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Balancing Calcium Signals through TRPC5 and TRPC6 in Podocytes

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ABSTRACT

Calcium (Ca^{2+}) ions are important mediators of cellular homeostasis owing to their ability to elicit a dynamic, transient, and tightly regulated range of biochemical responses. More than a decade ago, a nonselective, Ca^{2+}-permeable, cationic conductance was identified in podocytes downstream of angiotensin II (Ang II) signaling, but its molecular structure remained elusive. Six years ago, transient receptor potential canonical 6 (TRPC6) mutations were found in families with hereditary FSGS, and TRPC5 and TRPC6 channels are now known as the Ca^{2+} influx pathways for this previously described, nonselective, cationic current in podocytes. Ang II activation engages this Ca^{2+} influx to modulate the actin cytoskeleton in podocytes. These discoveries dovetail with previously described regulation of actin dynamics by the Ca^{2+}-activated phosphatase, calcineurin, and the emergence of Rho GTPases as critical regulators of podocyte function in health and disease. Understanding the interconnected signaling regulated by Ca^{2+} currents offers potential new therapeutic targets and highlights the notion that synergistic therapies targeting multiple levels of biochemistry may be useful in treating proteinuric kidney disease.

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A single ion channel can negotiate the passage of over 10 million ions per second across the plasma membrane,[1] and calcium (Ca^{2+}) ions, in particular, are important mediators of cellular homeostasis.[1,2] Ca^{2+} permeates the membrane of virtually every cell to regulate diverse vital processes such as muscle contraction, cytoskeletal structure, vesicle secretion, gene transcription, and programmed cell death, to name a few.[1,2] Thousands of Ca^{2+} channels on the plasma membrane precisely control the timing and entry of Ca^{2+} ions and cellular homeostatic mechanisms modulate the tight control and compartmentalization of these intracellular Ca^{2+} transients (Figure 1).[2]

Here, we review the emerging role of Ca^{2+} signaling in the regulation of podocyte function in health and disease (Figure 2). In particular, we explore recently uncovered insights into the activation of transient receptor potential canonical (TRPC) channels by Ang II and the resulting effects on podocyte signaling under physiologic and pathologic conditions (Figure 3). Finally, we highlight the implications of balancing Ca^{2+}-controlled signaling pathways in podocytes for the development of novel antiproteinuric therapies (Figure 4).

Similar to sodium (Na^+) and potassium (K^+) channels initially recorded in the classical Hodgkin-Huxley studies of the squid giant axon,[1,3,4] Ca^{2+} channels were identified in studies of excitable cells from crustaceans. In the early 1950s, when Na^+ was firmly established as the ion mediating action potentials, known as the Na^+ theory of the action potential,[1] Fatt and Katz accidentally stumbled on an important exception: the crayfish muscle.[1,5,6] In pioneering two microelectrode intracellular recordings, the investigators were surprised when Na^+ channel blockers did not abrogate action potentials, but instead engendered larger and more prolonged spikes.[5,6] Further experiments revealed that the mysterious action potentials were generated by the entry of divalent Ca^{2+} ions through voltage-gated Ca^{2+} channels.[1]

Because of these initial experiments, important insights have been gained into the electrogenic role of Ca^{2+} in excitable cells: Electricity is used to open (or gate) the channels, and in turn the channels are used to make electricity (action potentials). Importantly, most excitable cells ultimately translate their electric excitation into another form of activity or cellular function. Although the list of processes controlled by Ca^{2+} is lengthy, classical studies in excitable cells focus on three: contraction, secretion, and gating.[1] The role of Ca^{2+} in myocyte contraction is particularly important in the context of this review, as it provides insight into the Ca^{2+}-dependent mecha-

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nisms underlying all contractile cells, including kidney podocytes.[7]

How are cells capable of sensing Ca^{2+}? In a resting cell, the levels of free cytoplasmic Ca^{2+} are tightly controlled and held extremely low. The resting intracellular Ca^{2+} concentration [Ca^{2+}] lies in the 20 to 300 nM range in virtually all cells.[1] Calcium homeostasis mechanisms (Figure 1) such as the Na^{+}-Ca^{2+} exchanger (NCX) and the ATP-dependent plasma membrane Ca^{2+} pump (PMCA), along with Ca^{2+} buffers (such as calbindins or parvalbumin) and internal Ca^{2+} stores (endoplasmic reticulum [ER], mitochondria uniporter [MiCa]), labor to maintain the cytoplasmic Ca^{2+} levels low.[8] When a Ca^{2+}-permeable channel opens, whether in the plasma membrane or on a Ca^{2+}-loaded organelle (such as the inositol triphosphate receptor [IP3R] in the ER; Figure 1), Ca^{2+} ions flow transiently into the cytoplasm, until the homeostatic mechanisms prevail once again to tie up or extrude the excess Ca^{2+} ions. Ca^{2+} is a potent signaling molecule because of its ability to mediate a dynamic, dramatic, transient, and tightly regulated range of responses. Simply put, the entry or release of any amount of Ca^{2+} in the cytoplasm, large or small, is a call to action.[1,2,9]

PODOCYTES AND CALCIUM

The glomerular filtration unit of the kidney is a highly specialized corpuscle of capillaries capable of modulating hydrostatic ultrafiltration of blood plasma, allowing the passage of solutes but retaining vital proteins.[10–12] Among the three layers of the glomerular filtration barrier, the podocyte layer comprises unique cells with a complex organizing phenotype, including characteristic interdigitating foot processes with an interposed slit diaphragm that cover the outer aspect of the glomerular basement membrane.[10,12,13] Podocyte dysfunction and cytoskeletal disorganization leads to foot process effacement, disruption of the slit diaphragm, and proteinuria, and is often a starting point for progressive kidney disease.[7,12] Thus, proteins regulating the plasticity of the podocyte actin cytoskeleton are critical for sustained function of the glomerulus.[7,12] Mutations in genes encoding nephrin,[14] podocin,[15] or phospholipase C epsilon[16] give rise to congenital proteinuria.[14,15] Additionally, adult-onset focal segmental glomerulosclerosis (FSGS) is associated with podocyte mutations in genes encoding α-actinin 4,[17] CD2AP,[18] INF2,[19] TRPC6,[20] and synaptopodin.[21] Most recently, common variations in glypican 5 associate with acquired nephrotic syndrome.[22] Taken together, mutations in these moieties highlight the importance of the cytoskeleton in podocyte health and disease.

