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# Angiotensin II has acute effects on TRPC6 channels in podocytes of freshly isolated glomeruli

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## Abstract

A key role for podocytes in the pathogenesis of proteinuric renal diseases has been established. Angiotensin II causes depolarization and increased intracellular calcium concentration in podocytes; members of the cation TRPC channels family, particularly TRPC6, are proposed as proteins responsible for calcium flux. Angiotensin II evokes calcium transient through TRPC channels and mutations in the gene encoding the TRPC6 channel result in the development of focal segmental glomerulosclerosis. Here we examined the effects of angiotensin II on intracellular calcium ion levels and endogenous channels in intact podocytes of freshly isolated decapsulated mouse glomeruli. An ion channel with distinct TRPC6 properties was identified in wild type, but was absent in TRPC6 knockout mice. Single channel electrophysiological analysis found that angiotensin II acutely activated native TRPC-like channels in both podocytes of freshly isolated glomeruli and TRPC6 channels transiently overexpressed in CHO cells; the effect was mediated by changes in the channel open probability. Angiotensin II evoked intracellular calcium transients in the wild type podocytes, which was blunted in TRPC6 knockout glomeruli. Pan-TRPC inhibitors gadolinium and SKF 96365 reduced the response in wild type glomerular



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**DISCLOSURES**

The authors declared no competing interests.

**SUPPLEMENTARY MATERIAL**

**Supplementary Figure S1.** Effect of inhibition of NMDA receptors on Ang II-evoked calcium influx.

**Supplementary video 1.** Illustration of the effects of 1 μM and 10 μM of angiotensin II on the wild type mouse glomeruli volume.

**Supplementary video 2.** 3D video of a mouse glomerulus stained with Fluo4 and FuraRed fluorescent calcium indicators.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

epithelial cells, whereas the transient in TRPC6 knockout animals was not affected. Thus, angiotensin II-dependent activation of TRPC6 channels in podocytes may have a significant role in the development of kidney diseases.

## INTRODUCTION

Nephrotic syndrome is a group of kidney disease characterized by heavy proteinuria, hypoalbuminemia, edema, and dyslipidemia. Urinary losses of macromolecules in nephrotic syndrome reflect a dysfunction of the highly permselective glomerular filtration barrier. In the past decade, genetic studies have led to the identification of proteins playing a crucial role in slitdiaphragm signaling and maintenance of podocyte integrity and functions.[1] Particularly, the gene encoding transient receptor potential canonical channel 6 (TRPC6) was identified as the genetic basis for an autosomal dominant form of focal segmental glomerulosclerosis (FSGS).[2,3]

Interstitial angiotensin II (Ang II), a major bioactive product of the renin-angiotensin system, is found to be the key mediator of renal inflammation and fibrosis in progressive chronic nephropathies.[4] It was shown that expression of Ang II and its receptor is increased in patients with progressive glomerulopathies.[5] It was also demonstrated that Ang II application increased intracellular calcium ($[Ca^{2+}]_i$) in the podocytes.[6–8] Since TRPC6 channel mutations were found in patients with FSGS, members of the TRPC-family emerged as prime candidates for this raise of $[Ca^{2+}]_i$.

Ang II can act through two different types of receptors: $AT_1$ and $AT_2$, which are both involved in regulation of intracellular signals in podocytes.[9] However, the majority of Ang II actions in the glomerulus are mediated by $AT_1$. It was shown that increased $AT_1$ signaling in podocytes leads to proteinuria and FSGS.[10] Studies in models of chronic hypertension and protein-induced renal damages revealed that inhibition of AT receptors is effective against proteinuria.[11] $AT_1$ receptor antagonist candesartan ameliorates the peak level of proteinuria by preventing a reduction in the expression of slit diaphragm functional molecules.[12] Human trials demonstrated that the inhibition of $AT_1$ receptors delayed disease progression in patients with diabetic kidney disease.[13,14]

Recent studies demonstrated that Ang II enhances albuminuria by activating TRPC6 channels.[15] Furthermore, Zhang et al. showed that alteration of TRPC6 expression and $Ca^{2+}$ influx is involved in Ang II-induced apoptosis.[16] Besides, it was highlighted that the deleterious effects of Ang II on podocytes and its pathogenic role in glomerular diseases coincides with enhanced TRPC6 expression[17] and that Ang II activation of TRPC6 channels in rat podocytes requires generation of reactive oxygen species.[18] However, the exact mechanisms of action of Ang II in intact glomeruli remain unclear. Furthermore, it is not clear if this hormone mediates changes in the number of channels at the plasma membrane and/or channel gating.

We demonstrate here that Ang II up-regulates TRPC6 activity in intact podocytes of freshly isolated glomeruli and that this channel's activation further results in extensive $Ca^{2+}$ flux in podocytes. For these experiments recently developed single channel analysis of TRPC

channels[19,20] and calcium measurements[21] in their native setting, freshly isolated glomeruli, were performed. Transient overexpression of TRPC6 channels together with $AT_1$ receptor in CHO cells was also utilized to test effects of Ang II. Altogether, these techniques were employed to establish the effects of Ang II on TRPC channels in the podocytes of the glomeruli, and allowed hypothesizing that TRPC6 blockade and/or inhibition of ATRs may be of therapeutic benefit in the treatment of the nephrotic syndrome and particularly FSGS.

## RESULTS

### TRPC6 channels recordings in the freshly isolated mouse glomeruli

We have recently established a novel approach allowing us to perform single channel analysis of native TRPC-like channels in the podocytes of freshly isolated glomeruli.[19,20] After the glomeruli are isolated from the kidneys of the mice, cell bodies of the podocytes appear in the light microscope as oval structures on the surface of the glomerular capillary loops. Single channel analysis was used to assess TRPC activity in the podocytes in freshly isolated glomeruli of mice. TRPC channels typically show low levels of constitutive activity.[22] Figure 1a demonstrates the activity of a channel recorded in cell attached configuration in symmetric chloride solutions at different potentials. The channel has distinct TRPC family properties, including reverse potential close to zero, kinetics, slight voltage dependency and conductance of approximately 22 pS. The summarized current-voltage dependence for this channel is shown on Figure 1b. TRPC channels activity was also tested in the TRPC6 knockout mice; with the current solutions and conditions we were unable to record the activity of the channels similar to those recorded in the wild type mice.

