. 2B), this Ca^{2+} entry in response to Ca^{2+} radmission was significantly inhibited, but not abolished by knockdown of single TRPC1 or TRPC4. However, double knockdown of both subtypes of TRPC simultaneously had an additive effect and the reduction of SOCE in the double knockdown was significantly greater than that of either individual knockdown (Fig. 2D). Although there was residual Ca^{2+} entry in the double knockdown of cells, this change was not statistically significant. Combined with the evidence on the physical interaction of TRPC1 and TRPC4 (26), the results from the current study suggest that TRPC1 and TRPC4 might be acting in tandem, and that TRPC1/TRPC4 complexes constitute functional SOCs in MCs.

Store-depletion stimulates interaction between TRPC1 and TRPC4. The data described above indicate that TRPC1 and TRPC4 mediate SOCE by forming channel complexes in MCs. We speculated that the formation of TRPC1/TRPC4 heteromers is dynamic, dependent on store depletion. To test this speculation, reciprocal co-IP was

performed with and without TG stimulation. As shown in Fig. 3 A, IP of TRPC1 increased co-IP with TRPC4 after 3 min-TG treatment, compared to untreated cells. The increased co-IP by TG was also seen when reversed, with pull-down of TRPC4 followed by western blot of TRPC1 (Fig. 3B). This biochemical evidence strongly indicates that store depletion promotes formation of TRPC1/TRPC4 complexes that might underlie SOCE across the cell membrane.

Store depletion triggered plasma membrane translocation of TRPC1 Two possibilities could exist to result in an increase in TRPC1/TRPC4 complexes in the plasma membrane upon store depletion. One mechanism might be that depletion of internal Ca^{2+} stores stimulates TRPC1-TRPC4 interaction in the cytoplasm and the entire complexes are then inserted into the plasma membrane as functional channels. The other possibility is that store depletion triggers one of the two TRPC isoforms to migrate to the plasma membrane where it incorporates with its binding partner to form functional channel complexes. We favored the latter and proposed that TRPC1 translocated to the plasma membrane upon store depletion and bound to the existing TRPC4 in the plasma membrane. This hypothesis was tested in the current study with confocal microscopy and biotinylation of membrane proteins followed by Western blot. Fig. 4A shows the specific TRPC1 staining probed with 1F1 TRPC1 antibody in MCs with and without TG treatment. In control MCs (no TG treatment), TRPC1 was diffusely distributed in the cell (Fig. 4Aa). However, depleting stores with TG clearly increased the expression of TRPC1 in the peripheral region (the plasma membrane or sub-plasma membrane) of the cell (Fig. 4Ac). This finding was further validated in a live cell imaging assay. In this set

of experiments, we transiently transfected EYFP-TRPC1 constructs into MCs. At the time of the experiment, the successfully transfected cells were identified with green signals and the cell membrane of which was stained with 15 μM FM4-64 dye (red signal), a powerful and fast plasma membrane marker for live cells. The time course change in TRPC1 distribution in response to TG was continuously monitored with confocal microscopy. As shown in Fig. 4B, in the two adjacent EYFP-TRPC1-transfected cells, TRPC1 was expressed in both cytoplasm and the plasma membrane before TG treatment. After 3 min incubation with TG, more TRPC1 appeared in the plasma membrane, indicated by more and stronger yellow signals in the plasma membrane.

We also examined the membrane translocation of TRPC1 responding to TG treatment using a nonmembrane permeant agent (sulfo-NHS-SS-biotin) that biotinylates surface proteins, and precipitated the proteins with avidin-linked streptavidin beads. As shown in Fig. 5, the expression of biotinylated TRPC1, but not TRPC4, was significantly increased in the cells treated with TG (Fig. 5Aa and b, and Fig. 5B). The TRPC1 response appeared to be time-related, reaching a peak 3 min after treatment, which was consistent with electrophysiological data (Fig. 1). The enrichment of cell membrane proteins in the biotinylated fractions and equal amounts of loaded proteins in different samples were confirmed by reprobing the blots with anti-integrin β1 antibody, a membrane marker (Fig. 5Ac). The biotinylated membrane fraction was not contaminated by non-plasma membrane proteins because calnexin, an ER protein, was not detected in the biotinylated fractions (Fig. 5Ad).

Taken together, all confocal microscopy and biochemical data support the idea that depletion of internal Ca^{2+} stores specifically triggered translocation of cytosolic TRPC1, but not TRPC4, to the plasma membrane.

STIM1 was required for SOCE in MCs. Recent evidence demonstrates that STIM1 acts as a regulator of SOCE by signaling store-depletion in the ER to SOC at the plasma membrane. STIM1 is ubiquitously expressed in a number of cell types, and MCs are no exception. Here we confirmed a critical role for STIM1 in SOCE in MCs using a cell-attached patch clamp approach. As shown in Fig. 6A and B, specific knockdown of STIM1 by transient transfection of specific RNAi constructs (hSTIM1-shRNA) dramatically attenuated TG-stimulated increase of NP_O. This inhibitory effect was even greater than that by knockdown of single TRPC1 or TRPC4 (Fig. 2B). Western blot verified that the RNAi constructs significantly reduced the expression of STIM1 protein in MCs (Fig. 6C).

Recent studies suggest that store depletion triggers STIM1 to cluster in a local region of the ER membrane which closely apposes to the plasma membrane in which SOC proteins cluster (18), If TRPC1/TRPC4 complexes are the components of SOC, it is possible that there is a physical interaction between the TRPC proteins and STIM1. We tested this postulation using co-IP assay and found that STIM1 was able to pull-down TRPC4 (Fig. 7A). The estimated amount of TRPC4 interacting with STIM1 was ~3% of total TRPC4 protein based on the Input. Although this is a small percentage, considering the influence from the affinity of antibody and protein beads, and experimental procedures of IP that could cause loss of the protein-protein interaction, this biochemical

data provide evidence of a physical interaction between TRPC4 and STIM1. However, we could not resolve a consistent interaction between STIM1 and TRPC1 by co-IP (Fig. 7B) suggesting that the TRPC4-STIM1 interaction might be specific in MCs.

Discussion

Although a good deal of recent evidence demonstrates that Orai protein is the long-sought molecular entity of CRAC channel, a unique SOC characterized in hemopoietic cells (9; 30; 43), distinct biophysical properties of SOC in non-hemopoietic cells underlie different molecular channel components (1; 2; 26). TRPC proteins, most likely TRPC heteromultimers, remain strong candidates of SOC in a variety of cell types (28). Since the distribution of TRPC subtypes and the abundance of single TRPC proteins are distinct in different types of cells (8; 10; 38), the compositions of TRPC complexes mediating SOCE might be cell type specific. Our findings in the current study suggest that TRPC1 and TRPC4 are critical components of SOC in glomerular MCs. Our data support the proposal that store depletion triggers cytosolic TRPC1 transcolation to the plasma membrane where TRPC1 incorporates into the existing TRPC4 to form heteromultimers mediating SOCE. The additive effect of knockdown of both subunits on SOCE (Fig. 2D) and a good correlation in the time course between the membrane recruitment of TRPC1 (Figs. 3-5) and the SOC activation (Fig. 1) further support this hypothesis. Wang, *et al* recently reported that TRPC1 and TRPC4 were only two isoforms of TRPC in mouse MCs based on RT-PCR assay, and speculated that TRPC4 constituted SOC by forming homotetramers (39). However, the role of TRPC1 in SOCE

was not examined in their study. It might be that the native SOC is solely composed of TRPC4 in mouse MCs, but requires both of TRPC1 and TRPC4 in human MCs.

Whether there is any other protein, particularly Orai1, contributing to the native SOC in human MCs is unknown. Interestingly, Liao, *et al* recently reported that Orai1 physically interacted with TRPC3 and TRPC6, and that Orai1 by interacting with the TRPCs acts as regulatory subunits that confer store depletion sensitivity to these channels (20). The similar interaction between Orai1 and TRPC1 and/or TRPC4 may also exist in human MCs in which TRPC1/TRPC4 complexes constitute the pore-forming subunits of SOC and Orai1 acts as an accessory subunit regulating channel activation.