From the cell biologist's perspective, the podocyte's refined repertoire of cytoskeletal adaptations to environmental cues[12,23–28] renders it an ideal model system to study Ca^{2+}-dependent actin dynamics in a physiologically relevant context. A central working hypothesis has been that podocyte foot process effacement is mediated by rearrangement of the actin cytoskeleton.[7,23,24] As early as 1978, elegant work by Kerjaschki proposed that an increase in [Ca^{2+}]i may be an early event in podocyte injury[29] (Figure 2). Complement C5b-9 complex-mediated podocyte damage also associates with an increase in [Ca^{2+}]i and activation of phospholipase C (PLC).[30] Protamine sulfate, which can cause foot process effacement *in vivo*,[31] also increases [Ca^{2+}]i *in vitro*[32] (Figure 2). Differentiated mouse podocytes in culture increase [Ca^{2+}]i in response to bradykinin,[33] an effect also observed in cultured rat podocytes in response to Ang II[34] (Figure 2). Importantly, in pioneering whole rat glomerular recordings, Pavenstadt and colleagues described how Ang II evokes a nonselective cationic current in podocytes[35] (Figure 2). The molecular identity of this current remained elusive until 2005, when Winn and colleagues provided the first clues by describing a TRPC6 channel mutation in familial FSGS,[20] followed by the identification of five additional mutations by Pollak and colleagues[36] (Figure 2). De-

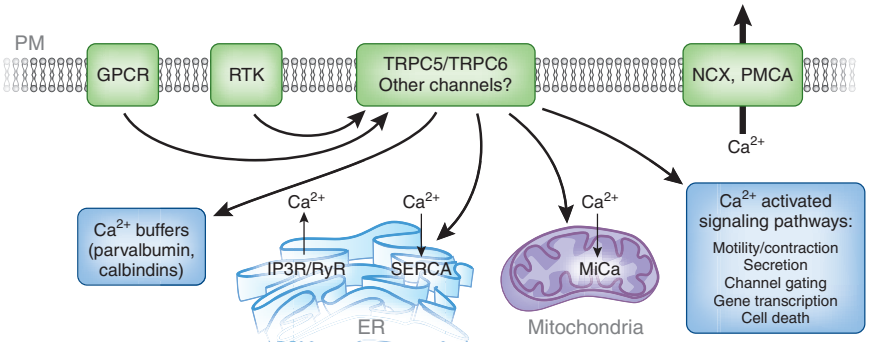

Figure 1. Schematic representation of Ca^{2+} homeostasis in podocytes Ca^{2+} is a potent signaling molecule because of its ability to mediate a dynamic, dramatic, transient, and tightly regulated range of intracellular responses[1,2,9] (PM: plasma membrane). Some proteins shown here have not yet been identified or studied in podocytes (calbindins, the mitochondrial uniporter or MiCa etc.), but they are likely to be present based on our understanding of calcium homeostasis in other cell types. The influx of Ca^{2+} is likely to be mediated by TRPC5 and TRPC6 channels, which were recorded at the single channel level in podocytes,[37] but other influx pathways cannot be excluded. TRPC5 and TRPC6 are activated by upstream receptors such as G-protein coupled receptors (GPCR), including the AT1R, and receptor tyrosine kinases (RTK), similar to other cell types.[62] Ca^{2+} is tightly regulated upon entry into the cytoplasm. Calcium homeostasis relies on the Na^{+}-Ca^{2+} exchanger (NCX), which has been described in podocytes,[110] the ATP-dependent plasma membrane Ca^{2+} pump (PMCA), plasma Ca^{2+} buffers (calbindins, parvalbumin, etc.) and internal Ca^{2+} stores (endoplasmic reticulum (ER), mitochondria) to maintain low cytoplasmic Ca^{2+} levels.[8] When a Ca^{2+}-permeable channel opens, whether in the plasma membrane or on a Ca^{2+}-loaded organelle (*e.g.* the IP3R in the ER), Ca^{2+} ions flow transiently into the cytoplasm, until the homeostatic mechanisms take over once again to buffer or extrude the excess Ca^{2+} ions.

TIMELINE I Evolution of calcium signaling in podocytes

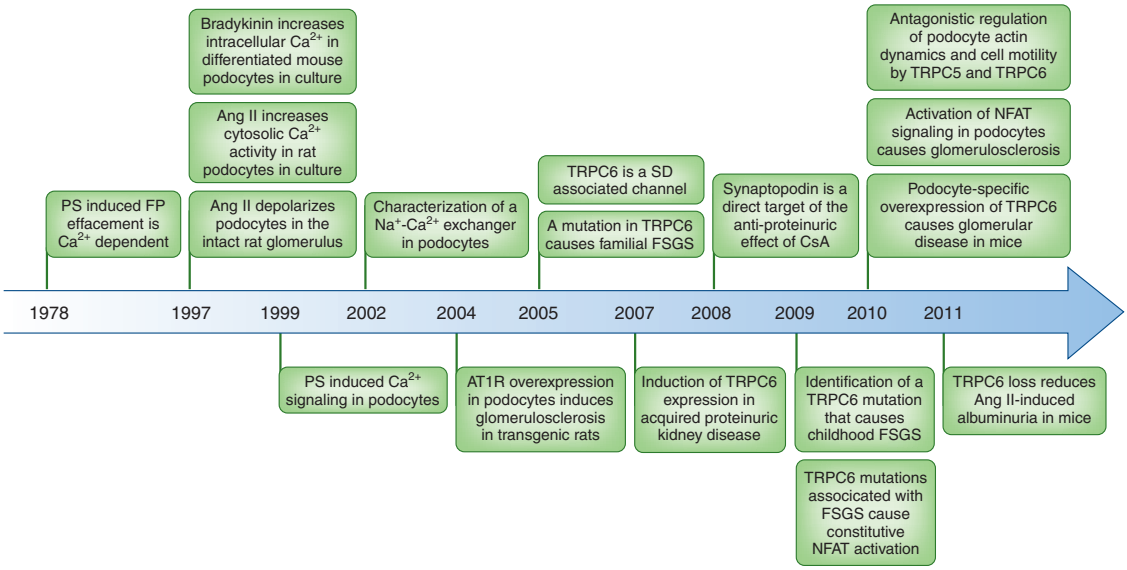

Figure 2. Evolution of calcium signaling in podocytes from 1978 to today.