### Angiotensin II activates TRPC6 channels in freshly isolated mouse glomeruli and in transfected CHO cells

Figure 2a illustrates the time course of TRPC6 channel activity in the isolated glomerulus following addition of Ang II (1 μM). As summarized in Figure 2b, application of Ang II resulted in the acute increase in the channel open probability ($P_o$) in this native preparation. We[20] and others[3,7,22,23] previously demonstrated that multiple members of the TRPC family are expressed in podocytes, but only TRPC6 is known as a cause of FSGS[2,3,24]. Thus, we tested an effect of Ang II specifically on the TRPC6 channel. For these experiments we analyzed activity of endogenous channels in response to treatment with Ang II in TRPC6 knockout mice. We did not observe any similar ion channel activity in the podocytes of the $TRPC6^{-/-}$ mice. Furthermore, application of Ang II did not result in the activation of any kind of ion channels in podocytes of the $TRPC6^{-/-}$ mice (Figure 2c). Occasionally, in both wild type and TRPC6 knockout mice, we were able to record the background activity of other ion channels, distinctly different from the TRPC6 (data not shown). However, identity of these channels requires further studies and none of them were activated by Ang II.

We further tested the involvement of TRPC6 channel in Ang II-mediated effects. For these experiments we overexpressed TRPC6 together with $AT_1$ receptor in CHO cells. Current-voltage dependence and representative current traces at different potentials recorded in the transfected CHO cells are shown in Figure 3; the conductance of the channels was 24.0 ±1.1 pS. As shown on Figure 3a and summarized in Figure 3b, Ang II significantly increased

TRPC6 $P_o$ in CHO cells, similar to native TRPC-like channels of the podocytes in the freshly isolated mouse glomeruli. Please note that Ang II washout resulted in fadeaway of the TRPC6 activity, whereas recurrent application of Ang II caused its restitution. Current-voltage relationship of the recorded channels is shown in Figure 3c. As a negative control we have used either untransfected CHO cells or cells transfected with the TRPC6 channel without $AT_1$ receptor. In both cases, we were unable to record the activation of the channels in response to Ang II (Figure 3d).

## Angiotensin II application in concentrations above 10 μM results in contraction of mouse glomeruli

Different concentrations of Ang II in the range of 100 nM to 25 μM were tested to establish the appropriate concentration for a detectable response both in patch-clamp experiments and in calcium concentration measurements. Ang II evoked the response at all studies concentrations (data not shown). However, the concentration of 1 μM was selected as the most suitable for the experimental procedures, as all the concentrations of Ang II above 10 μM resulted in contraction and subsequent relaxation of the glomeruli, which made electrophysiological studies impossible due to detachment of the patch pipette from the podocytes. Figure 4 summarizes the effect of 1 μM (Figure 4a) and 10 μM (Figure 4b) of Ang II on glomerular volume. Supplementary Video 1 illustrates the absence of glomerular contraction in response to 1 μM of Ang II (first mark) and a significant visible contraction after addition of 10 μM of Ang II (second mark).

## Intracellular calcium response is impaired in the podocytes of the TRPC6$^{-/-}$ mice compared to wild type animals

We tested the intracellular calcium response of the podocytes of the wild type and TRPC6$^{-/-}$ mouse glomeruli by the ratiometric confocal measurement with Fluo4/FuraRed fluorescent dyes. Figure 5a shows typical confocal images of the wild type and TRPC6 knockout mouse glomeruli in the calcium free solution and after the solution change to the one containing 2 mM $CaCl_2$; increase in the fluorescence intensity evoked by the calcium influx from the extracellular space can be clearly seen. Supplementary Video 2 shows a rotating confocal 3D reconstruction from a z-stack image collection (18 z-slices) of the glomerulus stained with Fluo4 and FuraRed. Fluorescent podocytes can be clearly seen on the surface of the glomerulus close to the glass.

Figure 5b summarizes the intracellular calcium response evoked by the extracellular calcium concentration change; the transient was significantly higher in the wild type podocytes compared to the podocytes from the TRPC6$^{-/-}$ animals. Therefore, this observed difference in the increase of the intracellular calcium concentration conforms to the expected blunted calcium influx in cells deficient for the TRPC6 channels. In all of the experiments calcium levels returned to the basal level after solution was changed for the calcium-free. In order to estimate the contribution of the TRPC channels into calcium influx in the podocytes, we performed ratiometric confocal calcium measurements with a pan-TRPC blocker $GdCl_3$, which is also known to potentiate TRPC5 channels.[25,26] Figure 5b illustrates the effect of 1 min pre-incubation of the wild type and TRCP6$^{-/-}$ glomeruli with 100 μM $GdCl_3$; as apparent from the calcium transient, $Gd^{3+}$ precluded the increase in the intracellular calcium

levels stimulated by the solution change. In the TRCP6$^{-/-}$ glomeruli, the same concentration of gadolinium resulted in a slightly less blunted decrease of the transient compared to control. Thus, while these data demonstrate that there are some compensatory changes in response to gadolinium, it also shows that TRPC6 is important for this response in wild type animals. We have also tested flufenamic acid (FFA) known to stimulate TRPC6 channels.[25,27] As seen from Figure 5c, FFA produced a fast and significant calcium transient in the podocytes.

### The effect of SKF 96365 on Ang II – stimulated increase in the intracellular calcium in wild type and TRPC6-deficient mouse podocytes

Figure 6a shows representative fluorescence images of the wild type mouse glomeruli stained with Fluo4 and FuraRed before and after application of 1 μM of Ang II. The right panel of Figure 6a demonstrated the responses of the Fluo4 and FuraRed signals separately. A region of interest (ROI) denoted on Figure 6b was used to create separate intensity profiles recorded from Fluo4 and FuraRed.