There has long been a debate as to what mechanism accounts for the communication of store-depletion in the ER to activation of SOC channels at the plasma membrane. Previously, the so-called Ca^{2+} influx factor (CIF) was proposed to be a diffusible signaling molecule that communicated between the ER and the plasma membrane, but the molecular identity of this factor could not be identified (4). Direct conformational coupling between IP_3 receptors that initiate store-depletion and SOC that facilitate store-refilling has also been proposed (28). More recently, there has been strong evidence for STIM1 as a modulator of SOC activity. In this proposed mechanism, STIM1 acts as a Ca^{2+} sensor in the ER. Upon store-depletion, STIM1 translocates into punctae directly apposed to the plasma membrane where it activates SOCE (18; 31). Thus, SOCs could be considered as the channels that are regulated by STIM1 and require the store depletion-mediated clustering of STIM1. In the current study, we confirmed that STIM1 was required for SOCE in human glomerular MCs as evidenced by dramatically inhibited

SOC activation with RNAi of STIM1. This finding is in agreement with an earlier report by Zhang, *et al* in rat MCs (44). Moreover, our results from the current study further indicate that the STIM1-mediated SOC activation might be through its interaction with TRPC4 protein. Indeed, STIM1 has been shown to selectively bind to certain isoforms of TRPC proteins, including TRPC1 and TRPC4 (16; 42). Different from the studies by Yuan, *et al*, and Huang, *et al*, who reported that STIM1 interacted with TRPC1 (16; 42), we were not able to demonstrate any physical association of the two proteins. This may simply be due to weak binding between STIM1 and TRPC1 in MCs, or distinct TRPC1 antibody used (Alomone in their study and 1F1 antibody in our study). Relatively low expression level of TRPC1 in MCs may also make it difficult to isolate by immunoprecipitation. This is supported by the co-IP data from Huang's study that showed a very weak interaction between the endogenous TRPC1 and STIM1 in native brain tissue and a clear interaction was only seen in a cell line overexpressing the two proteins (16).

In conclusion, the findings from the current study suggest that in human glomerular MCs, store depletion triggers membrane recruitment of cytosolic TRPC1 and heteromultimerization of the translocated TRPC1 with TRPC4 in the plasma membrane. The TRPC1/TRPC4 complexes constitute the functional subunits of SOC and are activated through interaction between STIM1 in the ER and TRPC4 of the channel complexes in the plasma membrane. How the TRPC1 translocation and interaction with TRPC4 and how STIM1-TRPC4 interaction are regulated upon store depletion remain unclear and further study is needed to explore the detailed regulatory mechanisms. Given

a significant role of MCs in the regulation of glomerular filtration, identification of Ca^{2+} signaling pathway and its regulatory mechanisms, such as SOCE, in MCs has important physiological and pathophysiological relevance.

Acknowledgements

We thank Dr. Leonidas Tsiokas (University of Oklahoma Health Sciences Center, Oklahoma City, OK) for generously providing us HA-TRPC1 expression plasmids, shRNA-TRPC1 constructs, and 1F1 TRPC1 antibody. We also thank Dr. Lutz. Birnbaumer (National Institutes of Health) for providing us bovine TRPC4-pcDNA3 expression plasmids.

Grants

This study was supported by Scientist Development Grant from American Heart Association (Ma) and Young Investigator Grant from National Kidney Foundation (Ma).

Figure 1. A. Representative traces, showing single channel activity before (Before TG) and 3 min after (TG, 3') thapsigargin (1 μM) stimulation in MCs in the presence and absence of MRS1845 (10 μM) or La^{3+} (2 μM). Downward deflections indicate inward currents. Holding potential was 80 mV (pipette). Dashed lines and numbers indicate the current levels. **B.** Open probability (NP_O) of single channels before and after application of TG in the presence and absence of MRS1845 or La^{3+}. "n" indicates the number of cells analyzed. "*" indicates significant difference between TG and Before TG. "#" indicates significant difference between Before TG in Control group and Before TG in La^{3+} group.

Figure 1.

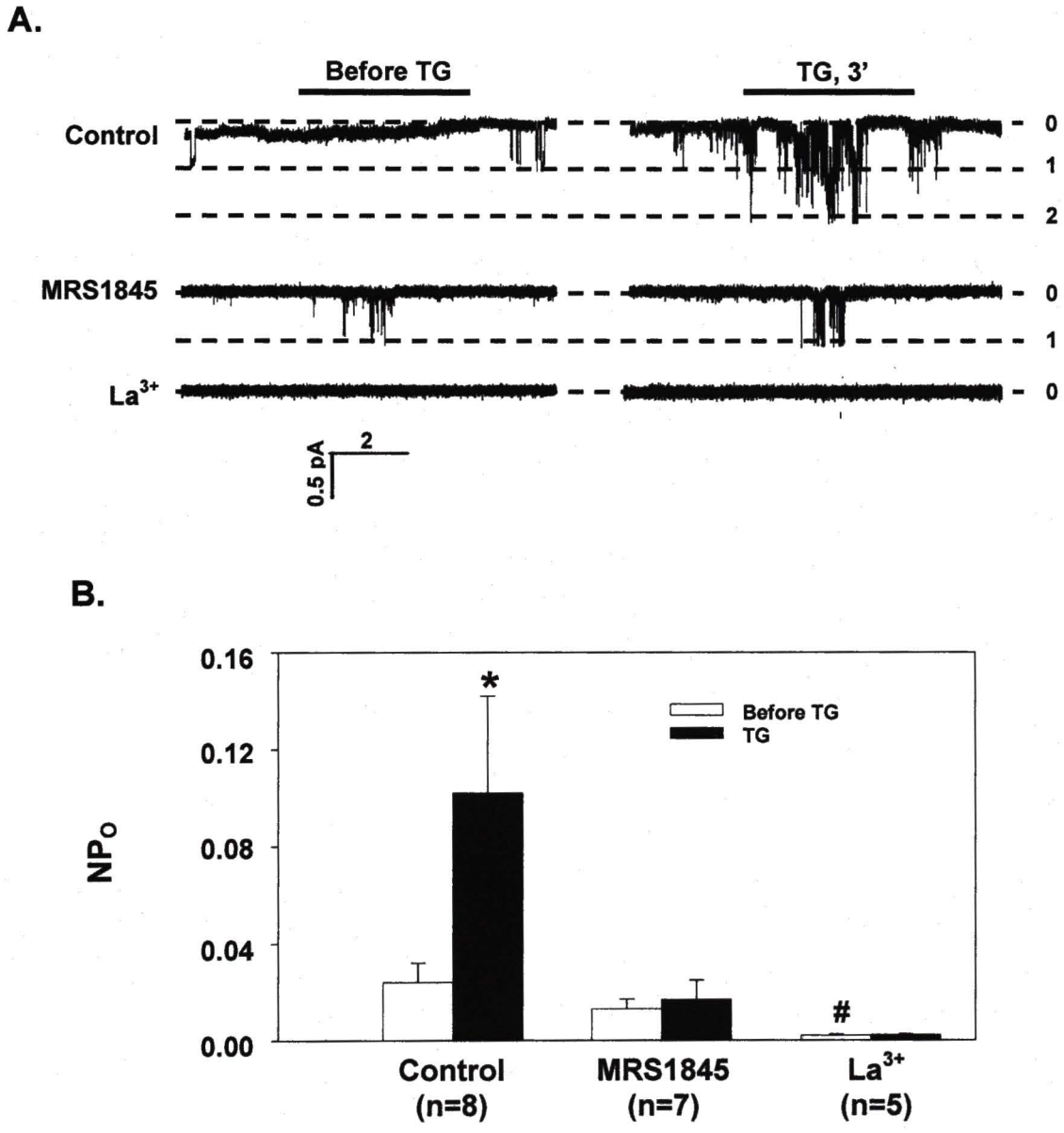
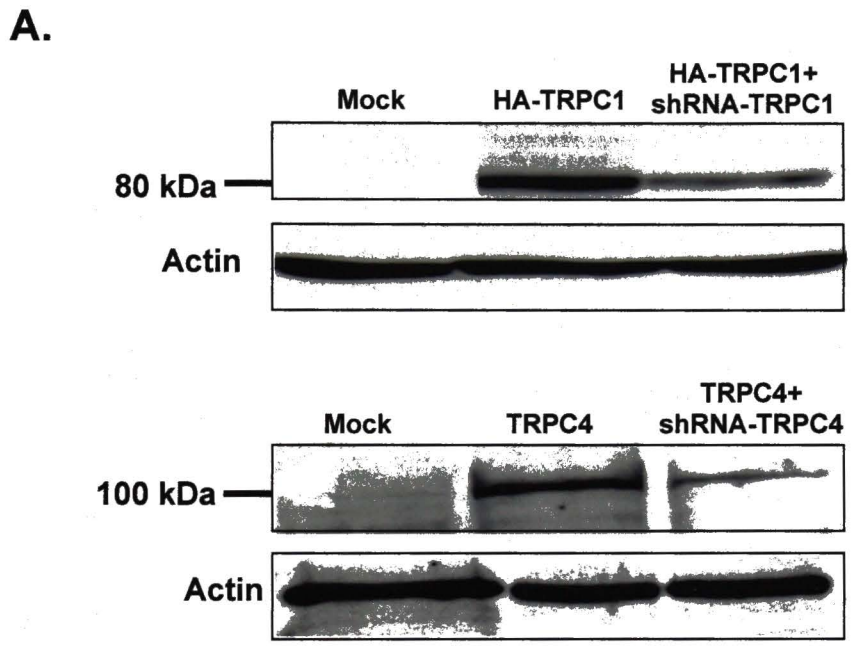

