

# Angiotensin II Depolarizes Podocytes in the Intact Glomerulus of the Rat

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## Abstract

The aim of this study was to examine the effects of angiotensin II (Ang II) on cellular functions of rat podocytes (pod) in the intact freshly isolated glomerulus and in culture. Membrane voltage ( $V_m$ ) and ion currents of pod were examined with the patch clamp technique in fast whole cell and whole cell nystatin configuration.  $V_m$  of pod was  $-38 \pm 1$  mV ( $n = 86$ ). Ang II led to a concentration-dependent depolarization of pod with an  $ED_{50}$  of  $10^{-8}$  mol/liter. In the presence of Ang II ( $10^{-7}$  mol/liter,  $n = 20$ ), pod depolarized by  $7 \pm 1$  mV. In an extracellular solution with a reduced  $Cl^-$  concentration of 32 mmol/liter, the effect of Ang II on  $V_m$  was significantly increased to  $14 \pm 4$  mV ( $n = 8$ ). The depolarization induced by Ang II was neither inhibited in an extracellular  $Na^+$ -free solution nor in a solution with a reduced extracellular  $Ca^{2+}$  (down to 1  $\mu$ mol/liter). Like Ang II, the calcium ionophore A23187 ( $10^{-5}$  mol/liter,  $n = 9$ ) depolarized pod by  $10 \pm 2$  mV, whereas forskolin ( $10^{-5}$  mol/liter), 8-(4-chlorophenylthio)-cAMP and N2,2'-*o*-dibutylryl-cGMP (both  $5 \times 10^{-4}$  mol/liter) did not alter  $V_m$  of pod. The angiotensin 1 receptor antagonist losartan ( $10^{-7}$  mol/liter) completely inhibited the Ang II-induced ( $10^{-7}$  mol/liter) depolarization ( $n = 5$ ). Like pod in the glomerulus, pod in short term culture depolarized in response to Ang II ( $10^{-8}$  mol/liter,  $n = 5$ ). Our results suggest that Ang II depolarizes podocytes directly by opening a  $Cl^-$  conductance. The activation of this ion conductance is mediated by an  $AT_1$  receptor and may be regulated by the intracellular  $Ca^{2+}$  activity. (*J. Clin. Invest.* 1997. 99:2772–2781.) Key words: podocytes • isolated glomeruli • angiotensin II • angiotensin 1 receptor • membrane voltage

## Introduction

Angiotensin II (Ang II)<sup>1</sup> can be locally produced in the glomerulus and it is known to cause a reduction of the ultrafiltration coefficient ( $K_f$ ), but the cellular basis for this glomerular effect remains unclear (1, 2). It has been suggested that Ang II re-

duces  $K_f$  by a contraction of mesangial cells leading to a reduction of peripheral capillary surface area, but morphometric studies have failed to support this hypothesis and it was questioned whether the contractile state of mesangial cells could affect GFR (1–3).

Besides its hemodynamic effects, Ang II is a growth hormone and contributes to the pathogenesis of glomerulosclerosis (4, 5). It is known that in contrast with other antihypertensive drugs, angiotensin-converting enzyme inhibitors ameliorate proteinuria and the progression of glomerulosclerosis in experimental and human diseases (6, 7). Concerning the influence of Ang II on proteinuria, it has been recently reported that it leads to an increase of urinary protein excretion rate and loss of glomerular size-selective functions in the rat isolated perfused kidney (8). The pathogenesis of glomerulosclerosis is not completely understood, but podocytes seem to play a major role in its initiation and progression (9). The podocyte is the most differentiated cell type in the glomerulus, which forms an important component of the glomerular filtration barrier and stabilizes the glomerular basement membrane. Its complete biological role is not understood. It has been speculated that the contractile foot of the podocyte process might not only have a static function, but it may also regulate glomerular filtration rate by changing filtration surface area and thereby  $K_f$ . It was further suggested that the contractile state of the foot processes is controlled by vasoactive hormones like Ang II (10).

On the basis of experiments with cultured glomerular epithelial cells, it has been assumed that podocytes possess receptors for various hormones such as endothelin and Ang II (11–13). However, in view of the high grade of podocyte differentiation, which does not allow cell division under physiological conditions, it is difficult to transfer the data obtained from cultured glomerular epithelial cells to the podocyte in vivo (9, 10). In addition, in the past it has been a matter of debate whether podocytes may proliferate at all and it has been difficult to determine whether glomerular epithelial cells in culture originate from the visceral or parietal epithelium (14, 15). In this regard, the morphological and immunohistochemical properties of glomerular epithelial cells in culture markedly differ from those observed in vivo (15, 16).

To come closer to the in vivo situation, we have developed an experimental approach that allows us to study podocytes in the intact glomerulus. With the help of the patch clamp technique, we have examined the effect of Ang II on membrane voltage and ion currents of podocytes in intact freshly isolated glomeruli. Our results demonstrate that it is possible to examine functional characteristics of podocytes in these intact glomeruli and suggest that Ang II alters the conductive properties of these cells.

## Methods

*Isolation and preparation of glomeruli.* Wistar rats of either sex (80–200 g body wt) were anaesthetized by injection of 100 mg/kg thiopen-

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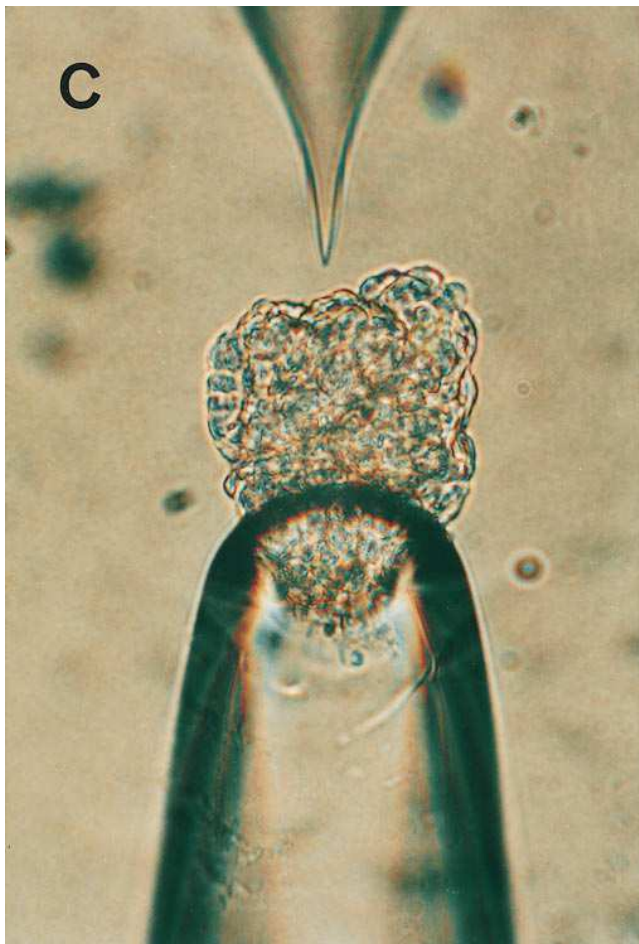
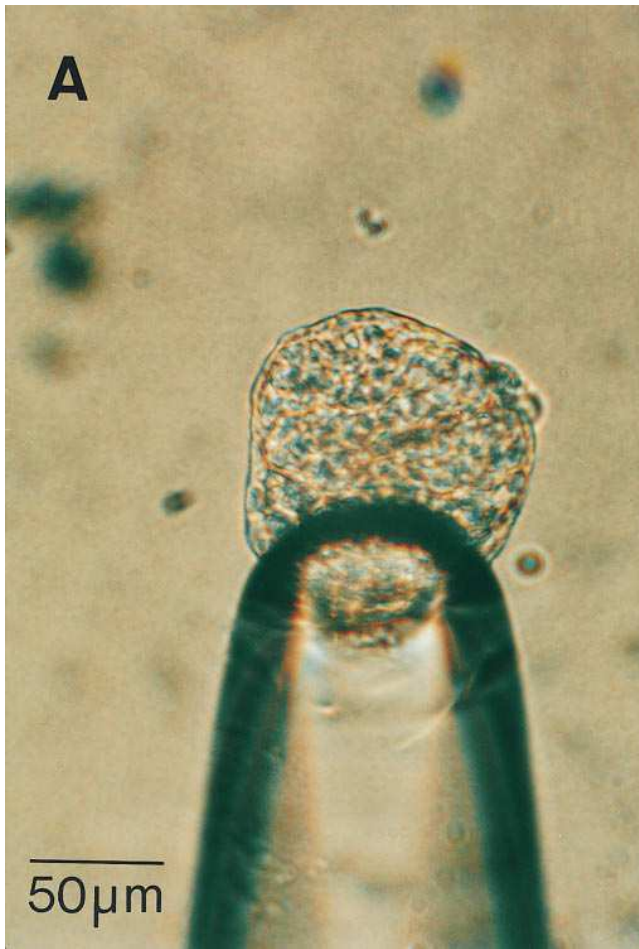
1. Abbreviations used in this paper: Ang II, angiotensin II;  $AT_1$ , angiotensin 1;  $G_m$ , cell conductance;  $V_m$ , membrane voltage.