A typical acute transient evoked by Ang II (1 μM) in the podocytes of the glomeruli isolated from wild type mice is shown on Figure 6b. The magnitude of the response to Ang II in podocytes isolated from wild type mice was decreased by the pretreatment with low concentrations of SKF 96365 (1 μM), which is a potent pan-TRPC inhibitor. The response to Ang II was significantly blunted in the podocytes of the TRPC6$^{-/-}$ mice, whereas SKF 96365 did not affect this Ang II – evoked transient. We also tested the effect of 1 μM Ang II in the wild type glomeruli in the absence of extracellular calcium. Podocytes still show an increase in calcium in response to Ang II in the absence of the extracellular calcium; thus, the transient recorded under these conditions most likely accounts for the release of calcium from the intracellular depot. The remaining response that we see after SKF96365 incubation has the same amplitude as the transient recorded in the calcium-free solution. It can be concluded that the remaining response after SKF96365 accounts for the depot depletion. This was further confirmed by testing the effect of Ang II in the TRPC6$^{-/-}$ podocytes in presence of 100 μM $Gd^{3+}$; the data showed that the effect of Ang II remains unchanged when the sample was pre-incubated with $Gd^{3+}$. Summarized responses to Ang II in wild type and TRPC6$^{-/-}$ mice are shown in Figure 6c and are represented by the maximum magnitude of the ratiometric transients. We further tested the effect of Ang II in the wild type podocytes in presence of 10 μM MK801, which is a non-competitive NMDA receptor antagonist, and found that the Ang II-mediated calcium transient is preserved when NMDA receptors are inhibited (Supplementary Figure S1).

## DISCUSSION

Whereas many studies have shown an important role of TRPC channels in podocytes and mediation of calcium flux by Ang II, the regulation of these channels has not yet been investigated in freshly isolated glomeruli except for the elegant study by Gloy et al., who demonstrated in the intact glomerulus that Ang II depolarizes podocytes directly by opening a $Cl^-$ conductance.[6] The authors proposed that the activation of $Cl^-$ conductance is mediated by an $AT_1$ receptor and may be regulated by the intracellular $Ca^{2+}$ activity.[6] Since it is

difficult to directly transfer data obtained from cultured glomerular epithelial cells to the responses of podocytes *in vivo*, we have established an experimental approach that allowed us to study podocytes in freshly isolated intact glomeruli. Calcium imaging and single channel analysis in the podocytes of intact glomeruli demonstrated that Ang II application results in calcium influx, which is regulated by the changes in the channel open probability of TRPC channels. Further electrophysiological and confocal experiments in the podocytes of the wild type and TRPC6$^{-/-}$ mice combined with the use of the pharmacological tools: TRPC blockers $Gd^{3+}$ and SKF 96365, TRPC6 activator FFA and NMDA blocker MK801, and studies in heterologous expression system confirmed that this effect is mediated by TRPC6 channels.

Recent studies utilizing TRPC6-deficient mice revealed that proteinuria is attenuated in TRPC6-deficient mice. Interestingly, elimination of TRPC6 has no effect on normal glomerular structure or function and does not affect blood pressure.[15] However, this could be potentially explained by compensatory mechanisms mediated by other TRPC-family members expressed in podocytes.[7,28] For instance recent studies identified that loss or inhibition of TRPC5 abrogates podocyte cytoskeletal remodeling and this channel is an important determinant of albuminuria.[29]

Nitschke et al. demonstrated that podocytes in the intact glomerulus respond to Ang II with an increase of $[Ca^{2+}]_i$ via an $AT_1$ receptor. Interestingly, this effect was specific since neither bradykinin, nor arginine vasopressin, thrombin, or serotonin influenced $[Ca^{2+}]_i$ in podocytes.[30] The authors demonstrated that the Ang II-induced increase of $[Ca^{2+}]_i$ was due to both a $Ca^{2+}$ release from the intracellular space and a $Ca^{2+}$ influx from the extracellular space.[30] Using available inhibitors, the authors attempted to identify a channel responsible for this extracellular calcium flux. However, neither the L-type $Ca^{2+}$ channel blocker nicardipine, nor an increase of the extracellular $K^+$ concentration changed $[Ca^{2+}]_i$ in the glomeruli.[30]

$Gd^{3+}$ and SKF 96365 used in this study for identification of the source of $Ca^{2+}$ flux are not specifically selective for TRPC6 but rather are pan-TRPC inhibitors. However, our calcium measurement data obtained in the knockout animals in combination with electrophysiological observations provide definite conclusions about the channel responsible for the Ang II-mediated increase of $[Ca^{2+}]_i$ in the podocytes of the intact glomeruli. Our data are in a good agreement with recent data published by Anderson et al[18] who reported that Ang II increases cationic currents in rat podocytes of an isolated glomerulus preparation and suggested that production of reactive oxygen species could permit activation of TRPC6 channels by G protein-coupled receptors and PLC-dependent cascades initiated by Ang II acting on $AT_1$ receptors in podocytes.[18] Role of NADPH oxidase Nox2 in this signaling mechanism was also proposed.[31] Nijenhuis et al. demonstrated that Ang II regulates TRPC6 mRNA and protein levels in cultured podocytes and that Ang II infusion enhances glomerular TRPC6 expression *in vivo* and that TRPC6 expression correlates with glomerular damage markers and glomerulosclerosis.[17] The authors proposed that the deleterious effects of Ang II on podocytes and its pathogenic role in glomerular disease involved enhanced TRPC6 expression via a calcineurin/NFAT positive feedback signaling pathway.[17] Our studies demonstrate that Ang II has acute effect on both increase of $[Ca^{2+}]_i$ and TRPC6

activity. Furthermore, the effect on channel activity is mediated by changes in the channel open probability. However, we cannot exclude that both mechanisms mediate TRPC6 activity with rapid changes in gating properties and long term effect on the level of transcription and protein expression.

Furthermore, role of the actin cytoskeleton as a downstream signaling event involved in Ang II-mediated increase of $[Ca^{2+}]_i$ was proposed. It was shown that Ang II caused a reactive oxygen species-dependent rearrangement of cortical F-actin and a migratory phenotype switch in cultured mouse podocytes with stable $AT_1$ receptor expression.[32] Small GTPases Rac1 and RhoA were identified as antagonistic regulators of actin remodeling in podocytes. The authors defined TRPC5 and TRPC6 as channels being downstream of the Ang II–evoked nonselective cationic conductance in podocytes.[33] The application of Ang II revealed three populations of channels with the corresponding conductances of 39, 68 and 80 pS. TRPC5 was identified as channel forming a molecular complex with Rac1, whereas TRPC6 was shown in a complex with RhoA.[33]

It is necessary to mention that apart from the Ang II-mediated calcium entry, which is now recognized to be an important part of the signal transduction events in the podocytes, there is a plethora of other processes in these cells, which involve calcium influx though various calcium conducting channels. For example, both TRPC5 and TRPC6 channels were identified to be antagonistic regulators of actin remodeling and cell motility in fibroblasts and kidney podocytes.[29,33] Functional NMDA receptors were also found to be expressed in the podocytes and to contribute to calcium entry in these cells.[34–36] However, our data are consistent with the idea that TRPC6 is the main channel responsible for Ang II-mediated calcium signaling.