tailed electrophysiology recordings demonstrating TRPC channel activity in podocytes were not available until recently, when Tian and colleagues[37] unveiled TRPC5 and TRPC6 as the channels downstream of Ang II–evoked nonselective cationic conductance initially identified more than a decade ago[35] (Figure 2).

ANG II–MEDIATED Ca^{2+} SIGNALING IN PODOCYTES

In the context of proteinuric kidney disease, angiotensin 1 receptor–mediated (AT1R-mediated) signaling is of particular importance. In classical studies of diabetic nephropathy, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) delay renal progression.[38,39] In 2004, Hoffmann and colleagues provided direct evidence that podocyte-specific overexpression of AT1R in transgenic rats is sufficient to cause proteinuria and FSGS-type lesions (Figure 2).[40] At a cellular level, AT1R signaling is upstream of a number of pathways that may be important to TRPC signaling. One is the AT1R-dependent activation of calcineurin.[41] Furthermore, AT1R signaling causes transactivation of the EGF receptor (EGFR) in tubular epithelia[42] and podocytes.[43] AT1R-EGFR interactions also activate downstream serine/threonine kinases such as the MAPK pathway in a process that is known to require an increase in cytosolic $[Ca^{2+}]$.[44] Ang II induces membrane ruffling and loss of stress fibers,[45] thereby phenocopying the depletion of synaptopodin[46,47] or TRPC6.[37]

TRPC CHANNELS AND Ca^{2+} SIGNALING

Transient receptor potential (TRP) channels are receptor-operated, nonselective, cationic channels first identified in Drosophila[48] (see below for some technical considerations regarding canonical TRP [TRPC] channels). Although nonselective for a particular cation, from a cellular signaling perspective, the central focus in the study of TRPC channels has been their ability to negotiate the influx of small but potent amounts of Ca^{2+} through the cell membrane.[9,49] The TRP superfamily consists of 28 members segregated into 7 families with diverse mechanisms of activation, tissue-specific distribution, and functional properties.[9,49] The canonical TRPC channels include seven members, TRPC1 to TRPC7.[9,49] On the basis of functional similarities and sequence alignment, two major subfamilies emerge, TRPC1/4/5 and TRPC3/6/7 (TRPC2 is a pseudogene in humans).[49] Four TRP subunits are required to form a functional channel, rendering a cation-conducting pore that is either a homomeric or heteromeric tetramer.[49] TRPC channel activation occurs primarily through activation of phospholipase C (PLC), and the cleavage of phosphatidylinositol bisphosphate (PIP_2) which gates the channels directly, as in the case of TRPC4 and TRPC5, or indirectly through its hydrolysis into diacylglycerol, as in the case of TRPC3,6,7.[9,50]

TRPC6 is closely related to TRPC7, with overlapping expression patterns in the kidney.[50] TRPC6 channels form homomers with a characteristic doubly rectifying current-voltage relationship and are six times more permeable for Ca^{2+} than Na^+ with a single-channel conductance of 35 pS.[49,51] TRPC6 channels, which inhibit endothelial cell migration,[52] are implicated in pulmonary arterial hypertension[53] and cardiac hypertrophy and fibrosis.[41,54–56] TRPC6 also increases smooth muscle cell contraction.[57–59] TRPC6 null mice were therefore expected to have decreased vascular

smooth muscle tone resulting in hypotension. Surprisingly, however, Birnbaumer and colleagues observed elevated BP and increased vascular smooth muscle contractility in aortic rings from TRPC6 null mice.[60] These unexpected effects were the result of compensatory upregulation of constitutively active TRPC3 channels.[60] In the kidney, TRPC channels came to the forefront when gain-of-function TRPC6 mutations were linked to familial FSGS[20,36] and TRPC6 was shown to be associated with the slit diaphragm.[36]

Recent work has also implicated TRPC5, in addition to TRPC6, as an important mediator of cytoskeletal changes in podocytes.[37] Although TRPC5 is ubiquitously expressed, its highest levels of expression are in brain and kidney.[9,50] TRPC5 channels generate Ca^{2+} transients implicated in neuronal growth cone motility[61,62] and vascular smooth muscle cell migration.[63] Vesicular insertion of TRPC5 from a reserve pool is downstream of EGF-RTK signaling in a pathway that involves Rac1 and phosphatidylinositol 4-phosphate 5-kinase.[62] TRPC5 subunits form homomeric channels with a unique current-voltage (I–V) signature curve in response to stimulation through G protein coupled receptors (GPCR).[64,65] TRPC5 channels conduct ten Ca^{2+} ions for one Na^{+} ion into cells with a single-channel conductance of 38 pS.[66] The current-voltage relation is doubly rectifying, with substantial inward current, little outward current up to +40 mV, and steeply outwardly rectifying current above +40 mV.