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*Figure 1.* Preparation of a glomerulus (interference microscopy, 400 $\times$ ). (A) An isolated rat glomerulus is fixed at the vascular pole by a holding pipette. (B) Removal of the Bowman's capsule by another glass pipette after incubation with 1 g/liter collagenase IV for 1 min. (C) Decapsulated glomerulus with a patch clamp pipette.

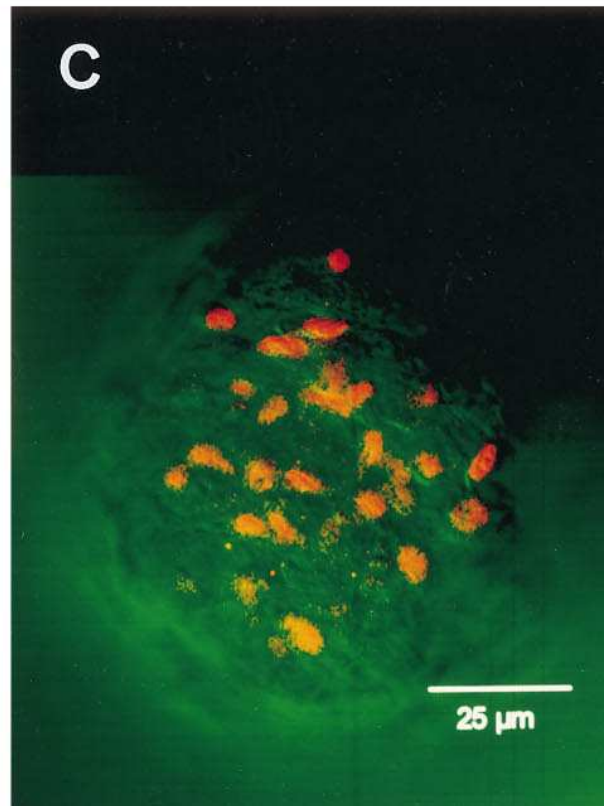
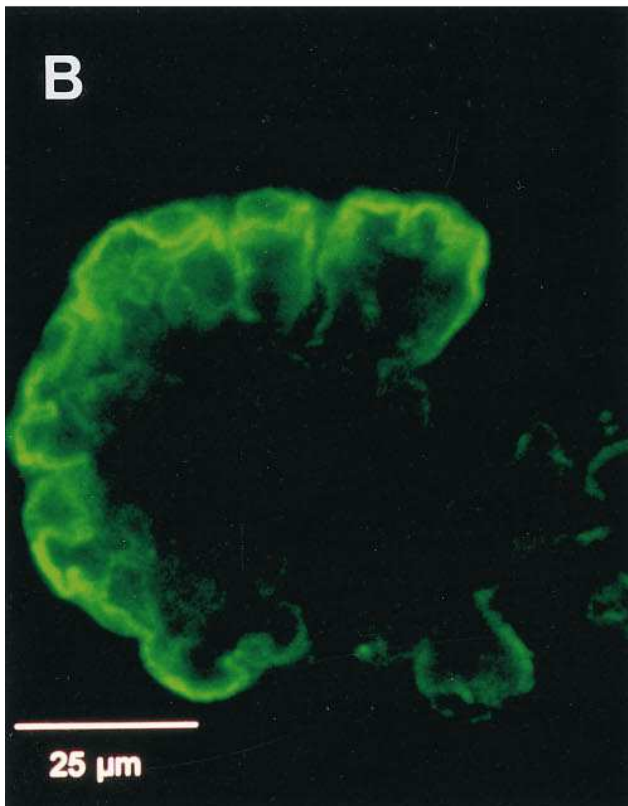
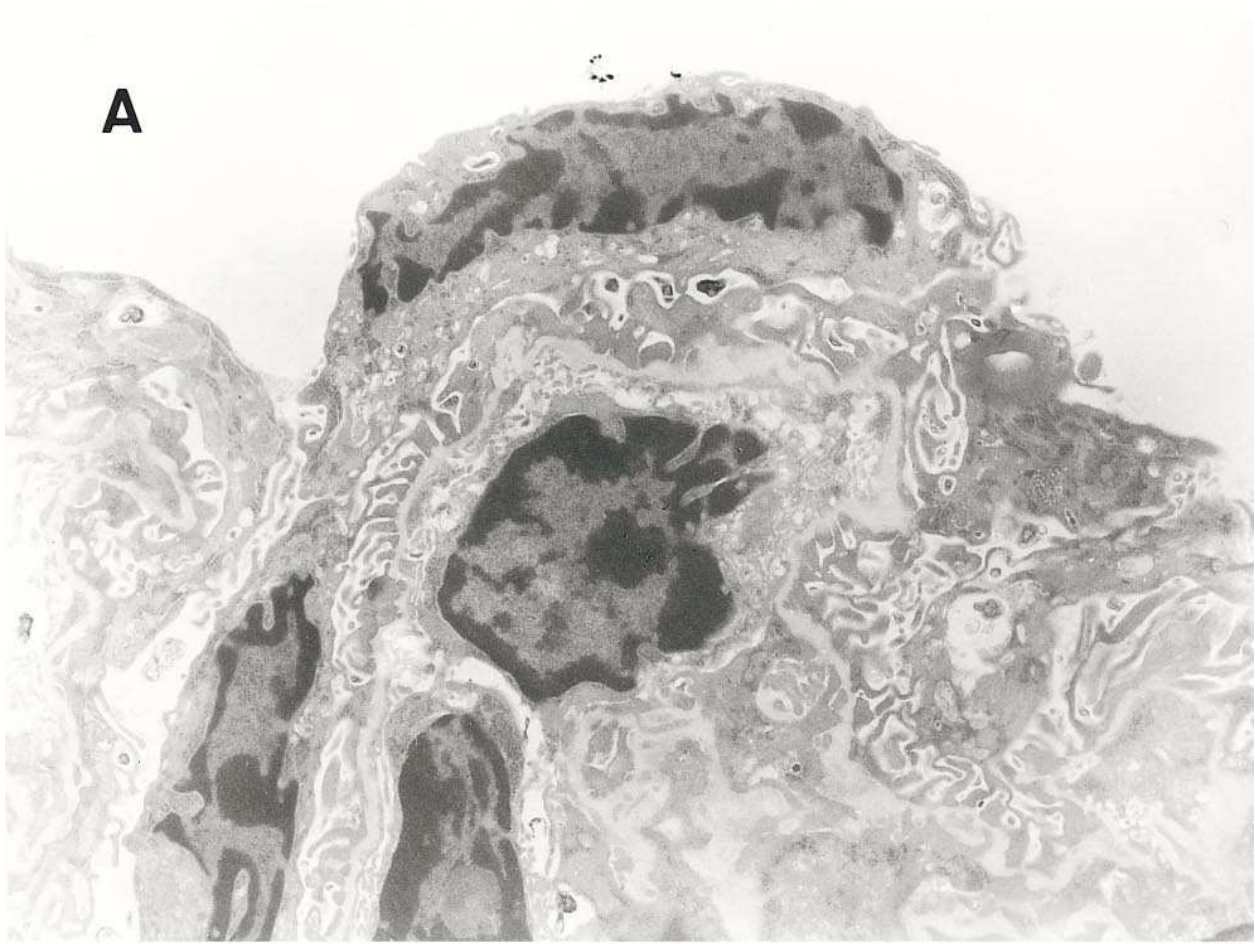