Selective inhibition or activation of signaling pathways may be an effective means of modulating proteinuria. However, the cellular mechanisms of these processes are still uncertain and require further investigation. Successful specific therapy of glomerular diseases depends on concurrent targeting of multiple signaling pathways. The ability of podocytes to precisely regulate intracellular $Ca^{2+}$ levels plays a central role in glomerular disease processes. Manipulating $Ca^{2+}$ levels by inhibiting TRPC channel activators may be a useful strategy for treating patients with FSGS and nephrotic syndrome.

## MATERIALS AND METHODS

### Animals

Animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the IACUC of the Medical College of Wisconsin. For experiments, male wild type (c57BL/6) or $TRPC6^{-/-}$[37,38] mice were used. Mice were provided with food and water *ad libitum*.

### Isolation of the mouse glomeruli

The kidneys of 8 to 12-weeks-old male mice were removed and then decapsulated; the cortex was isolated and minced as described previously for the rat kidney preparation.[19,20] The minced tissue was sequentially pushed through a steel sieve of 150 μm mesh and then

pipetted through a 106 μm sieve. This tissue homogenate was then pipetted onto a 75 μm sieve; the filtrate was then pushed through the 53 μm sieve and the glomeruli were rinsed from the both 75 and 53 μm sieves. For experiments, isolated glomeruli were allowed to settle onto cover glass chips coated with poly-*D*-lysine.

## Electrophysiology

Cover glasses that contained glomeruli were placed into a perfusion chamber mounted on an inverted Nikon Ti-S microscope and superfused with a physiologic saline solution (pH 7.4). Single-channel current data were acquired as described previously.[19,20] After a high resistance seal was obtained, cell-attached recording was performed immediately. The membrane resistance was monitored regularly to ensure the quality of recording. For measurements of acute effect only one experiment was performed per dish to avoid any possibility of examining cells whose properties might have been altered by extended exposure to Ang II. The recordings were made in symmetric chloride solutions. The bath solution consisted of 126 NaCl, 1 $CaCl_2$, 10 HEPES, 2 $MgCl_2$, 10 glucose, pH 7.4. The pipette solution contained 126 NaCl, 1.5 $CaCl_2$, 10 HEPES, 10 glucose; pH7.4; plus added directly before the patch-clamp experiments were 100 μM niflumic acid or DIDS (to block $Ca^{2+}$-activated $Cl^-$ channels), 10 mM TEA (to inhibit large-conductance $Ca^{2+}$-dependent $K^+$ channel), 10 nM iberiotoxin (to block $Ca^{2+}$-activated $K^+$ channels), 10 μM nicardipine (to block N-type $Ca^{2+}$ channels). During the patch-clamp measurements in the single-channel mode the activity of the ion channels was first monitored in response to the potential applied in steps of 10 or 20 mV in the range of – 90 mV to + 60 mV in order to estimate the channel's conductance and I-V relationship. After that, the voltage was clamped at – 60 mV and the channels' activity was recorded for several minutes before the drugs were applied.

## Glomerulus contraction measurements

For the volume response measurements freshly isolated mouse glomeruli were affixed to glass coverslips coated with poly-*L*-lysine. The glomerular responses were recorded using confocal microscopy (Nikon A1-R). Glomerular volume (V) was calculated at the maximum points before and after addition of the drugs from the surface area (S) of the glomerulus using the formula $V = 3/4\pi(S/\pi)3/4$ using the ImageJ software as previously described.[39] ΔV was calculated as (Vfinal−Vinitial)/Vinitial. At least ten glomeruli from three or more mice were studied under each condition. Control glomeruli were treated with equivalent volumes of buffer and no changes in the glomeruli volume was detected.

## cDNA constructs and cell culture

The wild type Chinese hamster ovary (CHO) cells were obtained from the American Tissue and Culture Collection and cultured in DMEM medium containing 10% FBS, 2 mM glutamine, and 80 μg/ml gentamicin. The plasmid encoding the wild type TRPC6 channel gene was previously described.[40] Plasmid encoding $AT_1$ receptor was obtained at Missouri S&T cDNA Resource Center (AGTR10TN01). Cell transfection with 1 μg of each plasmid encoding TRPC6 and $AT_1$ receptor and 0.5 μg of green fluorescent protein was performed 24–48 hrs before the experiments.

### Confocal laser-scanning fluorescence microscopy

Calcium imaging was performed with laser scanning confocal microscope system Nikon A1-R. Images were collected in time series (xyt, 4s per frame) with the Nikon imaging software. Changes in intracellular $Ca^{2+}$ concentration were estimated according to a protocol described previously[21] from ratiometric fluorescence images of Fluo-4 and Fura Red loaded glomeruli. Emitted light was collected by the objective lens Plan Apo ×60 oil DIC2. The glomeruli suspension was loaded with the dyes by adding Fluo-4 AM and Fura Red AM (5 μM of each; Invitrogen). Glomeruli were mounted on the poly-*D*-lysine covered glass in a registration chamber and washed for ~ 10 min with bath solution containing (in mM): 145 NaCl, 4.5 KCl, 2 MgCl2, 10 Hepes, pH 7.35. After stabilization of the fluorescence signal podocytes were identified on the basis of anatomic considerations, and fluorescence intensity ratios were recorded. Fluorescent signal was observed only from cells on the surface of glomeruli in area attached to glass. In each experiment, 4 to 7 podocytes of at least one glomerulus were selected; experiments for every compound tested were repeated at least 3 times.