TRPC5-mediated currents are increased in the presence of 100 μM La^{3+}, in contrast to virtually all other TRPC channels (except TRPC4), which are inhibited by La^{3+} at even lower concentrations.[67] TRPC5 is also strongly potentiated by intracellular Ca^{2+}.[66] Interestingly, coexpression of TRPC1 and TRPC5 in HEK293 cells results in heteromeric TRPC1/5 channels with voltage dependence similar to N-methyl-D-aspartate receptor channels: At polarized potentials the inward current is gently inwardly rectifying, but steeply outwardly rectifying above 0 mV. Loss of TRPC5 in the amygdala results in impaired fear responses in young mice,[68] suggesting a role for these channels in fear conditioning and anxiety during postnatal brain development. In podocytes, TRPC5 channels have been studied *in vitro*,[37] but no *in vivo* studies have been performed to date.

CALCINEURIN SIGNALING IN PODOCYTES

An important link between Ca^{2+} and podocyte injury is the finding that activation of the Ca^{2+}-dependent phosphatase, calcineurin, leads to cathepsin L-mediated cleavage of synaptopodin and proteinuria[47] (Figure 2). The calcineurin inhibitor, cyclosporine A (CsA), prevents synaptopodin degradation *in vitro*, and mice resistant to cathepsin-mediated synaptopodin degradation are protected from proteinuria *in vivo*.[47] Conversely, the activation of calcineurin in podocytes is sufficient to cause degradation of synaptopodin and proteinuria.[47] Taken together, these findings reveal a T cell and NFAT-independent mechanism for the long known antiproteinuric effect of CsA: Preservation of synaptopodin and the podocyte actin cytoskeleton.[47]

In light of the proposed central role of synaptopodin in podocytes, we must take a moment to revisit the question of synaptopodin in null mice. Synaptopodin mutant mice lacking Synpo-short and Synpo-long upregulate Synpo-T protein expression in podocytes, thereby rescuing kidney filter function during development.[69] Moreover, bigenic heretozygosity for synaptopodin and CD2AP results in proteinuria and FSGS-like glomerular damage, underscoring the importance of synaptopodin and CD2AP for sustained kidney filter function.[70] In humans, heterozygous mutations in the promoter of the *synpo* gene in patients with idiopathic FSGS reduce gene transcription *in vitro* and protein abundance *in vivo*.[21]

FSGS-causing TRPC6 mutations but not wild-type TRPC6 induce constitutive activation of calcineurin-NFAT–dependent gene transcription.[71] Although not clearly related to cytoskeletal regulation, this study supports an important role for TRPC channel-mediated Ca^{2+} influx in activating calcineurin and its downstream effectors in podocytes. One hypothesis involves a positive feedback loop, whereby calcineurin-NFAT–mediated increases in transcription lead to more TRPC channels on the membrane, thus more Ca^{2+} influx and perpetuation of the cycle, leading to podocyte injury. Such a feed forward cycle has been demonstrated in a mouse model of cardiac hypertrophy, where calcineurin-NFAT–mediated increases in TRPC6 transcription promote pathologic cardiac remodeling.[41] More recently, however, other TRPC channels, including TRPC4 (which is highly homologous and functionally similar to TRPC5[64,65]), were also shown to mediate calcineurin-NFAT activation in cardiac myocytes downstream of Ang II and AT1R signaling,[41,55,56] strongly suggesting this pathway is not specific to TRPC6. Recent work by Chen and colleagues also shows that activation of NFAT signaling in podocytes is sufficient to cause glomerulosclerosis in mice.[72] This finding begs the question of whether, by analogy to cardiac myocytes, podocytes have also evolved a feed forward loop linking TRPC channels to NFAT activation and subsequent TRPC transcriptional upregulation. As it stands now, however, there is no direct evidence showing that TRPC6 or another member of the TRPC family causes FSGS through the activation of NFAT in podocytes.

Ca^{2+}-DEPENDENT RHO GTPase SIGNALING IN PODOCYTES

The Rho family of GTPases (RhoA, Rac1, and Cdc42) controls pathways that modulate cytoskeletal dynamics.[73,74] Rac1 and Cdc42 promote cell motility through the formation of lamellipodia and filopodia at the leading edge, respectively.[74] In contrast, RhoA promotes the formation of stress fibers and focal contacts, generating a contractile phenotype.[74] In podocytes, predominance of RhoA activity produces a stationary phenotype, sug-

gesting stable foot processes, whereas predominance of Cdc42/Rac1 activity mediates a disease-associated motile phenotype, suggesting unstable or retracted foot processes.[7]

Synaptopodin promotes RhoA signaling through competitive inhibition of Smurf1-mediated ubiquitination of RhoA.[46] Synaptopodin thus protects RhoA from proteosomal degradation, and preserves stress fibers *in vitro*, while safeguarding against proteinuria *in vivo*.[46,47] This notion is supported by the observation that synaptopodin-depleted podocytes display loss of stress fiber formation and aberrant filopodia.[69] Synaptopodin also suppresses Cdc42 signaling through the inhibition of Cdc42:IRSp53:Mena complexes.[75] Overall, synaptopodin stabilizes the kidney filter by preventing the reorganization of the podocyte foot process cytoskeleton into a migratory phenotype.[47]

Elegant work by Fujita and colleagues[76] recently showed that Rho GDP dissociation inhibitor α null mice[77] also develop heavy albuminuria, which is attributed to increased, constitutively active Rac1 signaling in podocytes.[76] The proposed mechanism involves the Rac1-dependent accumulation of mineralocorticoid receptor into the podocyte nucleus through p21 activated kinase phosphorylation.[76] Pharmacologic intervention with a Rac1-specific small molecule inhibitor (NSC23766) diminishes mineralocorticoid receptor hyperactivity and ameliorates proteinuria and renal damage in this mouse model of proteinuria.[76]

Although Ca^{2+} and synaptopodin-mediated Rho GTPase signaling are important in the modulation of the podocyte cytoskeleton, the question remains as to how these pathways intersect. Previous studies in other cell types reveal that spatially and temporally restricted changes in the concentration of free calcium (Ca^{2+} flickers) are enriched near the leading edge of migrating cells.[78,79] Rho GTPases can be regulated by Ca^{2+} and by GTPase-activating proteins (GAPs), catalyzing the hydrolysis of GTP to GDP, and guanine nucleotide exchange factors (GEFs), catalyzing the exchange of bound GDP with free GTP.[74] In vascular smooth muscle cells, the Rho GEF, Arhgef1, mediates the effects of Ang II on vascular tone and BP in a Ca^{2+}-dependent manner.[80] Although these studies establish an intimate association between Ca^{2+} influx and Rho GTPase-mediated cytoskeletal reorganization in other cell types, the mechanisms by which the podocyte senses and transduces extracellular cues that modulate synaptopodin and Rho GTPase activity, cell shape, and motility remained unclear. In particular, the Ca^{2+} entry mechanism responsible for mediating these signaling pathways had been elusive.