Figure 2. (A) Electron microscopy of a section of a decapsulated glomerulus showing an intact podocyte with cell body and foot processes (9,000 $\times$ ). (B and C) Fluorescence images of one section of a single glomerulus, obtained by laser scanning microscopy. (B) Positive immunoflu-

tal-Na (Byk-Gulden, Konstanz, Germany) i.p., and the glomeruli were obtained by the perfusion technique with the rat placed on its right side on a heated operating table. The abdomen was opened and the aorta was prepared under a lens (40×). The left renal artery was identified and ligated near the aorta. With microscissors, the renal artery was cut and cannulated by a polyethylene catheter of appropriate size. The kidney was perfused with a cold (4°C) Ringer-like solution containing 5 mmol/liter Na<sup>+</sup> pyruvate and 5 g/liter fetal bovine serum albumin for 1–2 min to remove blood. After clamping the vascular pole, the kidney was excised and kept at 4°C. Rat glomeruli were obtained with the sieve technique as previously described (17). Approximately one third of the glomeruli obtained in this way possessed an intact Bowman's capsule and had diameters of 70–90 μm. The isolated glomeruli were kept in a medium without serum at 37°C under a moistened atmosphere containing 95% O<sub>2</sub>/5% CO<sub>2</sub> for up to 4 h. For the experiments, a single glomerulus with intact capsule was transferred into the bath chamber mounted on the stage of an inverted microscope. The glomerulus was kept at 37°C on the bottom of the bath and immobilized at the vascular pole by a holding glass pipette (Fig. 1 A). After incubation with 1g/liter collagenase IV for 1–2 min, the capsule was stripped off mechanically with a small broken glass pipette (Fig. 1, B and C). After 1–2 min of incubation, the glomerulus was superfused continuously at 37°C with a Ringer-like solution to remove the enzyme.

**Characterization of the podocytes.** After the glomeruli were decapsulated as described above, the cell bodies of the podocytes appeared in the light microscope (400×) as round or oval structures on the surface of the glomerular capillary loops. In three experiments, the integrity of the podocytes was further examined by electron microscopy. Glomeruli were fixed in glutaraldehyde (2 g/liter) in PBS for 24 h. Embedding and preparation of sections for electron microscopy was performed as described previously (18). Electron microscopy shows that after decapsulation the capillary loops were covered by intact podocytes that were attached to the glomerular basement membrane via numerous and intact foot processes (Fig. 2 A).

**Immunofluorescence staining and confocal microscopy.** In three sets of experiments, glomeruli were incubated with specific antibodies against specific podocyte markers, WT1 and pp44 (19, 20), located in the nucleus and foot processes, respectively. After rinsing with a Ringer-like solution, the glomeruli were preincubated for 30 min in a PBS buffer containing 5 g/liter gelatin and 10 g/liter bovine serum albumin to block nonspecific protein binding. Incubation with the above primary antibodies was followed by a 30-min incubation with secondary antibodies: rabbit anti-rat IgG for anti-WT1, and rabbit anti-mouse IgG for anti-pp44. The secondary antibodies were conjugated with Cy 3 and FITC, respectively. After rinsing, the glomeruli were fixed on a glass cover slip in glycerol (400 g/liter) and sealed by a cover slip. Fluorescence images were obtained using a confocal laser scanning microscope (LSM 4; Carl Zeiss, Jena, Germany) equipped with an argon laser (488 nm excitation). All emitted fluorescence light above 508 nm was collected using a plan Neofluar lens (100×/1.3 oil immersion, Carl Zeiss) and an emission pinhole setting of 30 U, corresponding to a measured optical z-resolution of 1 μm (full width half maximum). Fluorescence images of optical sections in variable z-distances through the glomerulus were recorded and analyzed together with simultaneously recorded pseudoconfocal transmitted light differential interference contrast images. Examples are shown in

Fig. 2, B and C. The location of the immunofluorescence staining in the periphery of the glomerulus indicates the presence of podocytes in their original anatomical location on the surface of the capillary loops.

**Cell culture of podocytes.** Differentiated podocytes were cultured according to the method described by Mundel et al. (20). In brief, isolated rat glomeruli were plated on collagen I-coated dishes in RPMI 1640 medium with 100 g/liter fetal calf serum, L-glutamine (2.5 mmol/liter), Na<sup>+</sup> pyruvate (0.1 mmol/liter), nonessential amino acids (0.2 g/liter) (Seromed, Berlin, Germany), and insulin-transferrin-sodium selenite supplement 5 mg/liter (Boehringer Mannheim, Mannheim, Germany). After 5–7 d of primary culture, outgrowing glomerular cells were trypsinized with trypsin-EDTA (0.5/0.2 g/liter) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS (Seromed) and passaged over a 30-μm sieve. This maneuver removed the remaining glomerular cores consisting mainly of mesangial and endothelial cells. Only cells passing through the sieve were replated on collagen I-coated glass dishes with a diameter of 1 cm. Cells were characterized with the following antibodies: rabbit anti-rat IgG for Wilm's tumor antigen WT-1 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-mouse IgG for anti-pp44 (1:1), vimentin (1:50), desmin (1:50), cytokeratin (1:20), and Factor VIII-related antigen (1:50) for 60 min (DAKOPATTS, Hamburg, Germany). The antigen-antibody complexes were visualized with fluorochrome-conjugated secondary antibodies (FITC or Cy 3; Santa Cruz Biotechnology). Cells showed positive staining for WT-1, pp44, vimentin, and desmin, thus indicating that they possess immunological characteristics of podocytes in vivo. For the experiments, only arborized cells between days 10 and 50 after the first subcloning were examined.