### Statistical analysis

Data are presented as mean ± s.e.m. The values of intracellular calcium ion concentration at every moment of time for individual cells were averaged by the number of regions registered in the experiment. Data are compared using the Wilcoxon signed-rank test and $P<0.05$ is considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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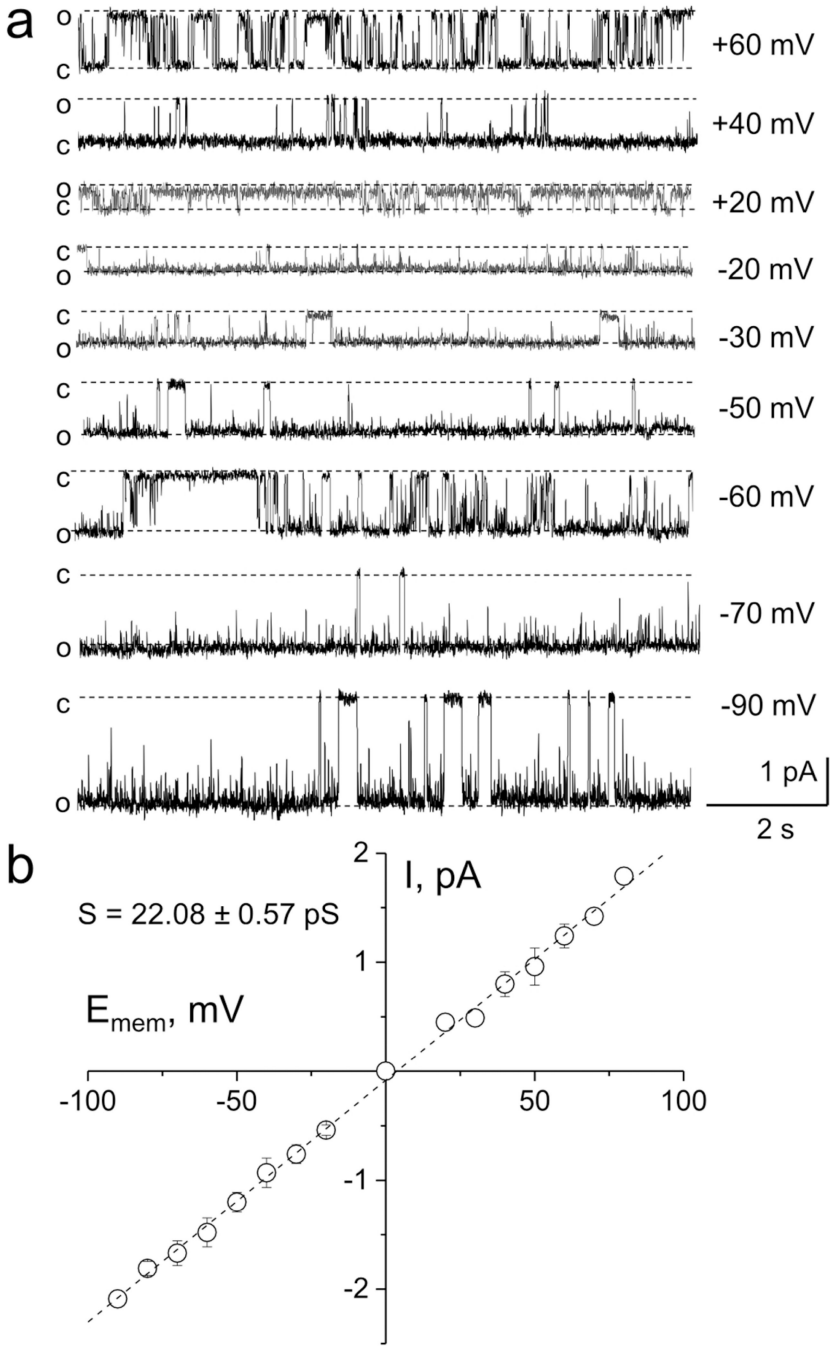


**Figure 1. Natvie TRPC6 channels in the freshly isolated mouse glomeruli**

**(a)** Representative current traces from the podocytes of the freshly isolated wild type mouse glomeruli. The activity of the identified TRPC6 channels is shown at different potentials. *c* and $o_i$ denote closed and open states of the channels. (**b**) A summarized current-voltage dependency of the identified TRPC6 channel in the podocytes of the freshly isolated glomeruli. Conductance (*S*) is shown on the graph. Each point is the mean of at least 6 independent observations made on 5 animals.

