Kidney International, Vol. 52 (1997), pp. 687–693

Angiotensin II increases the cytosolic calcium activity in rat podocytes in culture

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Angiotensin II increases the cytosolic calcium activity in rat podocytes in culture. In the glomerulus, angiotensin II (Ang II) reduces the ultrafiltration coefficient and enhances the filtration of macromolecules. During glomerular injury, inhibition of the renin-angiotensin system by angiotensin-converting-enzyme inhibitors reduces proteinuria and retards the progression to end-stage renal insufficiency. The mechanisms by which Ang II modulates glomerular function are still a matter of investigation. To study whether Ang II may regulate the cytosolic calcium activity ($[Ca^{2+}]_i$) in podocytes, these cells were propagated in short-term culture and the effect of Ang II was examined with the Fura-2 microfluorescence technique in single podocytes. The cellular identity of cultured podocytes was proven by the expression of WT-1 and pp44, specific antibodies against podocytes *in vivo*. Ang II led to a concentration-dependent, reversible and slow increase of $[Ca^{2+}]_i$ with an EC_{50} of 3 nmol/liter Ang II ($N = 229$). Ten nmol/liter Ang II increased $[Ca^{2+}]_i$ from 41 ± 9 to 260 ± 34 nmol/liter ($N = 210$). In a solution with an extracellular reduced Ca^{2+} concentration of 10 μmol/liter, Ang II-mediated $[Ca^{2+}]_i$ increase was significantly reduced by 60 ± 20% ($N = 12$), indicating that the $[Ca^{2+}]_i$ increase was due to a Ca^{2+} influx from the extracellular space and a release of Ca^{2+} from intracellular stores. Flufenamate, an inhibitor of non-selective ion channels, significantly inhibited Ang II-mediated increase of $[Ca^{2+}]_i$ ($IC_{50} = 20$ μmol/liter, $N = 29$), whereas the L-type Ca^{2+} channel blocker nicardipine even in high concentrations of > 1 μmol/liter had only a small inhibitory effect. The AT_1 receptor antagonist losartan inhibited Ang II-mediated $[Ca^{2+}]_i$ increase with an IC_{50} of about 0.3 nmol/liter ($N = 35$). The data suggest that Ang II increases $[Ca^{2+}]_i$ in podocytes by an influx of Ca^{2+} through non-selective channels and by a release of Ca^{2+} from intracellular stores. The effect of Ang II is mediated via an AT_1 receptor.

Angiotensin II (Ang II) plays a central role for controlling blood pressure, fluid balance and electrolyte homeostasis. In the glomerulus Ang II reduces the ultrafiltration coefficient (K_f) and enhances the filtration of macromolecules [1]. The mechanisms where by Ang II mediates these physiological functions are not completely understood. For example, it is still a matter of debate whether Ang II-induced decrease of K_f is due to a contraction of glomerular mesangial cells that leads to a reduction of capillary surface area [2–4]. Ang II modulates glomerular capillary permselectivity, and it has been recently shown that it increases urinary protein excretion rate and leads to a loss of glomerular size-selective function in the isolated perfused rat kidney. These effects of Ang II were not completely explained by an Ang II-mediated defect in glomerular size selectivity. Therefore, it has been speculated that Ang II may alter the charge selectivity of the glomerular barrier [5]. Ang II is not only a vasoactive hormone, but also acts as a growth hormone, which contributes to the pathogenesis of glomerulosclerosis [6]. The precise mechanisms responsible for the pathogenesis of glomerulosclerosis are poorly understood, but it has been related to primary or secondary podocyte injury [7, 8]. Podocytes are highly differentiated cells, which form the outer part of the filtration barrier. They possess foot processes with contractile filaments, which have been shown to counteract capillary wall distension. It has been speculated that hormone-induced contraction or dilation of podocytes foot processes regulate glomerular filtration rate (GFR) by changing filtration surface area and thereby K_f [9]. Under pathophysiological conditions, podocytes retract their foot processes resulting in a change of podocyte morphology towards a cuboidal cell type [7]. Since podocytes are involved in many physiological and pathophysiological processes within the glomerulus, studying cellular function of podocytes contributes to the understanding of the mechanisms of glomerular physiology and injury. Culturing of podocytes may be a helpful tool for studying their biological properties. However, it has been discussed controversially whether it is possible to culture podocytes and whether glomerular-derived epithelial cells are of visceral or parietal origin [10–12]. Recently, it has been shown that cultured podocytes can be induced to form processes and express pp44, which *in vivo* is only found in differentiated podocytes [13]. In this study we have propagated differentiated rat podocytes in short-term culture. We investigated the mechanisms of the effect of Ang II on the cytosolic calcium activity in podocytes.