**Nystatin patch clamp experiments.** The patch clamp methods used in these experiments have been described in detail in previous reports (21, 22). Decapsulated glomeruli were fixed with a holding pipette at the vascular pole (see above), kept at 37°C, and perfused with a solution containing (in mmol/liter): NaCl 145, K<sub>2</sub>HPO<sub>4</sub> 1.6, KH<sub>2</sub>PO<sub>4</sub> 0.4, MgCl<sub>2</sub> 1, Ca-gluconate 1.3, D-glucose 5, pH 7.4. The patch pipettes were filled with a solution containing (in mmol/liter): K-gluconate 95, KCl 30, Na<sub>2</sub>HPO<sub>4</sub> 4.8, NaH<sub>2</sub>PO<sub>4</sub> 1.2, Ca-gluconate 0.73, MgCl<sub>2</sub> 1.03, D-glucose 5, EGTA 1, ATP 1, pH 7.2, Ca<sup>2+</sup> activity 10<sup>-7</sup> mol/liter. The patch pipettes had input resistances of 4–8 MΩ. A flowing (10 μl/h) KCl (2.7 mol/liter) electrode was used as a reference.

After achieving a GΩ seal, the cell membrane was broken in some experiments by application of a short lasting suction via the pipette (fast whole cell configuration), and the membrane voltage (V<sub>m</sub>) was recorded continuously by a pen recorder (Gould, Seligenstadt, Germany). In most experiments, the cell was lost during this procedure. To overcome this problem, a relatively high concentration of nystatin (150 mg/liter) was added to the pipette solution in the majority of the experiments. Nystatin permeabilized the cell-attached membrane by inducing a nonselective cation conductance (reference 23; slow whole cell nystatin configuration). Due to the high concentration of nystatin, the membrane was usually permeabilized within a few minutes. The V<sub>m</sub> value was not different in either series (fast whole cell configuration, slow whole cell nystatin configuration). All experiments were performed after a control period of 3–5 min. V<sub>m</sub> and the membrane current (I) were monitored before, during, and after addition of agonists. Membrane currents were measured in the voltage clamp mode (V<sub>c</sub>) of the amplifier. V<sub>c</sub> was clamped to V<sub>m</sub> ± 40

Figure 2 legend (Continued)

orecence staining for podosynapsin (pp44), a podocyte-specific protein located in the foot processes. In this optical section of a glomerulus one can see the fluorescence (green) in the periphery of the glomerulus covering the capillary loops, where we would actually expect the foot processes of podocytes. (C) Immunofluorescence staining for WT-1, a podocyte-specific marker located in the nuclei of podocytes. Exemplary image (z-resolution of 1 μm) of a confocal z-scan series with sections taken every 15 μm. The fluorescence (yellow) in the periphery of the glomerulus strongly suggests the presence of intact podocyte cell bodies.

mV in steps of 10 mV with each step lasting for 100 ms, and current was measured to obtain estimates of cell conductance ( $G_m$ ) after correction for access conductance. In addition, membrane capacitance was estimated by the compensation circuitry of the patch clamp amplifier. In ion replacement experiments, NaCl was replaced isosmotically by the substituting ion. To vary the free  $Ca^{2+}$  activity, EGTA was added to the solution. The  $Ca^{2+}$  activity was calculated from a standard equation and was determined with a  $Ca^{2+}$  selective electrode (Radiometer, Copenhagen, Denmark).

The following reagents were used: angiotensin II, forskolin, A23187 (Sigma Chemical Co., Deisenhofen, Germany), 8-(4-chlorophenylthio)-cAMP, and N2,2'-*o*-dibutyl-*c*GMP from Boehringer (Ingelheim, Germany). Losartan was obtained from MSD (Haar, Germany).

**Statistics.** The data are given as mean values  $\pm$  SEM;  $n$  refers to the number of experiments. Paired  $t$  test was used to compare mean values within one experimental series.  $P < 0.05$  was accepted to indicate statistical significance (\* in the figures).

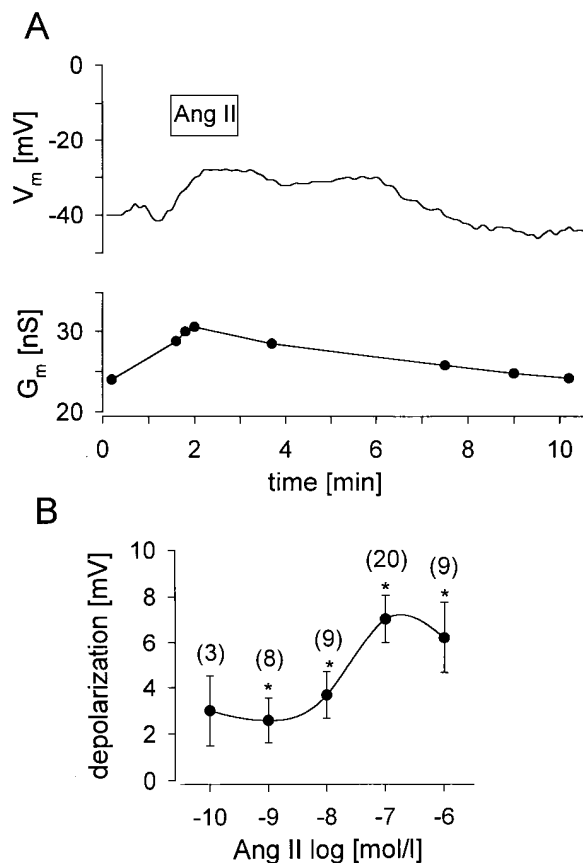
## Results

**Membrane voltage, input conductance, and membrane capacitance of podocytes.** In 86 experiments in the fast whole cell configuration or slow whole cell nystatin configuration, a stable resting  $V_m$  of  $-38 \pm 1$  mV was obtained for the control pe-

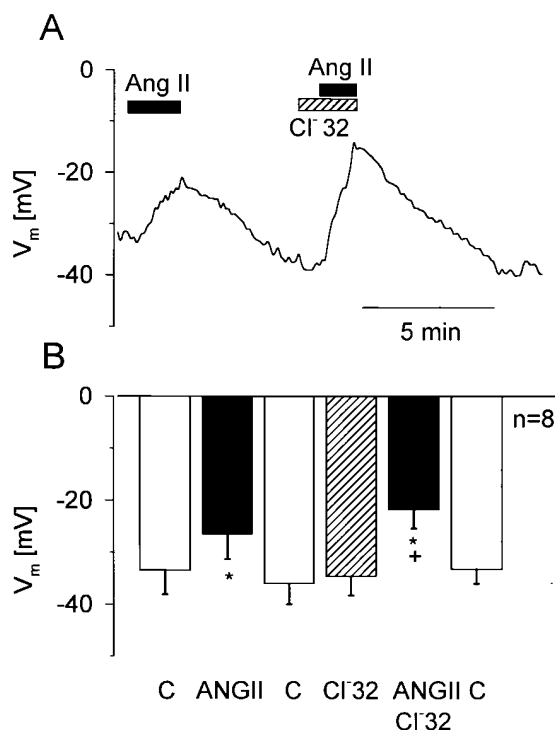
riod. After a stable  $V_m$  was reached, the podocytes had a resting membrane capacitance of  $67 \pm 4$  pF and access conductance of  $52 \pm 4$  nS ( $n = 66$ ).