A.
Before TG
TG, 3'
Control
MRS1845
La3+
0
1
2
0
1
0
0.5 pA
2
B.
NPO
0.16
0.12
0.08
0.04
0.00
*
#
Before TG
TG
Control
(n=8)
MRS1845
(n=7)
La3+
(n=5)

Figure 2. A. Western Blot, showing expression levels of TRPC1 (upper panel) and TRPC4 (lower panel) in HEK293T cells with transient transfection of pSHAG empty vector (Mock), TRPC1 or TRPC4 expression plasmids (HA-TRPC1 or TRPC4), and the expression plasmids co-transfected with corresponding RNAi constructs (HA-TRPC1+shRNA-TRPC1 or TRPC4+shRNA-TRPC4). Actin was used as a loading control. **B.** Effect of knockdown of TRPC1 or TRPC4 on the TG-evoked channel activation, expressed as percent changes in NP_O from baseline after TG treatment. UT: untransfected; Mock: mock transfection; TRPC1 KD: knockdown of TRPC1 with shRNA-TRPC1; TRPC4 KD: knockdown of TRPC4 with shRNA-TRPC4. **C** and **D**: Fura-2 fluorescence ratiometry measurement of [Ca2+]i, indicated by the ratio of 340/380 nm fluorescence, showing the role of TRPC1 and TRPC4 in the TG-induced SOCE. **C.** A representative experiment, showing TG-induced Ca2+ transients inside the cell and SOCE. Application of TG is indicated by the top horizontal bar. $[Ca^{2+}]_B$ indicates the Ca2+ concentration in the bathing solution. **D.** Effect of knockdown of TRPC1 (TRPC1 KD) or TRPC4 (TRPC4 KD) individually and simultaneously (Double KD) on SOCE in MCs, expressed as percent increase in ratio of 340/380 nm fluorescence upon Ca^{2+} readmission. Mock: mock-transfected, serving as a control. "n" indicates the number of cells analyzed. "*" indicates $P<0.05$, compared with Mock. "†" indicates $P<0.05$, compared with TRPC1 KD and TRPC4 KD.

Figure 2.


A.
Mock
HA-TRPC1
HA-TRPC1+
shRNA-TRPC1
80 kDa
Actin
Mock
TRPC4
TRPC4+
shRNA-TRPC4
100 kDa
Actin

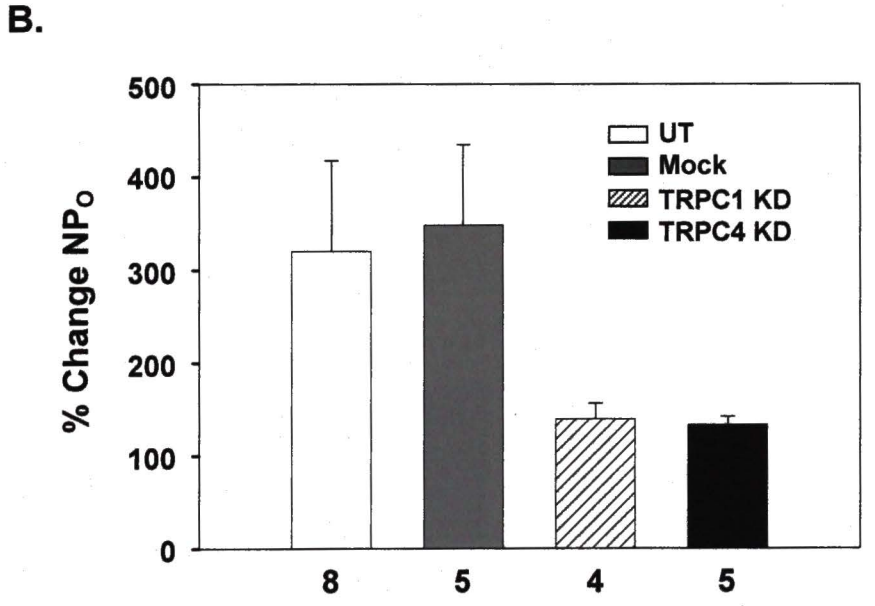
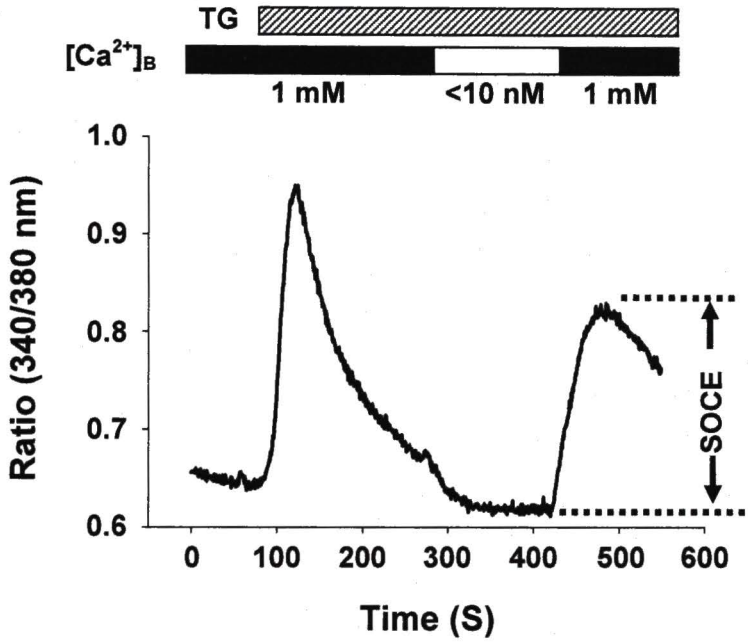

B.
% Change NPo
500
400
300
200
100
0
UT
Mock
TRPC1 KD
TRPC4 KD
8
5
4
5

Figure 2. (continued)

C.



D.

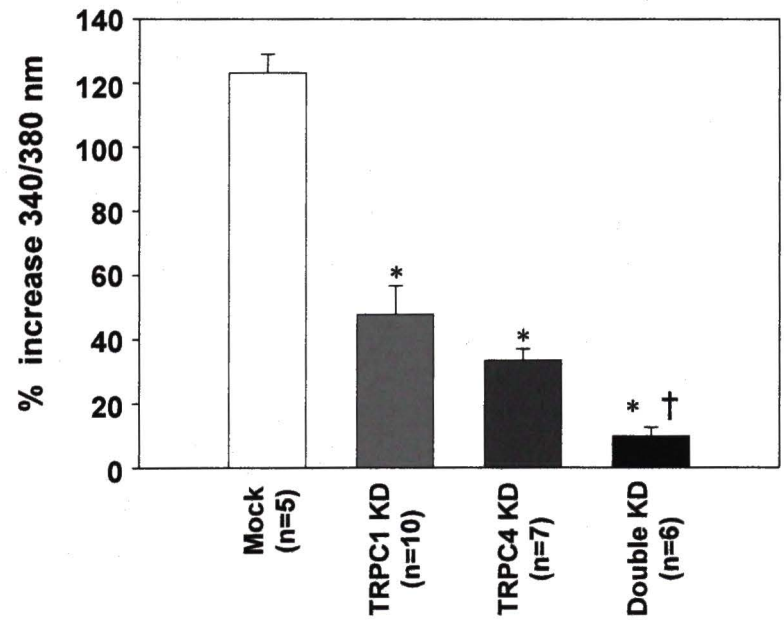


Figure 3. Co-immunoprecipitation showing interaction between TRPC1 and TRPC4, and the enhancement of the interaction by store depletion. **A.** Immunoprecipitation of TRPC1 followed by immunobloting TRPC4 in MCs with and without TG treatment for 3 min (Con and TG 3', respectively). Input indicates the cell lysates without TRPC1 pull down. **B.** Immunoprecipitation of TRPC4 and immunobloting TRPC1 in MCs with and without TG treatment for 3 min (Con and TG 3', respectively). Input indicates the cell lysates without TRPC4 pull down. **C** and **D.** Bar graph, showing the amounts of TRPC4 interacting with TRPC1 (C) in **A** and of TRPC1 interacting with TRPC4 (D) in **B**, expressed as the percentages of optical densitometry of the TRPC immunoblots in the precipitates to their corresponding inputs.

Figure 3.