METHODS

Cell culture

Differentiated podocytes were cultured according to the method described by Mundel, Reiser and Kriz [14]. 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^+ pyruvate 0.1 mmol/liter, nonessential amino acids (0.2

Key words: angiotensin II, cytosolic calcium, podocytes and calcium.

Received for publication January 27, 1997
and in revised form May 2, 1997
Accepted for publication May 5, 1997

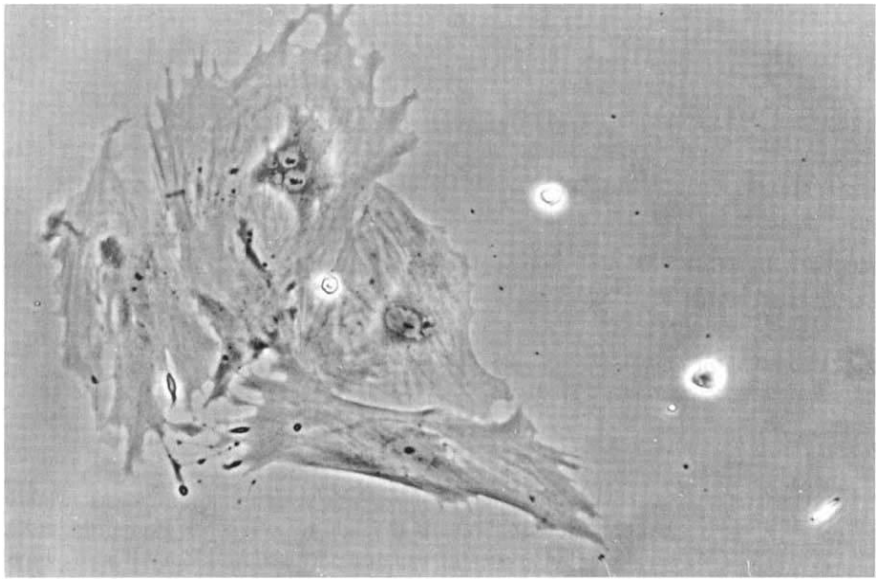

Fig. 1. Light micrograph of arborized podocytes in culture. Note that the large cell body has two nucleoli and shows multiple filament strands (×400).

g/liter; all Seromed, Berlin, Germany) and insulin-transferrin-sodium selenite supplement 5 mg/liter (Boehringer-Mannheim, Germany). After five to seven days of primary culture, outgrowing glomerular cells were trypsinized with trypsin-EDTA 0.5 g/0.2 g/liter in Ca^{2+}/Mg^{2+} free PBS (Seromed, Berlin, Germany) 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.

Immunochemical studies

Immunological characterization of cells has been performed in 19 different cell cultures. Cells were washed three times with PBS and then fixed with acetone at −20°C for five minutes or with 20 g/liter paraformaldehyde in PBS followed by permeabilization with 3 g/liter Triton X-100 in PBS. After rinsing with PBS, non-specific binding sites were blocked for 30 minutes with 20 g/liter FCS, 20 g/liter BSA, and 2 g/liter fish gelantine in PBS. Thereafter cells were incubated with the primary antibodies: anti-rat IgG for Wilm's tumor antigen WT-1 (1:100, Santa Cruz, 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 minutes (Dakopatts, Hamburg, Germany). After washing with PBS antigen-antibody complexes were visualized with fluorochrome-conjugated secondary antibodies FITC or Cy 3 (Santa Cruz, Heidelberg, Germany).