**Angiotensin II depolarizes the membrane voltage of glomerular podocytes.** Addition of  $10^{-7}$  mol/liter Ang II to podocytes for 1–3 min led to a long lasting and significant depolarization from  $-36 \pm 3$  mV to  $-28 \pm 3$  mV ( $n = 20$ ). The depolarization occurred with some delay ( $22 \pm 4$  s after the agonist was given) and the maximum depolarization was reached after  $104 \pm 16$  s (Fig. 3 A). The depolarization was completely reversible and  $V_m$  returned to the control value 2–3 min after the removal of Ang II. The effect was concentration dependent with an  $EC_{50}$  of  $\sim 1.5 \cdot 10^{-8}$  mol/liter Ang II (Fig. 3 B). During the depolarization, the conductances for the in- and outward current increased significantly from  $27 \pm 3$  to  $36 \pm 5$  nS and  $25 \pm 4$  to  $31 \pm 5$  nS, respectively ( $n = 15$ ). In contrast with Ang II, addition of arginin vasopressin ( $10^{-6}$  mol/liter) for 3–5 min did not induce a depolarization of podocytes ( $n = 5$ ).

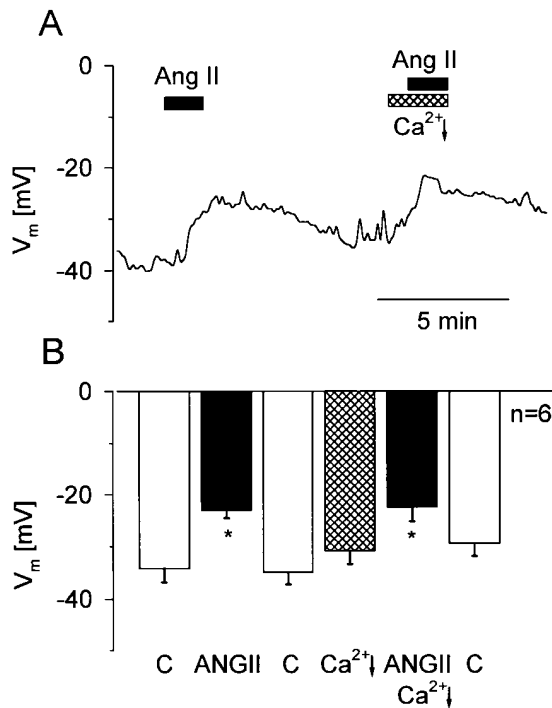
**Influence of a reduced extracellular chloride concentration on the depolarization induced by Ang II.** Under resting conditions, a reduction of the extracellular  $Cl^-$  concentration (from 147 to 32 mmol/liter) for 1 min did not significantly alter  $V_m$  or  $G_m$  of podocytes. In the presence of 32 mmol/liter  $Cl^-$ , the depolarization induced by Ang II ( $10^{-7}$  mol/liter) was significantly augmented to  $-22 \pm 4$  mV (paired experiments,  $n = 8$ , Fig. 4). Under these experimental conditions, the conductances for the in- and outward current were not significantly al-



**Figure 3.** (A) Effect of angiotensin II (Ang II,  $10^{-7}$  mol/liter) on membrane voltage and whole cell conductance of a glomerular podocyte, measured in the whole cell nystatin configuration. (B) Concentration response curve for the depolarizing effect of Ang II. Mean values  $\pm$  SEM (number of observations). \*Statistically significant depolarization.



**Figure 4.** Original recording (A) and summary (B) of the effect of a reduction of the extracellular  $Cl^-$  concentration (from 147 to 32 mmol/liter) on  $V_m$  of glomerular podocytes in the absence of the agonist and under stimulation with Ang II ( $10^{-7}$  mol/liter). Mean values  $\pm$  SEM. Note that the depolarization of  $V_m$  induced by Ang II (\*) was significantly increased (+) in the presence of a low extracellular  $Cl^-$  concentration.

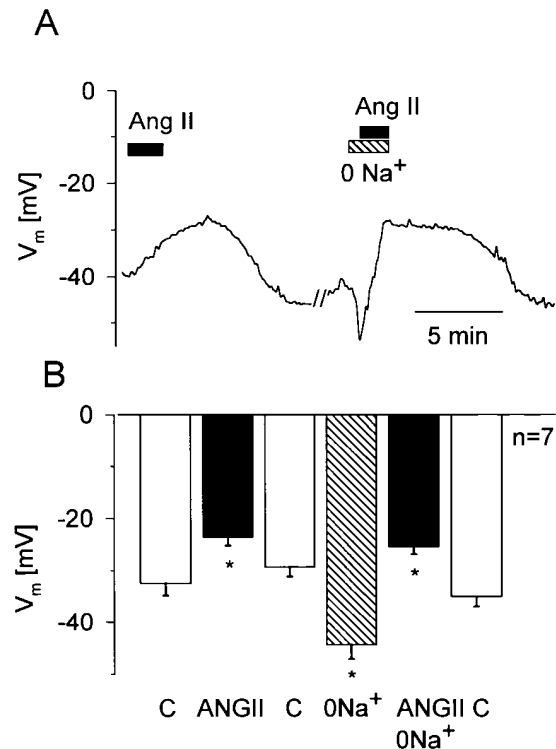


**Figure 5.** Influence of the extracellular  $\text{Ca}^{2+}$  activity ( $[\text{Ca}^{2+}]_e$ ) on the depolarization induced by Ang II ( $10^{-7}$  mol/liter). (A) Original recording. (B) Summary of six paired experiments given as mean values  $\pm$  SEM. (C) Control ( $10^{-3}$  mol/liter  $[\text{Ca}^{2+}]_e$ ),  $\text{Ca}^{2+}\downarrow$ , and cross-hatched bar =  $10^{-6}$  mol/liter  $[\text{Ca}^{2+}]_e$ . Note that Ang II depolarizes  $V_m$  of podocytes to the same extent in the presence of low extracellular  $\text{Ca}^{2+}$ . \*Statistically significant effects.

tered ( $n = 7$ ). Hence the membrane voltage change induced by Ang II followed the Nernst potential of  $\text{Cl}^-$ , suggesting that Ang II activates a  $\text{Cl}^-$  conductance in these cells.