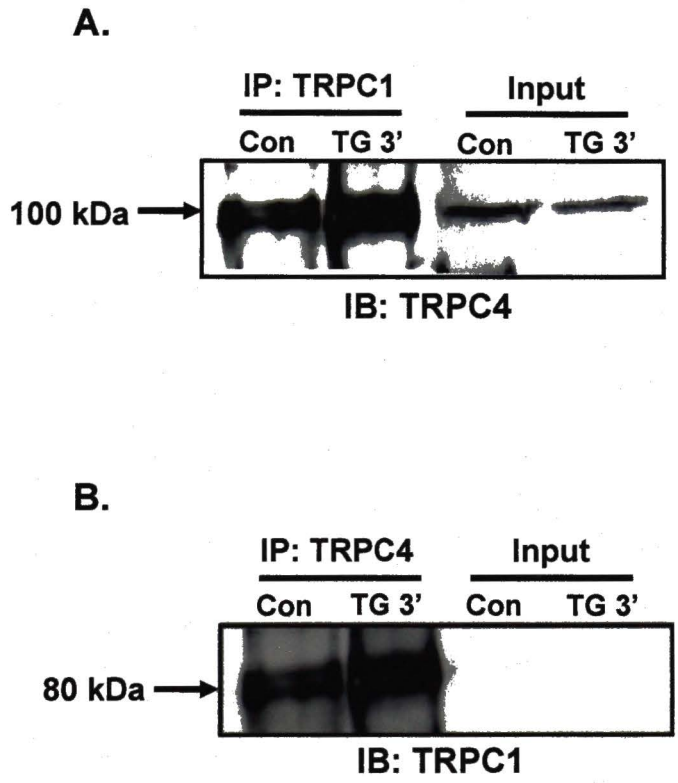
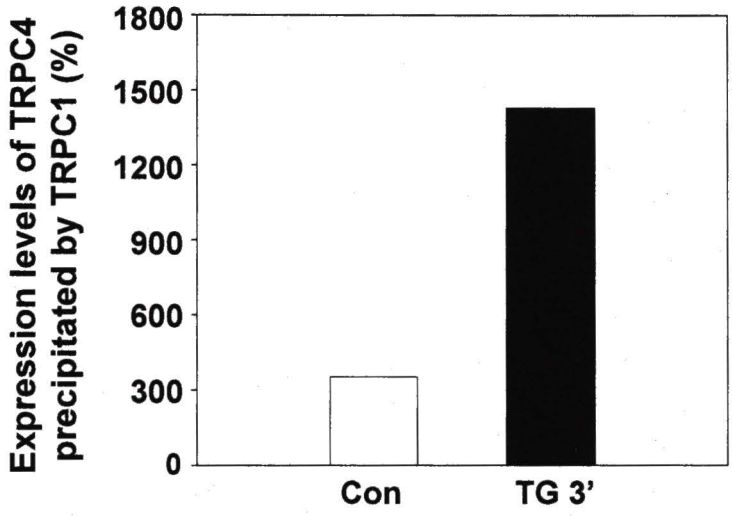

A.
IP: TRPC1
Input
Con
TG 3'
Con
TG 3'
100 kDa
IB: TRPC4
B.
IP: TRPC4
Input
Con
TG 3'
Con
TG 3'
80 kDa
IB: TRPC1

Figure 3. (continued)

C.



D.

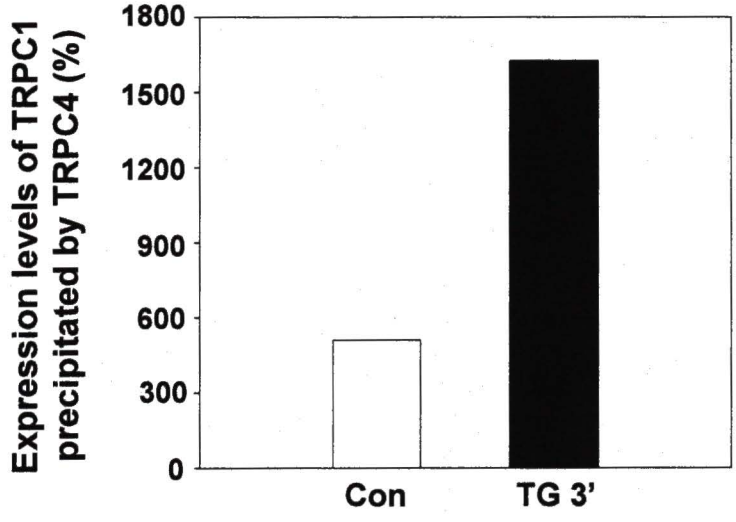


Figure 4. A. Influence of store depletion on TRPC1 distribution in MCs. TRPC1 was identified in MCs by immunofluorescence staining under confocal microscopy. TRPC1 was probed with 1F1 TRPC1 antibody followed by an Alexa Fluor 568-conjugated secondary antibody. a and c: TRPC1 stainings in control MCs (no TG treatment) and MCs with TG treatment (1 μM for 3 min), respectively; b and d: bright fields of a and c, respectively, showing morphology of MCs. **B.** TRPC1 trafficking stimulated by TG in live MCs. Two adjacent EYFP-TRPC1 transfected cells were seen in this representative field. The cell membrane was stained with 10 μM FM4-64 dye (red). The distribution of expressed TRPC1 is shown by green fluorescence. Yellow signals represent the plasma membrane localization of TRPC1. White arrows indicate accumulation of EYFP-TRPC1 in the plasma membrane in response to TG. All images were captured at the same focal plane with identical settings.

Figure 4.

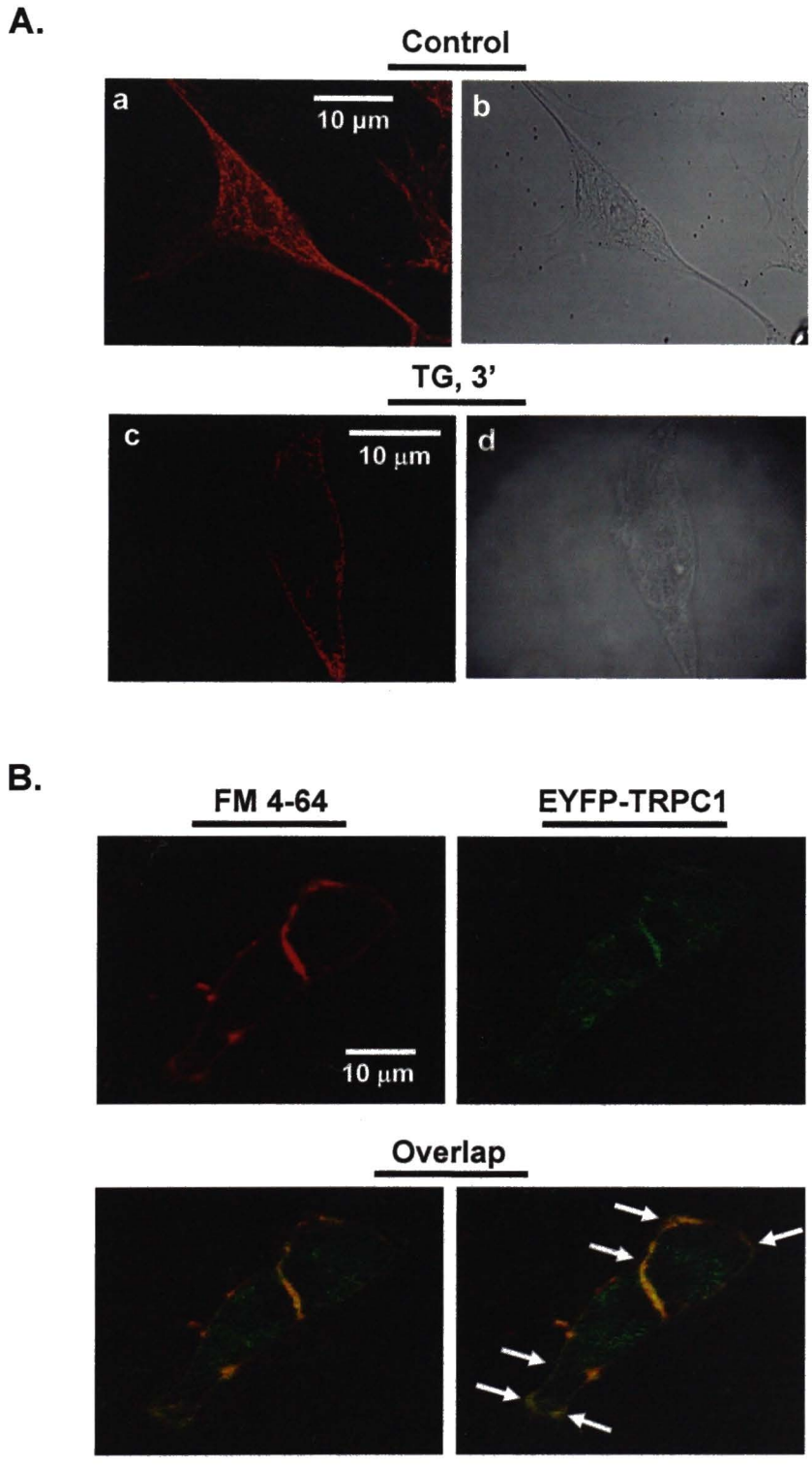


Figure. 5 A. Biotinylation of cell surface proteins of MCs with and without TG treatment. **a:** expression of TRPC1 proteins in the plasma membrane of MCs without TG treatment (Control) and MCs treated with TG for 3 and 5 min. **b:** expression levels of cell surface TRPC4 in MCs with and without TG treatment. **c:** a cell membrane marker, integrin-β1 was used as a positive control and loading control. **d:** an ER marker, calnexin, was used as a negative control. **B.** Relative amounts of cell surface TRPC1 and TRPC4 proteins, calculated by optical density of surface TRPC1 (a) or TRPC4 (b) divided by corresponding membrane integrin-β1 protein (c), expressed as percentages. "*" indicates significant difference compared with Pre-TG. Values are averaged from 3 independent experiments.