ANTAGONISTIC REGULATION OF PODOCYTE ACTIN DYNAMICS BY TRPC CHANNELS: A BALANCING ACT?

Recently, TRPC5 and TRPC6 were unveiled as conserved antagonistic regulators of actin dynamics and cell motility in podocytes and fibroblasts through the regulation of RhoA and Rac1, respectively.[37] The latter study identified conserved, antagonistic, mutually inhibitory signaling pathways triggered by AT1R-mediated TRPC5 and TRPC6 channel activity to control the balance between motile and contractile phenotypes. Gene silencing of *TRPC6* results in loss of stress fibers, activation of Rac1, and increased motility, which is rescued by constitutively active RhoA.[37] Conversely, gene silencing of *TRPC5* results in enhanced stress fiber formation, activation of RhoA, and decreased motility, which is reversed by constitutively active Rac1.[37] Remarkably, TRPC5 specifically interacts with and activates Rac1, whereas TRPC6 specifically interacts with and activates RhoA in another, distinct molecular complex.[37] Consistent with previous studies,[47] CsA rescues the loss of synaptopodin in TRPC6-depleted cells.[37] In contrast, synaptopodin expression is preserved in TRPC5-depleted podocytes.[37] The antagonistic relationship between TRPC5 and TRPC6 suggests that unopposed TRPC5 activity is responsible for the Ca^{2+}-calcineurin–dependent degradation of synaptopodin and loss of stress fibers in TRPC6-depleted podocytes. These results significantly extended our mechanistic understanding of TRPC channelopathies in podocytes by revealing that rather than focusing on a single channel, we have to consider the *balance* between TRPC5 and TRPC6 in understanding the effects of Ca^{2+} signaling in podocytes (Figure 3).

How can this new insight be placed in the context of what we have collectively learned thus far? Biochemical and detailed electrophysiology experiments identify TRPC6 as the predominant TRPC channel in the plasma membrane of podocytes, which supports the notion that at baseline podocytes maintain an adaptive TRPC6 and RhoA-predominant, contractile actin cytoskeleton[37] (Figure 3). This baseline condition, characterized by relative TRPC6 predominance, is likely to become severely biased toward TRPC6 in the presence of gain of function *TRPC6* mutations, where the podocyte's homeostatic mechanisms may be overwhelmed, leading to Ca^{2+} overload–mediated cellular injury or death, and ultimately, to FSGS (Figure 3). Alternatively, an unopposed or overactive TRPC6-RhoA pathway may tip the balance too far in favor of a contractile phenotype: Podocytes that are too stiff may have trouble adapting to fluctuations in glomerular filtration pressure, and may suffer from a disrupted cytoskeleton, disassembly of the actin filament network, and cell death. Interestingly, TRPC6 activity above or below physiologic levels may lead to disease, a notion supported by the observation that *TRPC6* overexpression in podocytes results in loss of stress fibers,[81] similarly to the silenced TRPC6 morphology.[37] This concept is in keeping with neuronal plasticity models, where either increased or decreased Ca^{2+} channel activity ultimately has the same effect on the cytoskeleton, resulting in the weakening or pruning of a synaptic contact.[82] Whatever the precise mechanism for podocyte loss may

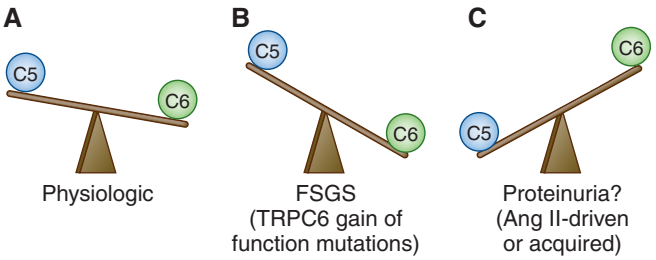

Figure 3. Antagonistic activities of TRPC5 *versus* TRPC6 signaling in podocytes in health and disease: Is it a balancing act? This working model attempts to synthesize published data and underscore the areas in which future experiments are likely to enhance our understanding of TRPC signaling in podocytes. (A) Under physiologic conditions, active TRPC6 channels are more abundant on the podocyte cell membrane, as demonstrated on the single channel level,[37] which underscores their importance for maintaining podocyte integrity, through their selective activation of RhoA.[37,46] (B) TRPC6 gain of function mutations[20,36,111] result in overactive TRPC6 channels, the cell is overwhelmed by TRPC6-mediated Ca^{2+} influx, which ultimately leads to FSGS.[83] The observed podocyte injury may result either broadly from Ca^{2+} cytotoxicity and cell death or specifically from excessive RhoA-mediated contraction, for example, increased "stiffness" leading to a "broken" actin cytoskeleton, and ultimately, cell death. (C) Given the experimental evidence that (a) constitutive Rac1 activity leads to proteinuria,[76] (b) TRPC5 activates Rac1 in podocytes,[37] and (c) Rac1 is required for TRPC5 insertion into the plasma membrane in podocytes,[37] it is reasonable to hypothesize that, in states of excess AngII, TRPC5/Rac1–mediated overactivity drives proteinuria. This notion generates interest in TRPC5 channels as mediators of acquired, Ang II–driven proteinuria.

be, an *in vivo* study showed that mice overexpressing wild-type *TRPC6* or *TRPC6* gain-of-function mutants develop albuminuria and FSGS-type lesions.[83] Although proteinuria in these mice was modest with low and variable penetrance, and structural abnormalities were not observed before 6 to 8 months of age,[83] these results are consistent with a dominant TRPC6 hypothesis (Figure 3). Clearly, more mechanistic work is required to delineate the precise mechanisms leading to FSGS in *TRPC6* transgenic mice.