*The  $V_m$  response to Ang II is independent of extracellular  $\text{Ca}^{2+}$  and  $\text{Na}^+$ .* To examine whether a  $\text{Ca}^{2+}$  influx may be responsible for the  $V_m$  response to Ang II, the extracellular  $\text{Ca}^{2+}$  activity was reduced from 1 mmol/liter to 1  $\mu\text{mol/liter}$ , and the effect of Ang II was examined in the presence of high (1 mmol/liter, control) and low (1  $\mu\text{mol/liter}$ ) extracellular  $\text{Ca}^{2+}$  activity. Reduction of extracellular  $\text{Ca}^{2+}$  for at least 1 min before addition of Ang II led to a small but not significant depolarization under control conditions. In the presence of low extracellular  $\text{Ca}^{2+}$  activity (1  $\mu\text{mol/liter}$ ), the depolarization by Ang II was unaltered compared with high  $\text{Ca}^{2+}$  conditions (Fig. 5). In addition, the small but significant increase of  $G_m$  under stimulation with Ang II was still observed. An original recording and the data of six paired experiments are shown in Fig. 5.

In another series of paired experiments, the influence of the extracellular  $\text{Na}^+$  concentration on the  $V_m$  response to Ang II was examined. When  $\text{Na}^+$  was removed from the extracellular bath solution (0 vs. 145 mmol/liter  $\text{Na}^+$ ) for 1–3 min, the cells hyperpolarized significantly from  $-25 \pm 2$  mV to  $-45 \pm 3$  mV ( $n = 15$ ) and the inward conductance fell significantly from  $22 \pm 4$  to  $16 \pm 2$  nS. In the absence of extracellular  $\text{Na}^+$ , Ang II ( $10^{-7}$  mol/liter) depolarized glomerular podocytes to the same value ( $-25 \pm 1$  mV vs.  $-24 \pm 2$  mV,  $n = 7$ ) as under control conditions (Fig. 6), suggesting that  $\text{Na}^+$  influx is not responsible for the depolarization under stimulation with Ang II.

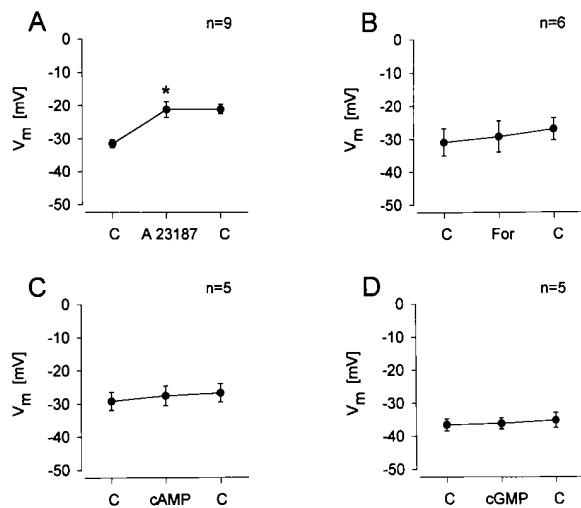


**Figure 6.** Effect of Ang II ( $10^{-7}$  mol/liter) on  $V_m$  of podocytes in the presence and absence of extracellular  $\text{Na}^+$ . (A) Original recording. (B) Summary of seven paired experiments, mean values  $\pm$  SEM. (C) Control (145 mmol/liter  $\text{Na}^+$ ), 0  $\text{Na}^+$  and hatched bar, removal of  $\text{Na}^+$  and replacement by NMDG. Removal of the extracellular  $\text{Na}^+$  leads to a hyperpolarization of these cells. In the absence of extracellular  $\text{Na}^+$ , Ang II depolarizes  $V_m$  of podocytes to the same value as under control conditions. \*Statistically significant effects.

*The calcium ionophore A23187 depolarizes  $V_m$  of glomerular podocytes by activating a  $\text{Cl}^-$  conductance.* A23187 ( $10^{-5}$  mol/liter) depolarized glomerular podocytes significantly from  $-31 \pm 1$  to  $-21 \pm 2$  mV ( $n = 9$ , Fig. 7 A). In a solution with a reduced extracellular  $\text{Cl}^-$  concentration (32 mmol/liter), the depolarization induced by A23187 was increased to  $-8 \pm 5$  mV ( $n = 3$ ). In this concentration ( $10^{-5}$  mol/liter), the depolarization induced by A23187 was sustained and not reversible. In contrast, forskolin ( $10^{-5}$  mol/liter), 8-(4-chlorophenylthio)-cAMP, and N2,2'-*o*-dibutyryl-cGMP (both  $5 \times 10^{-4}$  mol/liter, 5-min application time) did not alter  $V_m$  significantly (Fig. 7, B–D).

*Inhibition of the Ang II response by the receptor antagonist losartan.* Pretreatment of the cells with the Ang II receptor antagonist losartan ( $10^{-7}$  mol/liter) for 2–3 min completely inhibited the voltage response to Ang II ( $10^{-7}$  mol/liter,  $n = 5$ ) (Fig. 8, A and B). Ang II depolarized podocytes from  $-27 \pm 2$  mV to  $-20 \pm 2$  mV, whereas in the presence of losartan  $V_m$  was not significantly altered ( $-27 \pm 1$  vs.  $-26 \pm 1$  mV). An original recording and the summary of the data are shown in Fig. 8, A and B.

*Ang II depolarizes podocytes in culture.* Podocytes in short term culture showed positive immunofluorescence staining for WT-1, pp44, vimentin, and desmin ( $n = 8-19$ ). Fig. 9 shows podocytes in culture, positively stained for the podocyte-spe-



**Figure 7.** Summary of the effects of the calcium ionophore A23187 ( $10^{-5}$  mol/liter) (A), forskolin (*For*,  $10^{-5}$  mol/liter) (B), 8-(4-chlorophenylthio)-cAMP (*cAMP*,  $5 \times 10^{-4}$  mol/liter) (C), and N2,2'-*o*-dibutyryl-cGMP (*cGMP*,  $5 \times 10^{-4}$  mol/liter) (D) on  $V_m$  of glomerular podocytes. Mean values  $\pm$  SEM. C, control. Note that A23187 leads to a significant depolarization (\*), whereas forskolin, 8-(4-chlorophenylthio)-cAMP and N2,2'-*o*-dibutyryl-cGMP did not influence  $V_m$  significantly.

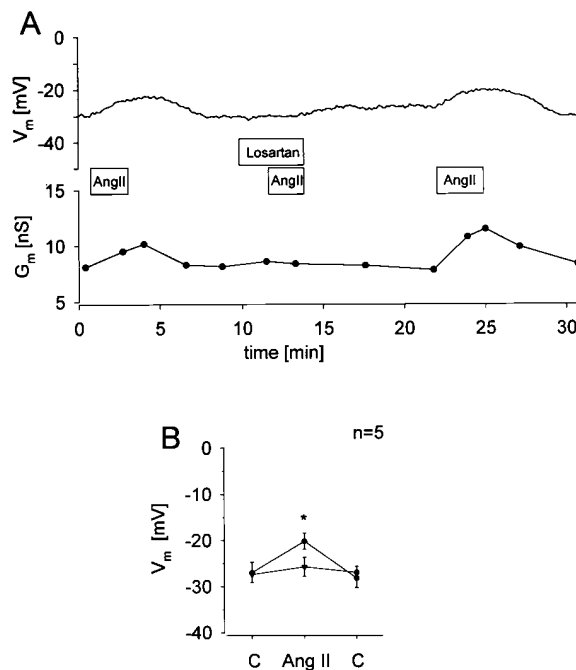
cific protein WT-1. Addition of  $10^{-8}$  mol/liter Ang II to podocytes in culture for 1–3 min resulted in a depolarization of the cells from  $-42 \pm 6$  to  $-19 \pm 4$  mV ( $n = 8$ ). In the presence of losartan ( $10^{-8}$  mol/liter), the effect of Ang II was significantly and reversibly inhibited by  $80 \pm 6\%$  ( $n = 8$ ). Fig. 10 A shows an original recording of the effect of Ang II in the absence and presence of losartan. Fig. 10 B summarizes the data.