Figure 5.

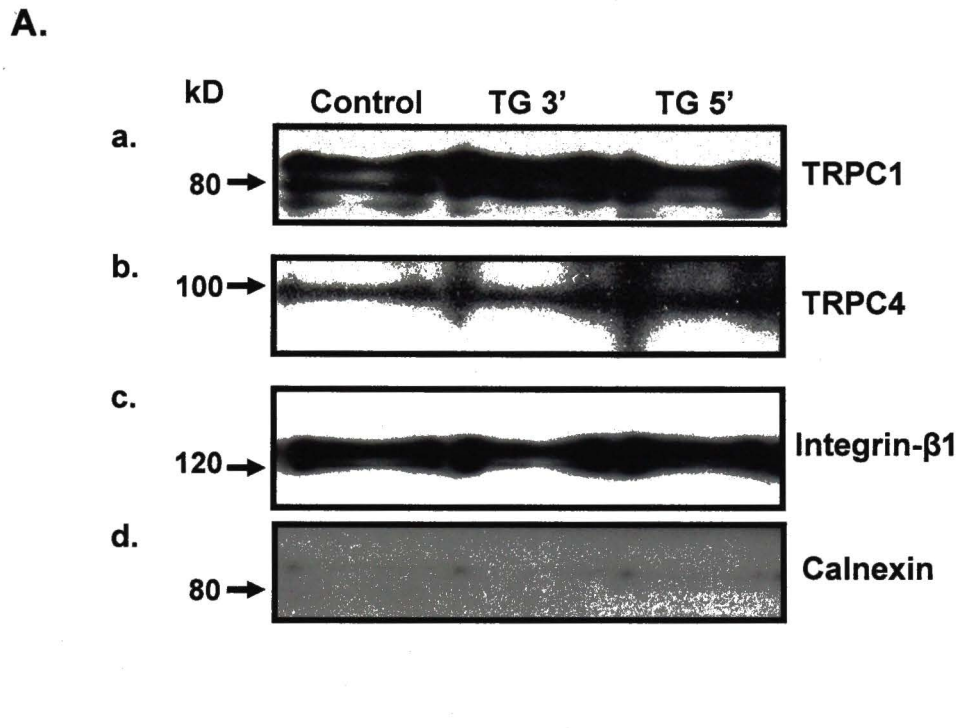

A.
kD
Control
TG 3'
TG 5'
a.
80
TRPC1
b.
100
TRPC4
c.
120
Integrin-β1
d.
80
Calnexin
B.
Percentage
200
160
120
80
40
0
*
*
Pre-TG
TG-3'
TG-5'
TRPC1
TRPC4

Figure 6. **A.** Representative traces, showing single channel activity before (Before TG) and after (After TG) TG (1 μM) stimulation in MCs of mock control (Mock) and STIM1 knockdwon (hSTIM1-shRNA). Arrows indicate the closed state of channels. Downward deflections indicate inward currents. Holding potential was 80 mV (pipette). **B.** NP_O of the channels before and after application of TG summarized from the two groups described in A. "n" indicates the number of cells analyzed. "*" indicates significant difference between hSTIM1-shRNA and Mock groups. **C.** Western blot in MCs, showing STIM1 protein expression in untransfected cells (UT) and the cells with transient transfection of pSHAG empty vectors (Mock) or hSTIM1-shRNA constructs. Actin was used as a loading control.

Figure 6.

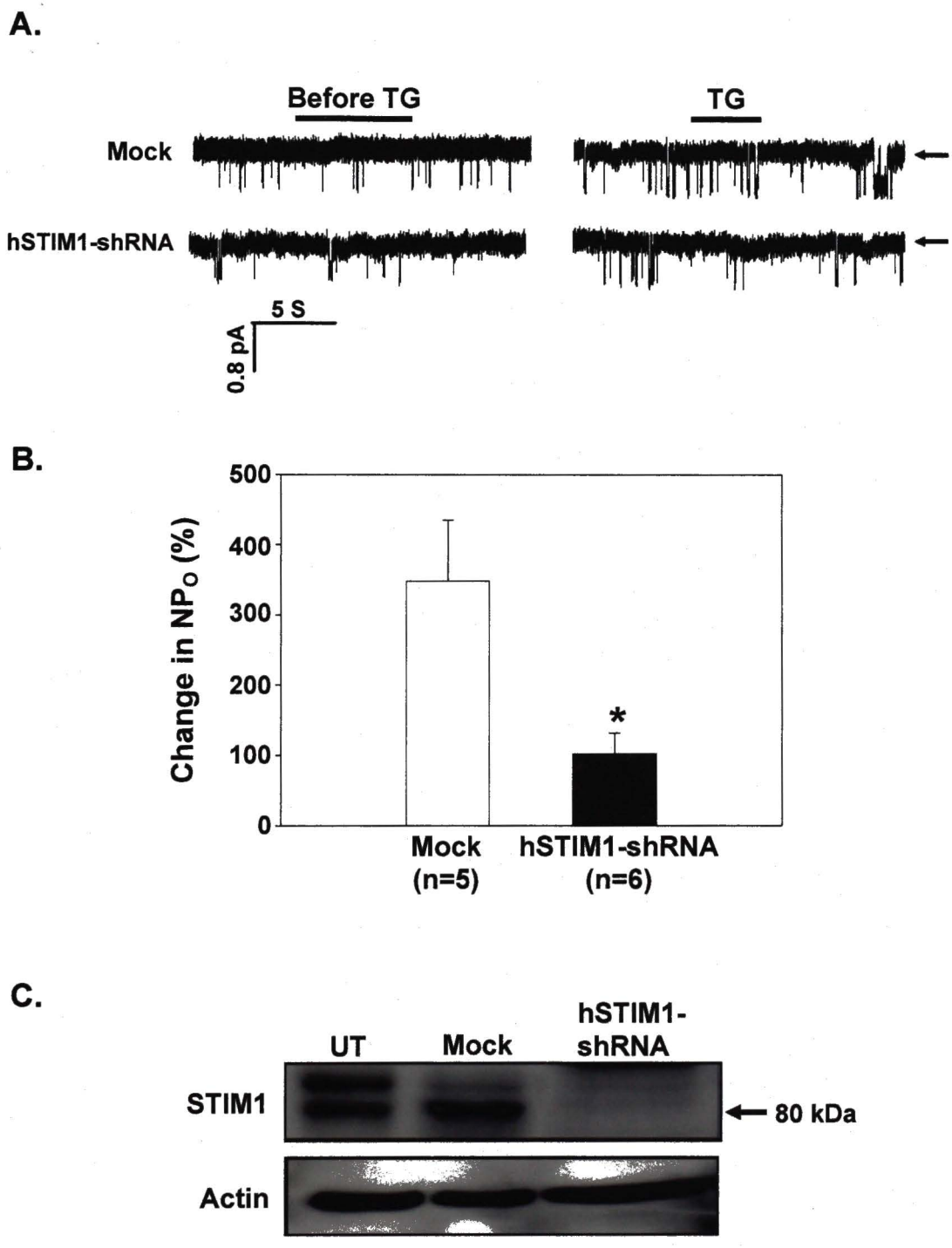
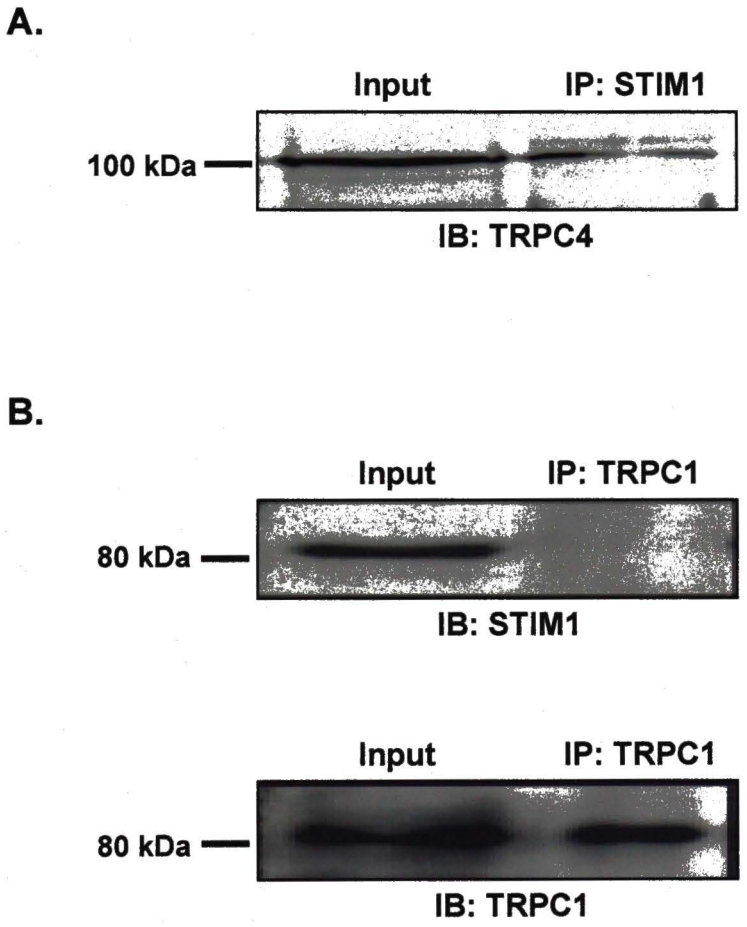