Constitutively active Rac1 signaling in podocytes results in enhanced translocation of the mineralocorticoid receptor to the nucleus, an effect efficiently blocked by a Rac1 inhibitor.[76] Intriguingly, TRPC5 channels depend on Rac1 for their insertion into the plasma membrane.[62] If TRPC5 is indeed an important mediator of Ca^{2+} signaling in podocytes, could the enhanced insertion and overactivity of TRPC5 channels in the podocyte plasma membrane contribute to the proteinuria observed in RhoGDIα null mice (Figure 3)? Clearly, future *in vivo* studies are needed to delineate the role of TRPC5 in podocytes in health and disease.

A recent study reported that *TRPC6* null mice were significantly protected from the proteinuric effects of Ang II.[84] A well-designed protocol included the analysis of 18 null animals, showing significantly mitigated proteinuria to Ang II infusion for the first 4 weeks of infusion. The observed protective role of *TRPC6* deletion is surprising given that *TRPC6* null mice were initially reported to be hypertensive at baseline,[60] and thus one would expect increased albuminuria, if not at baseline, certainly after Ang II infusion. In the original *TRPC6* deletion study, TRPC3 (over)compensated for the loss of TRPC6.[60] Significant upregulation of mRNA encoding TRPC3 was also found in the latter study.[84] Indeed, the well-known compensation by the various TRPC channels has plagued the TRPC deletion field for more than a decade, making it impossible to assign specific functions to specific channels.[85]

Although this study has extended our understanding of TRPC6 channels in podocytes *in vivo*, it also raises some interesting questions: Is the significance of the protective effect lost because of the inherent variability in proteinuria that some animal models display or could this be the sign of a biologic phenomenon? Is perhaps TRPC3 (over)compensating for the loss of TRPC6, thus conferring a protective effect from exposure to AngII? In light of the antagonistic effects of TRPC5 and TRPC6 on podocyte actin dynamics,[37] it will be interesting to study the role of TRPC5 in podocytes derived from TRPC6 knockout mice. Future studies will be necessary to elucidate the role of TRPC6 channels in podocytes *in vivo*, perhaps by developing podocyte-specific, inducible TRPC6 knockout mice.

TRPC CHANNEL TRAFFICKING IN PODOCYTES

Another important aspect of TRPC channel biology is the regulation by upstream receptor pathways. GPCR signaling promotes TRPC6 plasma membrane insertion in endothelial cells.[86] TRPC6 can traffic to the cell membrane as part of a large molecular complex that includes the large conductance Ca^{2+}-activated K^+ (BK) channel.[87,88] In neurons, BK conductance results in hyperpolarization, which terminates voltage gated Ca^{2+} channel activity.[89] The same inhibitory role for BK has been shown for TRPC1-BK interactions in vascular smooth muscle cells,[90] and TRPM4/5-BK interactions in mast cells.[91,92] In contrast, the speculation in podocytes is that BK promotes TRPC6 channel activity through membrane hyperpolarization, which allows for increased Ca^{2+} permeability through TRPC6 pores.[87,93] This would effectively prolong TRPC6 channel activity,[87,93] thereby tipping the balance in favor of a RhoA-dominant, contractile phenotype. This hypothesis has not yet been tested experimentally in podocytes, and thus we cannot exclude the alternate possibility that Ca^{2+}-mediated inactivation of TRPC6 channels precedes

or prevails over the effects of BK activity. Additionally, BK-TRPC5 interactions have not yet been tested in podocytes. Future studies will be required to address these unresolved issues.

Receptor-mediated insertion of TRPC5 into the plasma membrane depends on Rac1.[62] The reciprocal dependence between TRPC5 and Rac1 activities suggests there may be a feed forward loop: Rac1 promotes channel localization to the membrane,[37,62] which in turn triggers enhanced TRPC5-mediated Ca^{2+} influx, thereby increasing Rac1 activity.[37] The cycle may be terminated when the Ca^{2+} signal wanes, perhaps because of depletion of the pool of TRPC5-containing vesicles, or because of channel inactivation. What could be the physiologic significance of such a feed forward model? When the hydrostatic pressure across the glomerular filter changes, dictated by the cardiac cycle or under conditions of increased systemic BP, foot process remodeling is required to maintain an intact filter. According to this model, BP-driven increases in Ang II may signal to TRPC5 channels, triggering their insertion into the plasma membrane, thereby increasing Rac1 activity. This event would counter the effects of the more abundant TRPC6/RhoA pathway and therefore promote transient, adaptive foot process actin remodeling. Under maladaptive pathophysiology of excessive AT1R[40] or Rac1[76] signaling, excess TRPC5 channel insertion may promote excessive foot process remodeling, which causes podocyte injury (Figure 3). Further work is needed to elucidate the precise mechanisms for TRPC5 channel trafficking in podocyte health and disease.