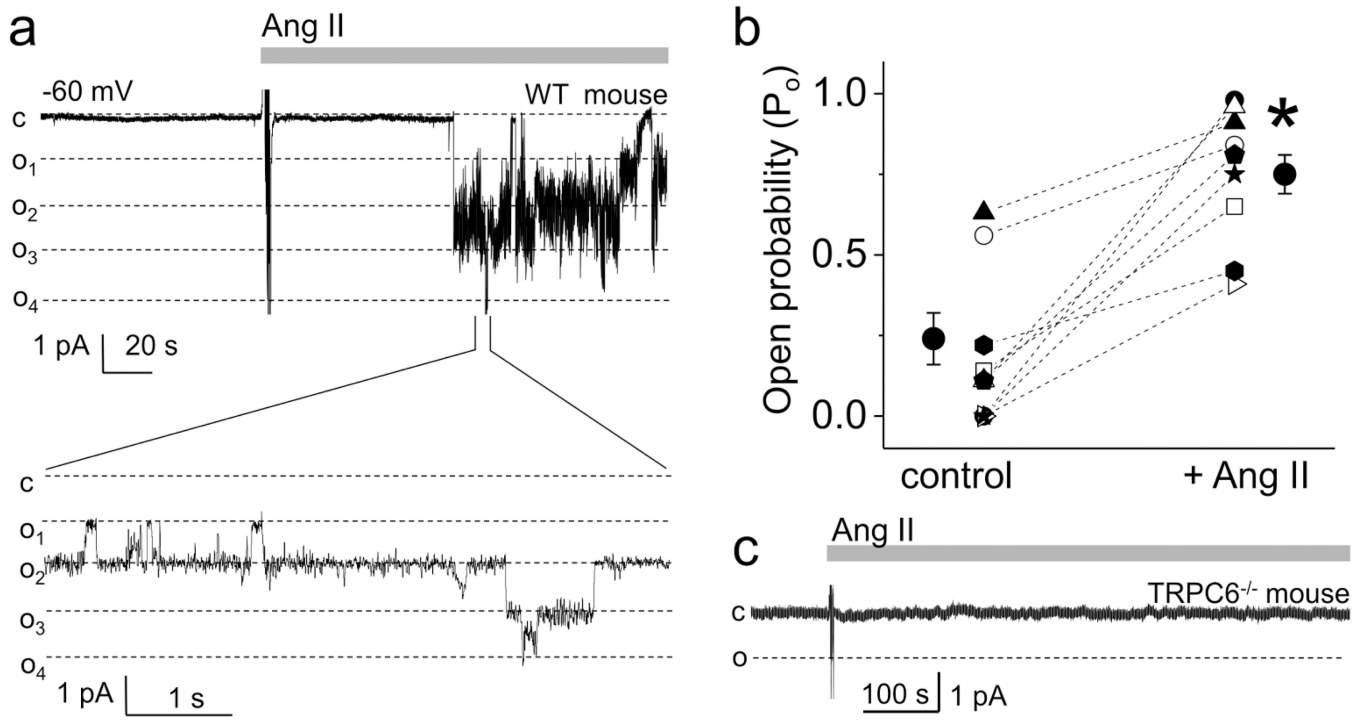


**Figure 2. Ang II activates TRPC6 channels in the podocytes of freshly isolated mouse glomeruli**

**(a)** Representative current traces of a TRPC6 channel from a cell-attached patch of a podocyte from a wild type mouse glomerulus. A continuous current trace is shown (upper row), arrow demonstrates addition of Ang II (1 μM) to the external bath solution. All patches were held at a −60 mV during the course of experiment. The *c* and $o_i$ denote closed and open current levels, respectively. (**b**) Summary graph for the channel's open probability before and after application of Ang II. ***P*<0.01 versus before Ang II, the number of patches tested was 10. (**c**) A representative recording made on the podocytes of the freshly isolated glomerulus of a TRPC6 knockout mouse. Arrow demonstrates addition of Ang II (1 μM) to the external bath solution. No TRPC6 channel activity was recorded in any of the patches before or after application of Ang II. The total number of animals used was 11 wild type and 7 $TRPC6^{-/-}$, respectively.

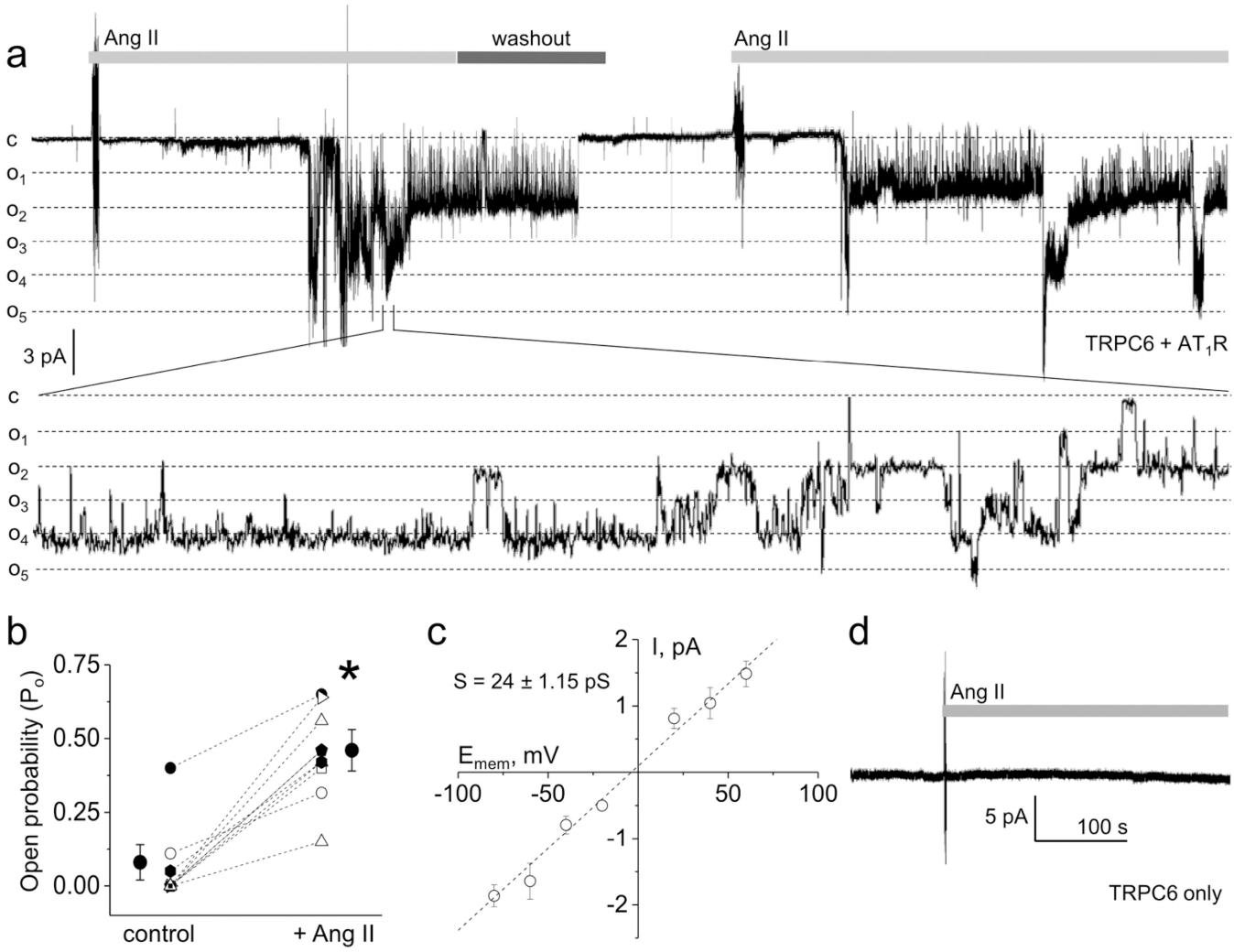


**Figure 3. Ang II activates TRPC6 channels in transfected CHO cells**

Shown is a representative current trace from the cells transfected with $AT_1$ receptor and TRPC6 before and after application of 1 μM Ang II and following washout and second Ang II application (total length 30 min) with an expanded region (10 s) showing the activity of the channel on an expanded scale (**a**) and a graph summarizing the channels' open probability before and after application of Ang II (**b**). *P<0.001 versus before Ang II. Cumulative current-voltage dependence of the recorded TRPC6 channel calculated from 6 independent patches is shown on (**c**). The number of independent experiments testing the effects of Ang II was 8. (**d**) A representative current trace illustrating the absence of the TRPC6 activity before or after application of Ang II in CHO cells transfected with the TRPC6 channel only.