A.
Before TG
TG
Mock
hSTIM1-shRNA
5 S
0.8 pA
B.
Change in NPO (%)
500
400
300
200
100
0
*
Mock
(n=5)
hSTIM1-shRNA
(n=6)
C.
UT
Mock
hSTIM1-
shRNA
STIM1
80 kDa
Actin

Figure 7. Co-immunoprecipitation, showing STIM1 interaction with TRPC4, but not TRPC1. **A.** Immunoprecipiation of STIM1 was followed by western blot (IB) for TRPC4. **B.** Immunoprecipiation of TRPC1 was followed by IB for STIM1 (left panel) or TRPC1 (right panel). In both A and B, non-precipitated lysates (Input) were loaded as controls.

Figure 7.


A.
Input
IP: STIM1
100 kDa
IB: TRPC4
B.
Input
IP: TRPC1
80 kDa
IB: STIM1
Input
IP: TRPC1
80 kDa
IB: TRPC1

Abbreviations

co-IP: co-immunoprecipitation

CRAC: Ca^{2+} release-activated Ca^{2+} channels

ER: endoplasmic reticulum

IP: immunoprecipitation

MC: mesangial cell

RNAi: RNA interference

SOC: store-operated channel

SOCE: store-operated Ca^{2+} entry

STIM1: stromal interaction molecule 1

TG: thapsigargin

TRPC: canonical transient receptor potential

References

1. **Albert AP and Large WA.** Store-operated Ca^{2+}-permeable non-selective cation channels in smooth muscle cells. *Cell Calcium* 33: 345-356, 2003.
2. **Albert AP and Large WA.** A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. *J Physiol* 538: 717-728, 2002.
3. **Baba Y, Hayashi K, Fujii Y, Mizushima A, Watarai H, Wakamori M, Numaga T, Mori Y, Lino M, Hikida M and Kurosaki T.** Coupling of STIM1 to store-operated Ca^{2+} entry through its constitutive and inducible movement in the endoplasmic reticulum. *PNAS* 103: 16704-16709, 2006.
4. **Bolotina VM and Csutora P.** CIF and other mysteries of the store-operated Ca^{2+}-entry pathway. *Trends in Biochemical Sciences* 30: 378-387, 2005.
5. **Broad LM, Cannon TR and Taylor CW.** A non-capacitive pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. *J Physiol (Lond)* 517: 121-134, 1999.
6. **Cioffi DL and Stevens T.** Regulation of endothelial cell barrier function by store-operated calcium entry. *Microcirculation* 13: 709-723, 2006.
7. **Du J, Sours-Brothers S, Coleman R, Ding M, Graham S, Kong D and Ma R.** TRPC1 channel is involved in contractile function of glomerular mesangial cells. *J Am Soc Nephrol* 18: 1437-1445, 2007.

8. **Facemire CS, Mohler PJ and Arendshorst WJ.** Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation. *Am J Physiol Renal Physiol* 286: F546-F551, 2004.

9. **Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M and Rao A.** A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441: 179-185, 2006.

10. **Garcia RL and Schilling WP.** Differential expression of mammalian TRP homologues across tissues and cell lines. *Biochem Biophys Res Comm* 239: 279-283, 1997.

11. **Goel M, Sinkins WG and Schilling WP.** Selective association of TRPC channel subunits in rat brain synaptosomes. *J Biol Chem* 277: 48303-48310, 2002.

12. **Graham S, Ding M, Sours-Brothers S, Yorio T, Ma JX and Ma R.** Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca^{2+} signaling in glomerular mesangial cells. *Am J Physiol Renal Physiol* 293: F1381-F1390, 2007.

13. **Harper JL, Camerini-Otero CS, Li AH, Kim SA, Jacobson KA and Daly JW.** Dihydropyridines as inhibitors of capacitative calcium entry in leukemic HL-60 cells. *Biochem Pharmacol* 65: 329-338, 2003.

14. **Hofmann T, Schaefer M, Schultz G and Gudermann T.** Subunit composition of mammalian transient receptor potential channels in living cells. *PNAS* 99: 7461-7466, 2002.

15. **Hoth M and Penner R.** Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355: 353-356, 1992.

16. **Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S and Worley PF.** STIM1 carboxyl-terminus activates native SOC, ICRAC and TRPC1 channels. *Nat Cell Biol* 8: 1003-1010, 2006.

17. **Kiselyov K, Xu X, Mozhayeva G, Kuo T, Pessah I, Mignery G, Zhu X, Birnbaumer L and Muallem S.** Functional interaction between $InsP_3$ receptors and store-operated Htrp3 channels. *Nature* 396: 478-482, 1998.

18. **Lewis RS.** The molecular choreography of a store-operated calcium channel. *Nature* 446: 284-287, 2007.

19. **Lewis RS.** Store-operated calcium channels. *Adv Second Messenger Phos Res* 33: 279-307, 1999.

20. **Liao Y, Erxleben C, Yildirim E, Abramowitz J, Armstrong DL and Birnbaumer L.** Orai proteins interact with TRPC channels and confer responsiveness to store depletion. *PNAS* 104: 4682-4687, 2007.

21. **Liou J, Fivaz M, Inoue T and Meyer T.** Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. *PNAS* 104: 9301-9306, 2007.

22. **Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE and Meyer T.** STIM is a Ca^{2+} store sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. *Current Biol* 15: 1235-1241, 2005.

23. **Liu XB, Bandyopadhyay BC, Singh BB, Groschner K and Ambudkar IS.** Molecular analysis of a store-operated and OAG sensitive non-selective cation channel: heteromeric assembly of TRPC1-TRPC3. *J Biol Chem* 280: 21600-21606, 2005.

24. **Ma R, Pluznick JL and Sansom SC.** Ion channels in mesangial cells: function, malfunction, or fiction. *Physiology* 20: 102-111, 2005.

25. **Ma R, Rundle D, Jacks J, Koch M, Downs T and Tsiokas L.** Inhibitor of myogenic family: a novel suppressor of store-operated Ca^{2+} currents through an interaction with TRPC1. *J Biol Chem* 278: 52763-52772, 2003.

26. **Ma R, Smith S, Child A, Carmines PK and Sansom SC.** Store-operated Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol* 278: F954-F961, 2000.

27. **Nilius B, Owsianik G, Voets T and Peters JA.** Transient receptor potential cation channels in disease. *Physiol Rev* 87: 165-217, 2007.

28. **Parekh AB and Putney Jr. JW.** Store-operated calcium channels. *Physiol Rev* 85: 757-810, 2005.

29. **Philipp S.** A novel capacitative calcium entry channel expressed in excitable cells. *EMBO J* 17: 4274-4282, 1998.

30. **Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A and Hogan PG.** Orail1 is an essential pore subunit of the CRAC channel. *Nature* 443: 230-233, 2006.

31. **Putney Jr JW.** Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42: 103-110, 2007.

32. **Riccio A, Mattei C, Kelsell RE, Medhurst AD, Calver AR, Randall AD, Davis JB, Benham CD and Pangalos MN.** Cloning and functional expression of human short TRP7, a candidate protein for store-operated Ca^{2+} influx. *J Biol Chem* 277: 12302-12309, 2002.

33. **Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G and Stauderman KA.** STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol* 169: 435-445, 2005.