TRPC CHANNELS IN PODOCYTES: A FEW TECHNICAL CONSIDERATIONS

Although TRPC channels are firmly established as receptor-operated channels (ROCs)[50] in a wide array of cell types including podocytes,[37] some investigators have implicated TRPC-mediated store-operated Ca^{2+} entry in podocytes.[93,94] In the past decade, there were indeed reports suggesting that various TRPC channels could serve as store-operated channels (SOCs). However, the identification of Orai-STIM as the best-characterized SOC channel complex and the channel responsible for Ca^{2+} release activated current (I_{CRAC})[95–97] has cast serious doubt on the idea that TRPC channels are components of SOCs.[9,50] It is worth noting that most data postulating TRPCs as SOCs are solely based on Ca^{2+} imaging, whereas most of the electrophysiology studies refute the idea that TRPCs are SOCs.[9] Ca^{2+} imaging alone is prone to false positives, and electrophysiology alone is prone to false negatives.[9] Ca^{2+} imaging is an indirect readout of channel function because it reflects the total cytosolic [Ca^{2+}] regardless of the cause. Ca^{2+} entry into the unbuffered cytoplasm can affect the activity of numerous Ca^{2+}-activated channels, transporters, or other molecules, which in turn influence the imaging results.[9] In Ca^{2+} imaging experiments, voltage levels, which fuel the entry of Ca^{2+} ions into the cell, are uncontrolled. In contrast, patch clamp electrophysiology offers a direct measurement of channel activity if and when these channels are present in the plasma membrane.[9] Perfusion of the membrane with defined solutions, tight control (clamping) of the voltage, and the opportunity to apply compounds on either side of the membrane, coupled with excellent time and current resolution, are clear advantages of this technique.[9] However, in standard patch clamp, the intracellular contents are dialyzed out, removing important diffusible signaling molecules.[9] Perforated patch recordings can be performed to overcome this difficulty, but even in their absence, electrophysiology is the technique of choice to record TRP channel activity.[9] Thus, a combination of Ca^{2+} imaging and electrophysiology may ultimately be the most illuminating approach for the characterization of TRP channels in podocytes, but Ca^{2+} imaging alone should be avoided, as experience in other cell types has shown that it is prone to erroneous conclusions. As it stands, there is compelling evidence that TRPC channels are receptor-operated and there is no need to evoke them as components of SOCs.

Another important consideration involves the idea that TRPC6 channels may act as mechanosensors in podocytes.[98] The term mechanosensor describes a channel *directly gated* by mechanical force, thus acting as its own force sensor, to be distinguished from a mechanically *sensitive* channel that is activated by second messengers downstream of true mechanical force sensors.[99] By this definition, a review of the literature reveals little evidence to support the notion that TRPC6 channels are mechanosensors. One study reported that the interaction of podocin with TRPC6 results in enhanced TRPC6 channel activity in oocytes in a cholesterol-dependent manner.[100] Another report suggested that receptor and mechanically mediated TRPC6 activity share a common underlying mechanism, which involves lipid sensing by the channel.[101] Similarly, in an elegant study, Mederos y Schnitzler and colleagues[102] showed that TRPC6 is not a mechanosensitive channel, but rather, that the AT1R imparts mechanosensitivity to vascular smooth muscle cells through its effects on TRPC6 channels, to mediate the Bayliss effect,[103] the intrinsic property of arterial blood vessels to constrict in response to rises in intraluminal pressure. Thus, with regard to TRPC6, the weight of the evidence suggests that, rather than direct mechanical gating, there is likely indirect sensitivity to mechanical forces through signaling mechanisms involving plasma membrane lipids. In light of the discovery of TRPC5 channels as important mediators of Ca^{2+} influx in podocytes,[37] it is also worth considering this question more broadly. There is one report of osmomechanical stimulation of TRPC5, which was however dependent on alterations in PIP_2 levels,[104] and thereby

not directly gated by mechanical forces.

In general, TRP ion channels are emerging as candidate transduction channels in a wide variety of sensory systems, particularly those involved in sensing mechanical stimuli. As reviewed in great detail by Christensen and Corey,[99] for these TRP channels, and generally for all mechanically gated channel candidates, it is essential to differentiate between channels that are directly gated by mechanical forces and those that are downstream of a force sensor. It is also essential to show these candidate proteins fulfill a number of specific criteria (reviewed in detail[99]). To date, therefore, the question of whether TRPC5, TRPC6, or any other ion channel in podocytes serves as a mechanosensor lacks experimental evidence and is thus in need of further investigation.