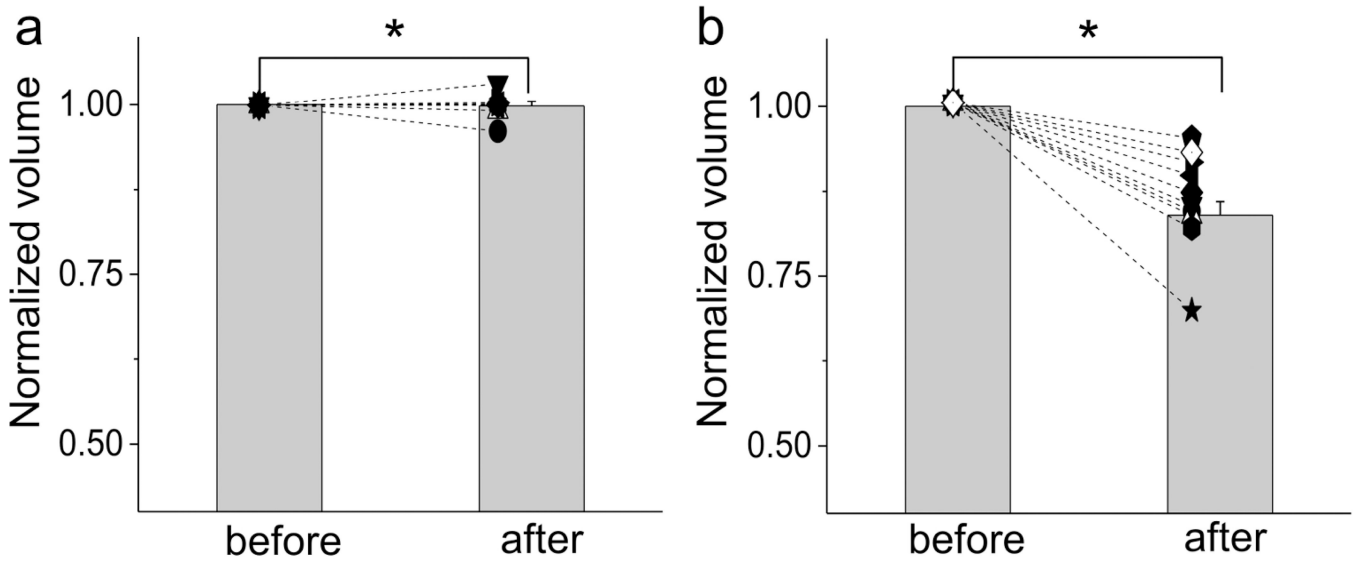


**Figure 4. Angiotensin II application results in the changes of the glomerular volume**

The graph summarizes the effect of Ang II in concentration 1 (**a**) and 10 μM (**b**) on the glomerular volume. * denotes $P<0.05$ vs before application of Ang II. The summary was calculated from 12 independent glomeruli from 5 different wild type mice.

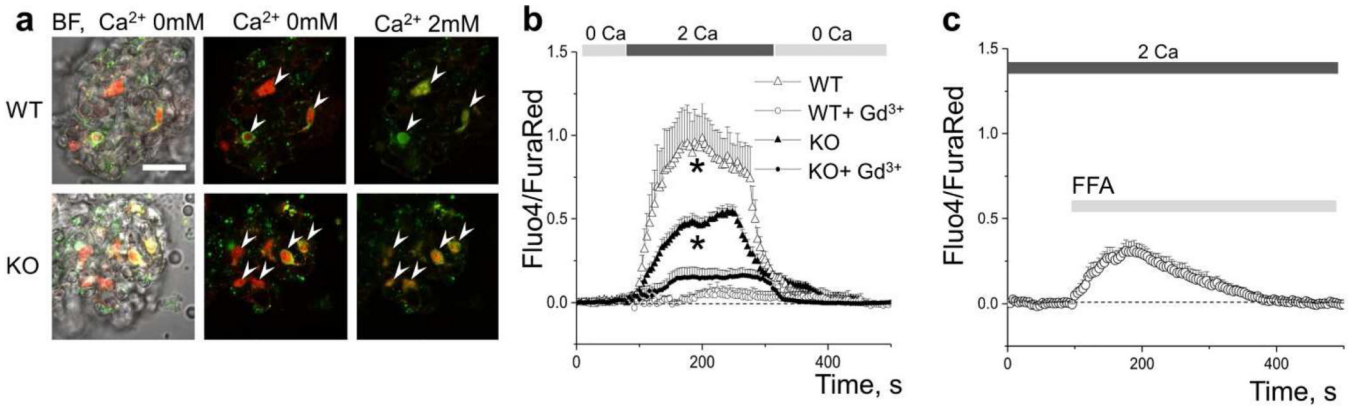


**Figure 5. Intracellular calcium response in the podocytes of the freshly isolated glomeruli of TRPC6$^{-/-}$ mice compared to wild type animals**

(**a**) Representative images illustrating the wild type (upper row, WT) and TRPC6$^{-/-}$ (bottom row) mouse glomeruli stained with Fluo4 (green pseudocolor) and FuraRed (red pseudocolor) in the calcium-free solution (bright field (BF) merged with fluorescence and fluorescence only) and after solution change to the calciumcontaining solution (2 mM $Ca^{2+}$). The lower intensity green-colored fluorescence in the knockout podocytes after the solution change should be noted. Arrows denote the typical regions of interest (ROIs) where the fluorescence signal intensity was recorded. Scale bar shown is 50 μm. (**b**) Shown is the summarized intracellular calcium transient in the podocytes of the freshly isolated glomeruli of the wild type and TRPC6 knockout mice in response to the extracellular solution change from calcium-free (nominal 0 mM $Ca^{2+}$) to calcium-containing (2 $Ca^{2+}$) and back; representative traces recorded in the wild type and TRPC6$^{-/-}$ glomeruli in presence of 100 μM $Gd^{3+}$ are also shown. The calcium response is shown as the ratio of the signal from the fluorescent dyes Fluo4/FuraRed. The number of animals used was 7 and 9 (total number of ROIs was 89 and 106) for the knockout and wild type mice, respectively. (**c**) The effect of flufenamic acid (FFA, 30 μM) on intracellular calcium concentration in podocytes of wild type glomeruli. Asterisk denotes $p \leq 0.05$.