34. **Sours S, Du J, Chu S, Zhou JX, Ding M and Ma R.** Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 290: F1507-F1515, 2006.

35. **Trebak M, Bird GStJ, Mckay RR and Putney Jr JW.** Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. *J Biol Chem* 277: 21617-21623, 2002.

36. **Vig M, Beck A, Billingsley JM, Lis A, Parvez S, Peineit C, Koomoa DL, Soboloff J, Gill DL, Fleig A, Kinet JP and Penner R.** CRACM1 multimers form the ion-selective pore of the CRAC channel. *Curr Biol* 16: 2073-2079, 2006.

37. **Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, Kraft S, Turner H, Fleig A, Penner R and Kinet JP.** CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. *Science* 312: 1220-1223, 2006.

38. **Walker RL, Hume JR and Horowitz B.** Differential expression and alternative splicing of TRP channel genes in smooth muscles. *Am J Physiol Cell Physiol* 280: C1184-C1192, 2001.

39. **Wang X, Pluznick JL, Wei P, Padanilam BJ and Sansom SC.** TRPC4 forms store-operated Ca^{2+} channels in mouse mesangial cells. *Am J Physiol Cell Physiol* 287: C357-C364, 2004.

40. **Wu X, Zagranichnaya TK, Gurda GT, Eves EM and Villereal ML.** A TRPC1/TRPC3 mediated increase in store-operated calcium entry is required for differentiation of H19-7 hippocampal neuronal cells. *J Biol Chem* 279: 43392-43402, 2004.

41. **Yeromin AV, Zhang SL, Jiang W, Yu Y, Safrina O and Cahalan MD.** Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature* 443: 226-229, 2006.

42. **Yuan JP, Zeng W, Huang GN, Worley PF and Muallem S.** STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* 6: 636-645, 2007.

43. **Zhang SL, Yeromin AV, Zhang XHF, Yu Y, Safrina O, Penna A, Roos J, Stauderman KA and Cahalan MD.** Genome-wide RNAi screen of Ca^{2+} influx identifies genes that regulate Ca^{2+} release-activated Ca^{2+} channel activity. *PNAS* 103: 9357-9362, 2006.

44. **Zhang W, Meng H, Li ZH, Shu Z, Ma X and Zhang BX.** Regulation of STIM1, store-operated Ca^{2+} influx, and nitric oxide generation by retinoic acid in rat mesangial cells. *Am J Physiol Renal Physiol* 292: F1054-F1064, 2007.

CHAPTER V

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

Summary

Glomerular mesangial cells (MCs) play an important role in the physiological regulation of glomerular filtration rate (GFR) and renal function. Loss of mesangial cell function is known to play a role in the dysregulation of GFR in a number of diseases, including diabetic nephropathy and glomerulosclerosis. Meanwhile, there is growing evidence that store-operated Ca^{2+} entry (SOCE), including that which is mediated by canonical transient receptor potential (TRPC) proteins, plays a significant role in the regulation of Ca^{2+} homeostasis in normal function and pathophysiology of many cells (8). While store-operated channel (SOC) function has been identified in MCs (5), the role of this mode of Ca^{2+} entry in MC physiology and the molecular components that make up SOCs in MCs remains poorly defined.

The first study described here was aimed at identifying the expression of TRPCs in MCs. TRPC1, 3, 4, and 6 were found in cultured human mesangial cells as well as in tissue sections from both rat and human kidneys, while TRPC5 and 7 could not be identified at the protein level. Because TRPCs are thought to form heteromeric channels, the interaction between TRPC isoforms was also assessed. TRPC1 was found to interact with both TRPC4 and 6 by co-immunoprecipitation. While interaction between TRPCs within the same structural subgroups (TRPC1, 4 and 5 or TRPC 3, 6, and 7) has previously been demonstrated, the interaction of TRPC1 with TRPC6 is the first evidence

of such interaction across subgroups. This may indicate a complex formation that is specific to human MCs.

The next aim of the first study was to assess the role of TRPC1 specifically in SOCE in MCs, as TRPC1 currently has the strongest evidence for SOC function in a number of cell types (3). Fluorescent Ca^{2+} imaging experiments demonstrate that knockdown of TRPC1 expression in MCs using RNAi significantly attenuates SOCE triggered by thapsigargin- (TG-) mediated store-depletion, while overexpression of TRPC1 had the opposite effect to enhance SOCE. This evidence argues that TRPC1, at least in part, mediates SOCE in MCs.

The next study was aimed at assessing the role of TRPC1-mediated SOCE in the contractile function of MCs, whose response to vasoactive substances contributes to the regulation of glomerular function as a whole. In cultured MCs, knockdown of TRPC1 expression or antibody blockade of TRPC1 channel activity were both shown to inhibit angiotensin II- (Ang II-), or endothelin-1-mediated contraction. Further experiments demonstrated that this loss of contractile response was due to TRPC1-mediated SOCE. Antibody blockade of TRPC1 channel activity resulted in an attenuation of Ang II-stimulated membrane currents as measured by single channel patch clamp, while TRPC1 antibody blockade as well as knockdown of TRPC1 expression using RNAi both blocked Ang II-mediated SOCE as measured by fura-2 Ca^{2+} imaging.

The potential role of TRPC1-mediated MC contraction was also examined *in vivo*. In rats, infusion of TRPC1 antibody attenuated the Ang II-mediated reduction in GFR while not affecting mean arterial pressure or renal blood flow. This indicates that TRPC1

blockade had intraglomerular effects on glomerular function, likely by blocking Ang II-induced MC contraction.

These results suggest that TRPC1 mediates SOCE in response to receptor-mediated store-depletion, in addition to TG-mediated receptor-independent store-depletion demonstrated in the first study. This mode of Ca^{2+} entry plays a role in the Ang II-stimulated contractile function of MCs, and thereby contributes to MC-mediated regulation of glomerular filtration.

While these studies indicate an important role for TRPC1 in MC function, it is unlikely to act alone. Indeed, TRPC1 has been shown to interact with other TRPC isoforms to form functional channels (4; 9; 10). In the first study, immunocytochemistry demonstrated that both TRPC1 and TRPC4 are expressed at the plasma membrane and interact with each other by co-immunoprecipitation. The final study was aimed at determining if TRPC1 and TRPC4 combine to form a functional channel in MCs. Knockdown of either TRPC1 or TRPC4 attenuated TG-mediated increase in channel open probability, as measured by single-channel patch clamp. This knockdown had a similar effect on TG-mediated SOCE, while simultaneous knockdown of both TRPC1 and TRPC4 had an additive effect, as measured by fura-2 Ca^{2+} imaging. Co-immunoprecipitation experiments demonstrate that TG-induced store-depletion increases the interaction between TRPC1 and TRPC4, while also increasing TRPC1 localization to the plasma membrane. This may point a mechanism of SOC activation by which increase channel activity is attributed to a translocation of TRPC proteins to the plasma

membrane. In this case, translocation of TRPC1 may increase complex formation with TRPC4 to increase functional channel activity.

Recently, the stromal interaction molecule-1 (STIM1), has gained much attention as a Ca^{2+} sensor in the endoplasmic reticulum which activates SOCs, including TRPCs and Orai proteins, another candidate for SOC. The final study here identified the presence of STIM1 in MCs, and co-immunoprecipitation indicates that STIM1 interacts with TRPC4 but not TRPC1. Since store-depletion increases the localization of TRPC1 but not TRPC4 to the plasma membrane, this may indicate another role for TRPC4. It may be that TRPC4 is acting as an adapter to convey the store-depletion signal from STIM1 to TRPC1, rather than complexing directly with TRPC1 (**Figure 1**). As discussed in the introduction, this may indicated a cell-type specific function of STIM1 and TRPC-mediated SOCE in MCs.

Collectively, these studies demonstrate that TRPC1 in particular mediated SOCE in MCs, contributing to the regulation of contractile function in these cells. The interaction of TRPC1 with TRPC4 may serve to form functional channels, or contribute to a signaling complex between STIM1 in the ER and TRPCs at the plasma membrane. These studies further the basic knowledge of Ca^{2+} signaling mechanisms in MCs, an important regulator of MC function in normal physiology and pathophysiology. They also contribute to the understanding of TRPC function in general- how these proteins form channels activated by store-depletion and the potential mechanism of activation involving STIM1.

Discussion and future directions

Role of TRPC-mediated SOCE in other MC functions. Contraction of MCs is not their only physiologically relevant function. The secretion of extracellular matrix proteins by MCs also helps to support the glomerular tuft structure in general. In addition, MC proliferation and increase in extracellular matrix secretion both contribute to a decline in glomerular function associated with glomerulosclerosis and end-stage diabetic renal failure (2). Like MC contraction, Ca^{2+} homeostasis may play a role in both of these functions, and may also be regulated by SOCE, particularly TRPC-mediated SOC activity demonstrated here. Further studies may examine the role of TRPCs in the proliferation and extracellular matrix secretion of MCs. This could be done by assessing the effects of TRPC knockdown or overexpression on both of these processes in MC culture. Growth factor stimulation is also known to contribute to MC expansion(1). It would be interesting to examine the effects of growth factor stimulation on the expression of TRPCs in MC culture, and to further examine the role of TRPCs in SOCE stimulated by growth factors.