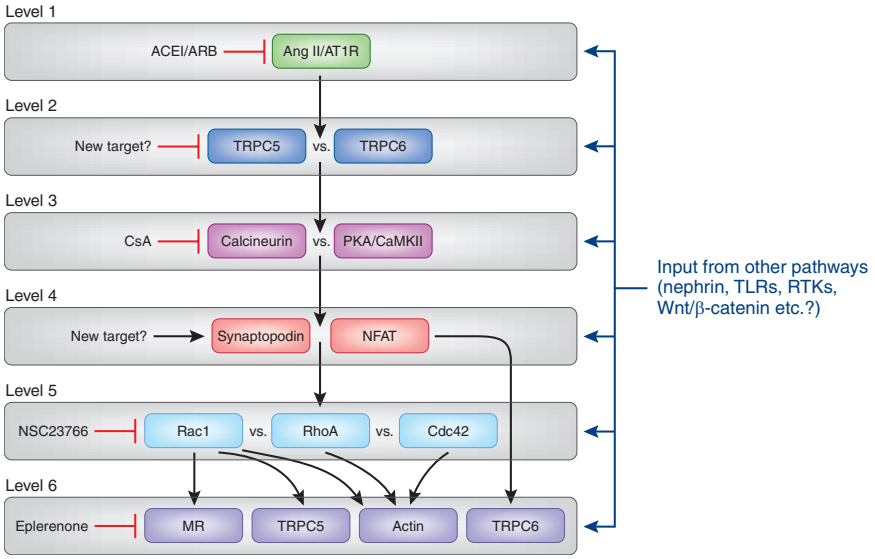

Figure 4. A model for multiple signaling pathways in podocyte injury: Is a multidrug, synergistic therapy the answer to proteinuria? A synthesis of work by many groups suggests that multiple signaling levels are involved in the Ang II–mediated regulation of podocyte function in health and disease. Level 1 consists of the binding of Ang II, whose availability is limited by ACE inhibitors, to AT1Rs, which are blocked by ARBs. Subsequent activation of TRPC5 and TRPC6 channels on level 2 results in Ca^{2+} influx into podocytes. TRPC5-driven signaling may predominate under pathologic conditions of excess Ang II and/or Rac1 activity. TRPC5-targeted agents may therefore be a novel therapeutic approach to proteinuria. On level 3, Ca^{2+}-activated phosphatases (calcineurin) or kinases (PKA and CamKII) battle for downstream effects on their mutual targets, synaptopodin and NFAT (level 4). Synaptopodin-preserving agents such as CsA exert their antiproteinuric effect by blocking the calcineurin-initiated degradation of synaptopodin. Stabilization of synaptopodin protein abundance or inhibition of NFAT signaling may be another therapeutic approach to proteinuria. On level 5, Rac1, RhoA, and Cdc42 battle for downstream effects on the actin cytoskeleton. Inhibition of Rac1 has an antiproteinuric effect *in vivo*, perhaps because of the decreased activity of mineralocorticoid receptors (MR), similar to treatment with Eplerenone (level 6). Synergistic mechanisms on level 6 include (a) the Rac1-driven insertion of TRPC5 channels in the plasma membrane (level 6), which potentiates the activity of Rac1 in a positive feedback loop, and (b) the effects of Rac1 on the actin cytoskeleton, resulting in maladaptive podocyte foot process motility, which correlates with proteinuria (level 6). RhoA and Cdc42 also affect the actin cytoskeleton (level 6). NFAT promotes TRPC6 transcription (level 6). Although AngII and AT1Rs are centrally important in podocyte Ca^{2+} signaling, inputs from many other pathways are likely to modulate the molecular events in each of the signaling levels, for example, signaling through the Nephrin pathway. This model also offers an explanation why the combined inhibition of several levels is necessary for effective and sustained antiproteinuric treatment (TLRs, toll-like receptors; RTKs, receptor tyrosine kinases).

CONCLUSIONS AND CLINICAL IMPLICATIONS

A fundamental question in cell biology is how a signal maintains specificity in time and space. This is particularly important in contractile cells such as podocytes, where the continuous remodeling of the actin cytoskeleton is required to adapt to ever-changing environmental cues. This review highlights the critical role played by the tiny but potent Ca^{2+} ion in this dynamic process. Although more work is needed to develop a mechanistic understanding of TRPC channelopathies in podocytes, a novel emerging paradigm suggests the *balance* between TRPC5 and TRPC6 may be particularly important in understanding the effects of Ca^{2+} signaling in podocytes.

Unresolved questions of current interest include the role of synaptopodin in the TRPC-dependent regulation of Rho GTPase activity in podocytes. Long-term studies should reveal if and how, similar to myocytes, podocytes employ Ca^{2+} as a selective activator of kinases or phosphatases to mediate contraction *versus* relaxation or increased foot process motility. Importantly, a systematic investigation of all Ca^{2+} influx, release, and efflux mechanisms is needed to ultimately obtain a comprehensive view of Ca^{2+} signaling in podocytes (Figures 1 and 3).

Another open question is the relation of TRPC/Rho GTPase-mediated actin remodeling to nephrin and Nck-mediated remodeling of podocyte actin dynamics.[7,23,105,106] A recent report suggests that nephrin may suppress TRPC6 channel activity under physiologic conditions, whereas *TRPC6* mutations causing FSGS escape the inhibitory effect of nephrin.[107] Future studies should test whether slit diaphragm-mediated actin dynamics can modulate TRPC signaling, and *vice versa*, whether TRPC signaling can

modulate slit diaphragm protein function.

Proteinuria is a cardinal sign and a prognostic marker of kidney disease, and also an independent risk factor for cardiovascular morbidity and mortality.[108] For decades, proteinuric kidney disease, such as hypertensive and diabetic nephropathy, have been treated with essentially the same agents, targeting the renin-angiotensin system.[109] How can our enhanced knowledge of Ca^{2+} signaling in podocytes translate into new therapeutic interventions that may help our patients in the clinic? The discovery that Ang II acts through TRPC5 and TRPC6 to target Rho GTPase activity[37] offers a molecular, mechanistic understanding of how ACEi and ARB treat proteinuria in a podocyte-specific manner, above and beyond their systemic BP lowering effects (Figure 4).[39]

A new disease paradigm thus emerges whereby proteinuria arises as a result of dysfunctions in a multilevel signaling cascade, which bring together upstream receptor pathways, TRPCs, synaptopodin, NFAT, Rho GTPases, and downstream targets such as the actin cytoskeleton or the mineralocorticoid receptor (Figure 4). The detailed mechanistic understanding of podocyte-specific signaling validates the podocyte as the target of choice for the treatment of proteinuria. Although still speculative, this schematic understanding of key molecules provides a framework and should pave the road for the identification of novel drug targets in podocytes that may act synergistically with established agents (Figure 4). For example, we can imagine a new agent acting as an inhibitor of TRPC5 channels: This would abrogate Rac1 signaling, shut down Rac1-mediated TRPC5 membrane insertion, and thus act synergistically with Rac1 inhibitors or mineralocorticoid receptor blockers (eplerenone)[76] to stem proteinuria (Figure 4). Similarly, one can envision synaptopodin-stabilizing agents, which, much like CsA,[47] may act by promoting the RhoA-dependent preservation of the stationary actin cytoskeleton (Figure 4). The Ang II–mediated regulation of podocyte actin dynamics involves at least six signaling levels, each of which can receive additional input from multiple other signaling pathways (Figure 4). This model may help explain why ACEi and ARBs have only limited efficacy as antiproteinurics. It also highlights why the combined or synergistic effect of specific agents targeted to specific levels within the signaling cascade, similar to oncologic treatments, may ultimately be our best answer for the effective treatment of proteinuria.

NOTE ADDED IN PROOF

The following studies were published after acceptance of this manuscript: Vassiliadis J, Bracken C, Matthews D, O'Brien S, Schiavi S, Wawersik S: Calcium mediates glomerular filtration through calcineurin and mTORC2/Akt signaling. *J Am Soc Nephrol* 22: 1453–1461, 2011; Zhu L, Jiang R, Aoudjit L, Jones N, Takano T: Activation of RhoA in podocytes induces focal segmental glomerulosclerosis. *J Am Soc Nephrol* 22: 1621–1630, 2011.

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