Measurements of the intracellular Ca^{2+} activity $[Ca^{2+}]_i$

For measurements of $[Ca^{2+}]_i$ podocytes were preincubated for 40 minutes with 5 μmol/liter of the Ca^{2+} sensitive dye fura-2 (Sigma, Deisenhofen, Germany). Fluorescence measurements were performed in podocytes on an inverted fluorescence microscope set-up [15]. The system allows fluorescence measurements at the single cell level at three excitation wavelengths. The field of measurement could be chosen by an adjustable pinhole between 2 and 300 μm diameter. A time resolution of up to 200 Hz was achieved using a high-speed filter wheel [15] and a single photon counting tube (Hamamatsu H63460-04, Herrsching, Germany). The autofluorescence signal of cells, which had not been loaded with Fura-2, was measured and subtracted from the results obtained in Fura-2 loaded cells. This had no effect on the bandwidth of the measurements. A calibration of the Fura-2 fluorescence signal was attempted at the end of each experiment using the Ca^{2+} ionophore ionomycin (1 μmol/liter) and low and high Ca^{2+} buffers. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio 340/380 nm according to the equation described by Grynkiewicz, Poenie and Tsien [16]. For the experiments cells between day 5 and 50 after the first subcloning were examined.

Statistics

The data are presented as mean values ± SEM; N refers to the number of experiments. Paired t-test was used to compare mean values within one experimental series. A P value ≤ 0.05 was accepted to indicate statistical significance.

RESULTS

Characterization of arborized podocytes in culture

For Fura-2 measurements only single arborized cells with the following characteristics were examined. Cells were very large (100 to 300 μm), showed multiple filament strands radiating from the perinuclear region to the cytoplasm, and were often binucleated. Figure 1 shows a micrograph of typical arborized podocytes in culture. Cells exhibited very little proliferative activity and could be cultured in a medium without supplemented serum for more than four weeks. The cellular identity was proven by the expression of Wilm's tumor antigen (WT-1, $N = 19$) and pp 44 ($N = 8$). Both antigens are only expressed by podocytes of adult kidneys [17, 18]. In addition cells stained positively for desmin and vimentin ($N = 15$ for both), but did not stain for cytokeratin or factor VIII, thus excluding tubule or endothelial cells ($N = 15$ for both). Figure 2 shows the positive staining of podocytes for pp44, an actin-associated protein of the foot processes.