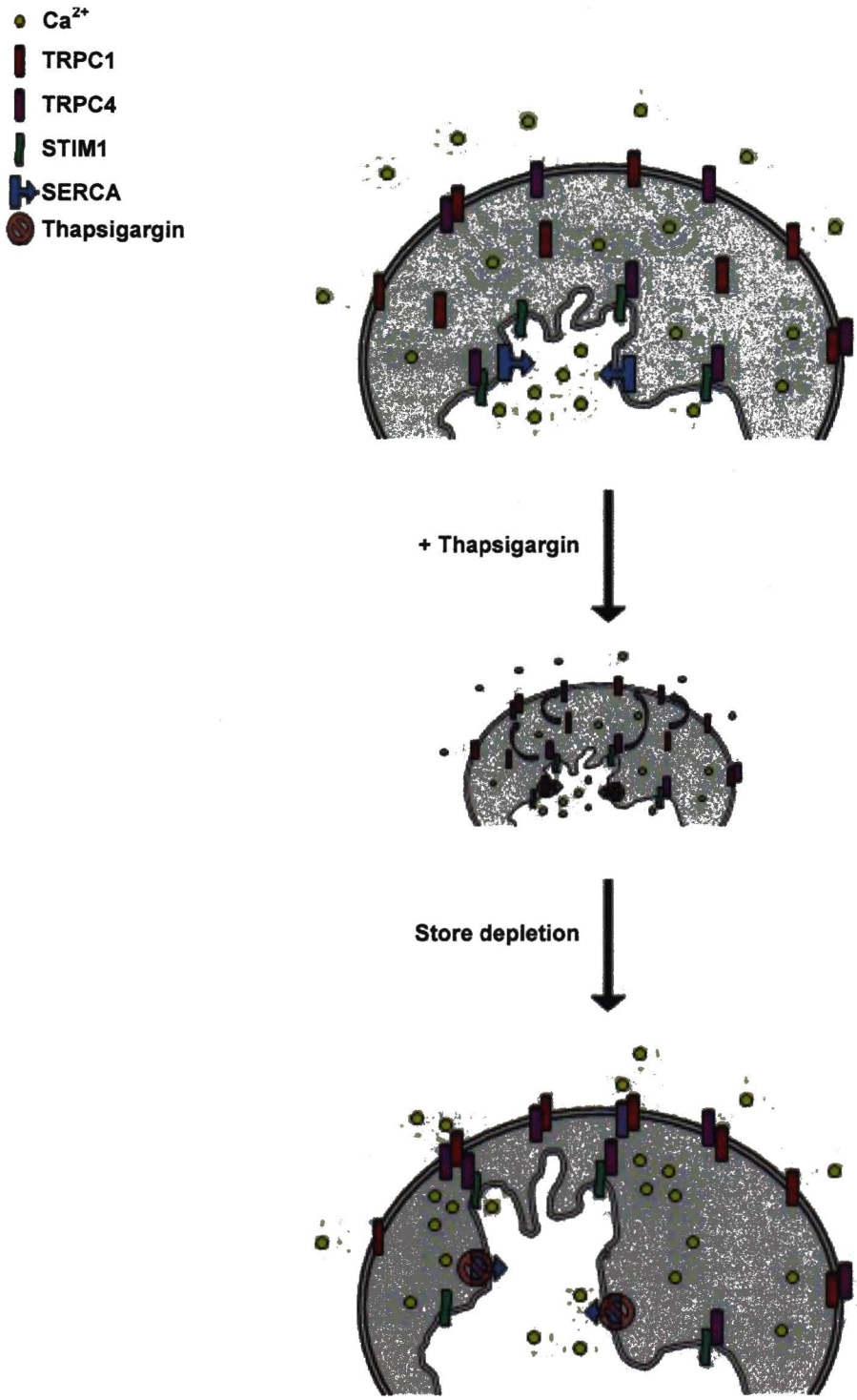
TRPC1/TRPC4 channel or "signalplex?" TRPCs are known to from heteromeric channels composed of different isoforms, in agreement with the interaction between TRPC1 and TRPC4 demonstrated here. These results suggest that store-depletion-triggered translocation of TRPC1 to the plasma membrane may account for increased channel activity by increasing channel formation with TRPC4 already present in the plasma membrane. However, while both proteins are also present in the cytosol, only TRPC4 was shown to interact with STIM1. If translocation of STIM1 activates TRPC

channels, one would expect that insertion of TRPC4 into the plasma membrane would also be required to increase channel formation and channel activity, yet, store-depletion-mediated increase in plasma membrane TRPC4 is not seen. It is possible that instead, TRPC4 bound to STIM1 may act as an adapter protein, signaling store-depletion from STIM1 to TRPC1 channels in the plasma membrane as discussed above. This would constitute a novel SOCE signaling mechanism. It is likely that these interactions may be mediated by the coiled-coil domains found on STIM1 as well as TRPC1 and TRPC4. Overexpression of deletion mutants lacking these coiled-coil domains could be used to explore this potential mechanism. FRET analysis could provide additional knowledge of the interactions between these proteins.

Role for TRPCs in MC pathophysiology. These studies have identified a role for TRPCs in the regulation of SOCE and contractile function in MCs. Both of these functions are known to be down-regulated during diabetic renal failure, resulting in a loss of MC-mediated regulation of GFR (6; 7). The effect of high glucose treatment of MC cultures as well as diabetic rat models could be used to examine the role of TRPCs during this pathophysiological state. It is possible that loss of SOC function may be attributed to alterations in TRPC activation or complex formation described here.

Figure 1. Translocation of TRPC1 and STIM1 regulates SOC activity. At rest, TRPC1 and TRPC4 are distributed throughout the plasma membrane and cytosol, while STIM1 on the ER membrane is bound to TRPC4. Upon store depletion, e.g. by SERCA inhibition with TG, TRPC1 translocates to the plasma membrane, increasing heteromeric channel formation with TRPC4, while STIM1 translocates to regions directly apposed to the plasma membrane. STIM1 may directly activate TRPC1/TRPC4 complexes or activation may occur indirectly via TRPC4.

Figure 1.


Ca^{2+}
TRPC1
TRPC4
STIM1
SERCA
Thapsigargin
+ Thapsigargin
Store depletion

Abbreviations

Ang II: angiotensin II

GFR: glomerular filtration rate

MC: mesangial cell

SOC: store-operated channel

SOCE: store-operated Ca^{2+} entry

STIM1: stromal interaction molecule 1

TG: thapsigargin

TRPC: canonical transient receptor potential

References

1. **Abboud HE**. Growth factors and the mesangium. *J Am Soc Nephrol* 2: S185-S189, 1992.
2. **Alsaad KO and Herzenberg AM**. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update. *J Clin Pathol* 60: 18-26, 2007.
3. **Beech DJ, Xu SZ, McHugh D and Flemming R**. TRPC1 store-operated cationic channel subunit. *Cell Calcium* 33: 433-440, 2003.
4. **Lintschinger B, Balzer-Geldsetzer M, Baskaran T, Graier WF, Romanin C, Zhu MX and Groschner K**. Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+}-sensitive cation channels. *J Biol Chem* 275: 27799-27805, 2000.
5. **Ma R, Smith S, Child A, Carmines PK and Sansom SC**. Store-operated Ca^{2+} channels in human glomerular mesangial cells. *Am J Physiol Renal Physiol* 278: F954-F961, 2000.
6. **Mene P, Pugliese G, Pricci F, Di Mario U, Cinotti GA and Pugliese F**. High glucose inhibits cytosolic calcium signaling in cultured rat mesangial cells. *Kidney Int* 43: 585-591, 1993.
7. **Mene P, Pascale C, Teti A, Bernardini S, Cinotti GA and Pugliese F**. Effects of advanced glycation end products on cytosolic Ca^{2+} signaling of cultured human mesangial cells. *J Am Soc Nephrol* 10: 1478-1486, 1999.
8. **Nilius B, Owsianik G, Voets T and Peters JA**. Transient receptor potential cation channels in disease. *Physiol Rev* 87: 165-217, 2007.

9. **Strubing C, Krapivinsky G, Krapivinsky L and Clapham DE**. TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* 29: 645-655, 2001.

10. **Zagranichnaya TK, Wu X and Villereal ML**. Endogenous TRPC1, TRPC3 and TRPC7 proteins combine to form native store-operated channels in HEK-293 cells. *J Biol Chem* M505842200, 2005.