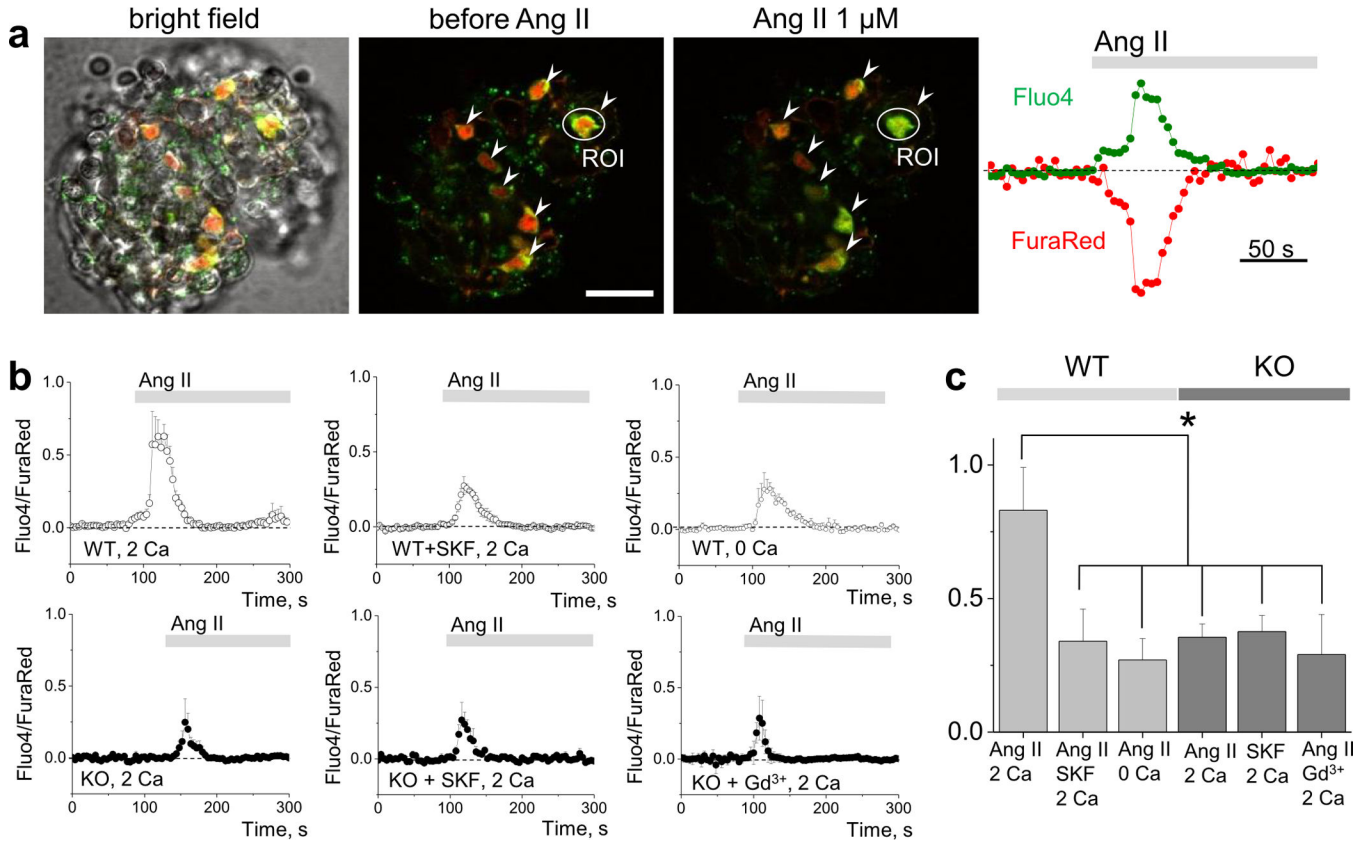


**Figure 6. Ang II-evoked calcium influx in the podocytes of the wild type and TRPC6-deficient mouse glomeruli**

(**a**) Representative images of the wild type mouse glomeruli stained with Fluo4 (green pseudocolor) and FuraRed (red pseudocolor) (shown are bright field merged with fluorescence and fluorescence only before and after application of Ang II). ROIs (regions of interest) are marked with arrows. Right panel demonstrates separate intensity signals recorded from Fluo4 (green) and FuraRed (red) from a ROI marked with a circle. Please note the typical increase and decrease in Fluo4 and FuraRed intensities, respectively. (**b**) Upper panel shows the representative calcium transients caused by the application of 1 μM of Ang II in the podocytes of the wild type mouse glomeruli which were treated with vehicle (WT) or 1 μM of the pan-TRPC channel blocker SKF 96365 (WT + SKF) in the presence of extracellular calcium, and with 1 μM of Ang II in the calcium-free solution (WT, 0 Ca). Bottom panel illustrates the effects of Ang II on the calcium transients in the podocytes of the TRPC6$^{-/-}$ mouse glomeruli treated and not treated with SKF 95365 (KO and KO + SKF, respectfully) and glomeruli pre-incubated with 100 μM $Gd^{3+}$ (KO + Gd); (**c**) Graph demonstrating the summarized responses of the wild type or knockout podocytes of mouse glomeruli treated or not treated with SKF 96365 or $Gd^{3+}$ to 1 μM of Ang II in calcium-free or calcium-containing solution. The data were summarized from at least 8 glomeruli for each column and the responses were recorded from at least 6 different animals for each group. Scale bar shown is 30 μm. Asterisk denotes $p \leq 0.05$ compared to application of Ang II in wild type mice in calciumcontaining buffer.