## Discussion

Glomerular visceral epithelial cells (podocytes) contribute to the initiation and progression of several glomerular diseases. Especially membranous nephropathy, minimal change disease, and focal segmental sclerosis have been related to primary or secondary podocyte injuries (24). The biological function of podocytes *in vivo* and their contribution to glomerular injury are poorly understood.

In this study, we have developed a new method allowing examination of electrophysiological properties of podocytes within intact glomeruli. The present data suggest that Ang II directly influences the conductive properties of these cells. Glomeruli were obtained by sieving renal cortex of rat kidneys perfused *in vivo*. Compared with the microdissection, this technique yields a large number of encapsulated and decapsulated glomeruli. Only the visually intact and encapsulated glomeruli were selected for the present experiments. We used only glomeruli with intact capsules because we argued that the capillary loop architecture and the podocytes would be protected by the surrounding Bowman's capsule during the sieving procedure.

The following arguments support our conclusion that podocytes were examined in this study. (a) After decapsulation of glomeruli, cell bodies of podocytes could be visually well de-



**Figure 8.** The Ang II receptor antagonist losartan reversibly inhibits the  $V_m$  response induced by Ang II in podocytes in the intact isolated glomerulus. (A) Original experiment of the effect of Ang II ( $10^{-7}$  mol/liter) on membrane voltage ( $V_m$ ) and whole cell conductance ( $G_m$ ) of a podocyte in the absence and presence of  $10^{-7}$  mol/liter losartan. (B) Summary of these experiments. In the presence of losartan ( $\blacktriangledown$ ), Ang II has no effect. Mean values  $\pm$  SEM; \*statistical significance.

tected with differential interference contrast microscopy, and the patch pipette was carefully placed onto the cells. (b) Methodologically, it is not possible to perforate the glomerular basement membrane without occluding the patch pipette and obtaining a tight seal (e.g., from a mesangial cell). Electron microscopy and laser scanning microscopy pictures, which had been performed after the patch clamp experiments, revealed the existence of intact glomerular basement membranes and podocytes. (c) In case of tearing off podocytes with the patch pipette from the glomerular basement membrane, only the denuded membrane but no other glomerular cells could be observed. (d) The results presented here are highly reproducible. In addition, characteristic ion currents (a  $\text{Na}^+$  permeable and an inward rectifying  $\text{K}^+$  conductance), which are not present in cultured mesangial or endothelial cells, could be detected in podocytes in the intact glomerulus and in podocytes in culture (J. Gloy, unpublished observations). (e) The studies in primary cultures of podocytes revealing qualitatively similar results support the conclusion that intact and *in situ* podocytes were examined (see below).

$V_m$  and  $G_m$  of podocytes were examined with a modified nystatin patch clamp technique allowing for the simultaneous recording of  $V_m$  and ion currents (21). The  $V_m$  value of  $-38 \pm 1$  mV measured with this technique is in agreement with results from cultured human glomerular epithelial cells (25). We and others (26, 27) have previously shown that mesangial cells have a resting  $V_m$  that is more negative ( $-49 \pm 1$  mV), whereas  $V_m$  of glomerular endothelial cells is near the equilibrium po-

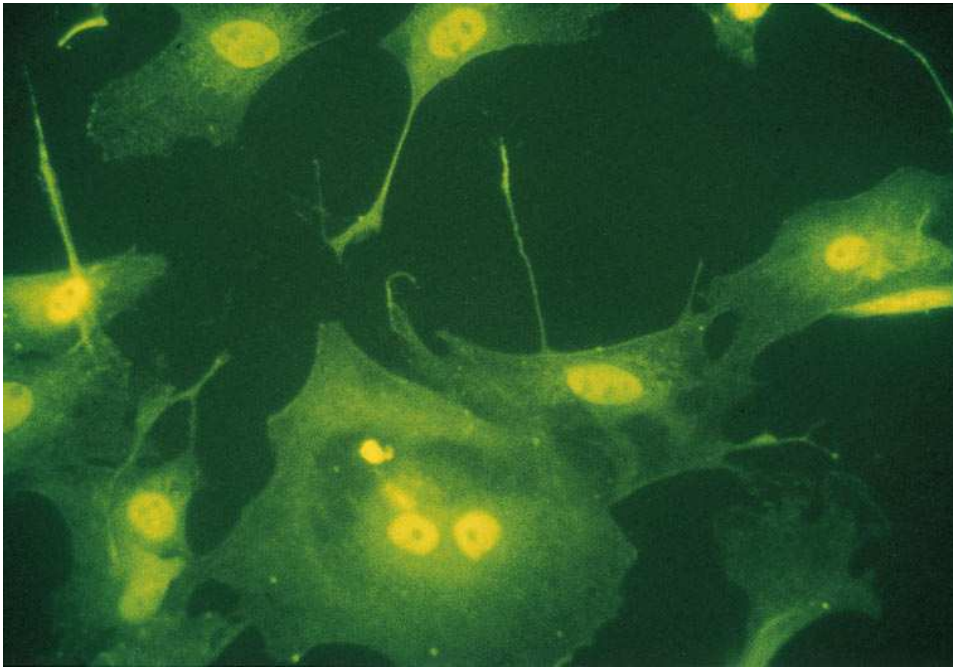


Figure 9. Rat podocytes in short term culture (320 $\times$ ): immunofluorescence staining for WT-1, a podocyte-specific protein located in the nuclei of podocytes.

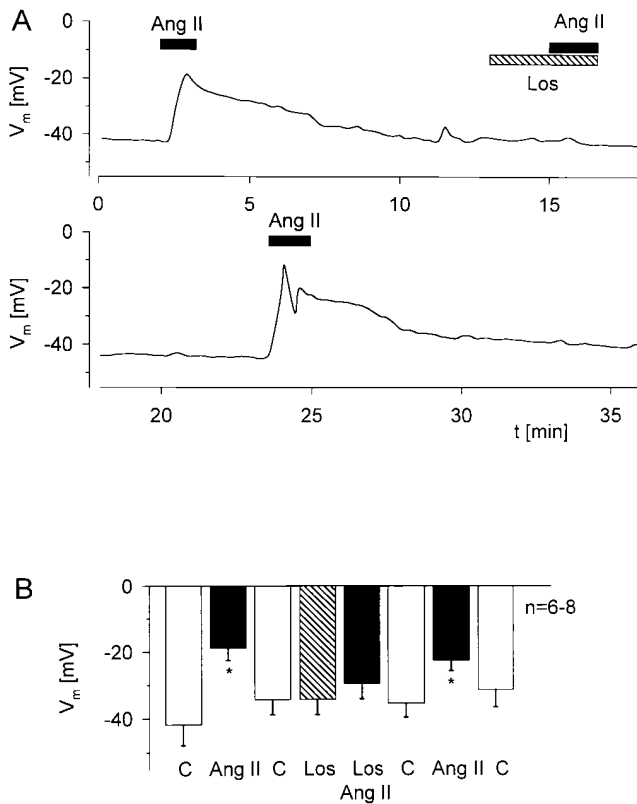


Figure 10. (A) Original recording of the effect of angiotensin II (Ang II,  $10^{-8}$  mol/liter) on membrane voltage ( $V_m$ ) of cultured podocytes in the absence and presence of losartan (Los,  $10^{-8}$  mol/liter). Note that Ang II leads to a sustained and reversible depolarization of a podocyte. In the presence of Los, the effect of Ang II was reversibly inhibited. (B) Summary of the data. \*statistical significance.

tential of  $K^+$  ( $-90$  mV; J. Gloy, unpublished results). The membrane capacitance of glomerular podocytes was  $67 \pm 4$  pF, which is substantially higher when compared with data obtained from other epithelial or mesangial cells. It probably reflects the large surface area of podocytes with their numerous interdigitating foot processes.

Ang II led to a sustained depolarization to a value that is probably close to the Nernst equilibrium of  $Cl^-$ . This is supported by the present finding that the depolarization was increased when the bath  $Cl^-$  concentration was reduced to 32 mmol/liter. Furthermore, Ang II increased the inward conductance. These three findings suggest that in glomerular podocytes the depolarization induced by Ang II is at least in part due to the activation of a  $Cl^-$  conductance. To examine the influence of other ions ( $Na^+$  and  $Ca^{2+}$ ), which might contribute to the depolarization, additional series were performed. We show that the voltage response to Ang II was not altered in the absence of  $Na^+$  or when  $Ca^{2+}$  in the bath was reduced to 1  $\mu$ mol/liter. Nevertheless, the cells hyperpolarized in the absence of  $Na^+$  and  $G_m$  fell significantly, suggesting that these cells possess a  $Na^+$  or a nonselective cation conductance. Whether this conductance is specific for  $Na^+$  or a nonselective cation conductance remains to be clarified.

The effect of Ang II on  $V_m$  of pod was not inhibited in the presence of low extracellular  $Ca^{2+}$  (1  $\mu$ mol/liter), indicating that  $Ca^{2+}$  influx is not a prerequisite for the depolarization. On the other hand, the effect of Ang II was mimicked by the  $Ca^{2+}$  ionophore A23187. Both findings can be explained by assuming that Ang II activates the  $Cl^-$  conductance by the release of  $Ca^{2+}$  from intracellular stores. The activation of other second messengers like cAMP and cGMP by Ang II does not appear likely since forskolin, 8-(4-chlorophenylthio)-cAMP, and N2,2'-o-dibutyl-*o*-cGMP did not affect  $V_m$  of glomerular podocytes. This is in contrast to findings by Sharma et al. (13),

who described an increase of cytosolic cAMP by Ang II in cultured podocytes, leading to disaggregation of actin filaments and to an alteration of the cytoskeleton, whereas atrial natriuretic peptide, via activation of cGMP, had an opposite effect. One explanation for these differences might be the fact that cAMP, if produced by Ang II in podocytes, would not alter  $V_m$ . Other explanations might include a different hormonal regulation of second messengers in cultured glomerular epithelial cells as compared with podocytes in the intact glomerulus. In this regard, in the latter study, cells had not been characterized by specific antibodies against podocytes, and they had a cobblestone appearance and stained positively for cytokeratin, whereas podocytes in the glomerulus possess an extremely differentiated cell shape and are cytokeratin negative (5, 14).

The effect of Ang II in several tissues is mediated by distinct angiotensin receptors that have been classified to angiotensin 1 and 2 ( $AT_1$  and  $AT_2$ ) receptors. It is known that Ang II increases the cytosolic  $Ca^{2+}$  activity in cells via an  $AT_1$  receptor, and this was related to the vasoconstrictive properties of Ang II. Losartan was characterized as a specific blocker of the  $AT_1$  receptor (28). In our study, losartan, in an appropriate concentration, completely inhibited the Ang II-induced depolarization of  $V_m$  of podocytes, thus indicating a specific effect of Ang II on cellular functions of podocytes via an  $AT_1$  receptor. Recently, it has been shown that a specific  $AT_1$  receptor antagonist inhibits Ang II-mediated modulation of glomerular capillary permselectivity (8).

Little is known about cellular mechanisms and actions of Ang II, the transduction pathways, and even the characterization of Ang II receptors in glomerular podocytes. In vitro autoradiography using labeled Ang II revealed a high density of Ang II binding sites in glomeruli, interpreted as a high density of Ang II receptors in mesangial cells (29). However, these data have been recently reinterpreted by Kriz et al. (10). They suggest a more widespread distribution of Ang II receptors, probably including podocytes. With respect to possible Ang II effects from the urinary space, Seikaly et al. (30) have measured Ang II concentrations in the Bowman's space of up to 1,000-fold higher than those in the vascular space and argued that this Ang II is produced by the local renin-angiotensin system.

Our present data show for the first time that Ang II influences cellular functions of podocytes in the intact glomerulus. Due to our approach, we cannot completely exclude that the depolarization induced by Ang II is indirectly influenced by some messenger or autakoid liberated from another cell type; e.g., mesangial cells. However, arginine vasopressin, which is known to modulate mesangial cell function in a similar way as Ang II (31), did not depolarize podocytes. To further examine whether Ang II may directly influence podocyte function, we tested the effect of Ang II on the membrane voltage of cultured rat podocytes. In the past there has been a debate whether glomerular epithelial cells in culture originate from visceral or parietal epithelium (15, 32). However, it has been shown very recently that podocytes with specific characteristic morphological and immunological properties can be propagated in cell culture (33). The cultured cells used in our experiments had a characteristic arborized morphology and stained positive for pp44, WT-1, desmin, and vimentin, but did not stain for cytokeratin or Factor VIII. Thus the cells expressed the immunological properties of podocytes in vivo (17, 33).

The resting membrane voltage of podocytes in culture did not differ from the membrane voltage of podocytes in the isolated glomerulus. Like podocytes in the intact glomerulus, cells in culture depolarized in the presence of Ang II. The  $AT_1$  antagonist losartan inhibited the response to Ang II, indicating that cultured podocytes also possess  $AT_1$  receptors. In comparison with the effect of Ang II on podocytes in the glomerulus, Ang II-induced depolarization of the cultured cells appeared to be stronger. This might be due to an overexpression of the  $AT_1$  receptor in the cultured cells, but subsequent studies have to clarify the exact mechanisms for this observation.

In conclusion, our results indicate that it is possible to examine the membrane voltage and conductance properties of podocytes in intact glomeruli and in cultured podocytes. They suggest that angiotensin II directly influences cellular functions of these cells. Ang II depolarizes podocytes by opening a  $Cl^-$  conductance via an  $AT_1$  receptor, and this ion conductance may be mediated by the intracellular  $Ca^{2+}$  activity.

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