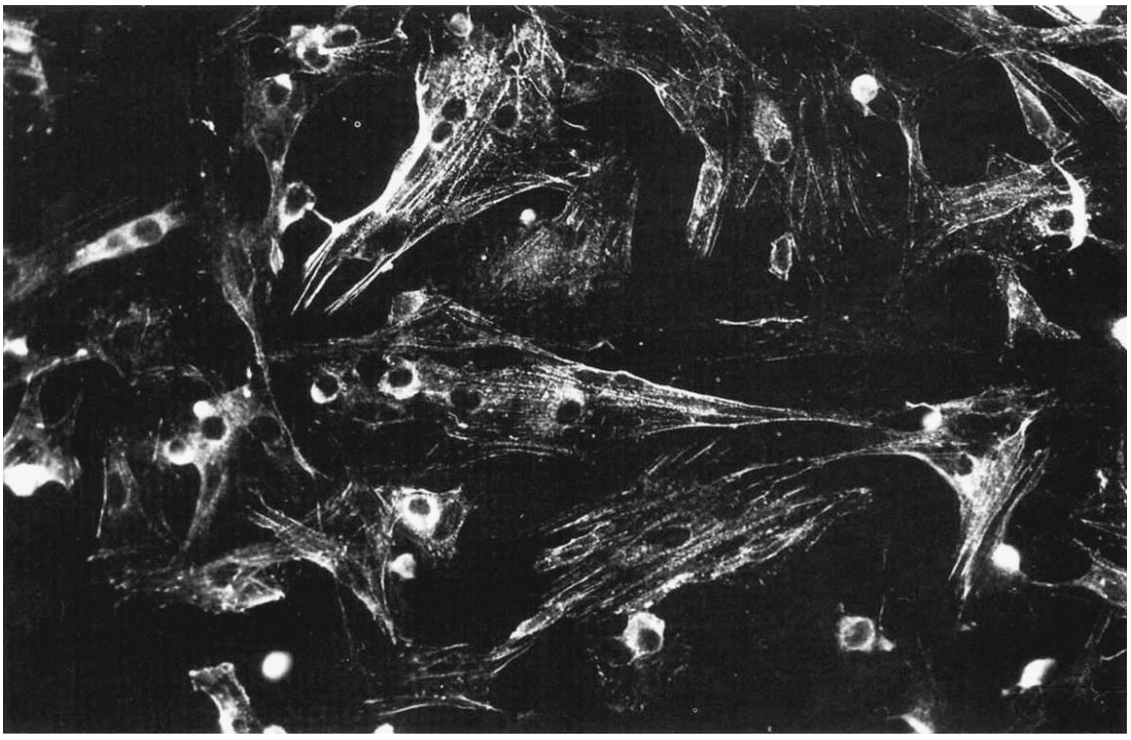

Fig. 2. Immunological characterization of cultured podocytes. Expression of pp44 in arborized podocytes. The protein is found along the actin filaments in a dotted pattern (×400).

Angiotensin II (Ang II) increased the cytosolic calcium activity $[Ca^{2+}]_i$ in podocytes

Addition of Ang II to single podocytes loaded with Fura-2 resulted in a slow and monophasic increase of $[Ca^{2+}]_i$ ($N = 229$). Ten nmol/liter Ang II increased $[Ca^{2+}]_i$ from 41 ± 9 to 260 ± 34 nmol/liter ($N = 210$). After removal of Ang II and rinsing with Ringer-like solution, $[Ca^{2+}]_i$ returned to the baseline value. Figure 3A shows a typical fluorescence recording obtained from a single podocyte exposed to 10 nmol/liter Ang II. The $[Ca^{2+}]_i$ response to Ang II was concentration-dependent with an ED_{50} of 3 nmol/liter Ang II (Fig. 3B).

In nine separate experiments podocytes cell morphology was studied by differential–interference-contrast microscopy in the absence and presence of 0.1 μmol/liter Ang II. No detectable contraction of the cells in response to Ang II could be observed (data not shown).

Reduction of extracellular Ca^{2+} inhibited the Ang II-induced $[Ca^{2+}]_i$ increase

In a solution with an extracellular reduced Ca^{2+} (10 μmol/liter) Ang II-mediated $[Ca^{2+}]_i$ increase was significantly reduced by 60 ± 10% ($N = 12$). In the presence of 1 μmol/liter extracellular Ca^{2+} the Ang II effect on $[Ca^{2+}]_i$ was reduced by 83 ± 13% ($N = 7$, data not shown). Figure 4 shows an original recording of the effect of Ang II on $[Ca^{2+}]_i$ in the presence and absence of an extracellular reduced Ca^{2+} (Fig. 4A) and the summary of 12 paired experiments (Fig. 4B).

Effect of flufenamate and nicardipine on the Ang II-induced $[Ca^{2+}]_i$ increase

Flufenamate, a blocker of non-selective ion channels [19], in paired experiments attenuated the Ang II (10 nmol/liter) mediated $[Ca^{2+}]_i$ increase in a concentration-dependent manner with an IC_{50} of 20 μmol/liter ($N = 5$ to 11). Figure 5A shows an original experiment of the effect of flufenamate (100 μmol/liter) on Ang II-mediated increase of $[Ca^{2+}]_i$. Figure 5B summarizes the data.

The L-type Ca^{2+} channel blocker nicardipine (≤ 100 nmol/liter) did not significantly inhibit the $[Ca^{2+}]_i$ increase induced by Ang II. Only higher concentrations of nicardipine (≥ 1 μmol/liter) had a slight, but significant inhibitory effect on the $[Ca^{2+}]_i$ response ($N = 4$ to 6). Figure 6 summarizes the effect of Ang II on $[Ca^{2+}]_i$ in the absence and presence of nicardipine.

Effect of Ang II on $[Ca^{2+}]_i$ in podocytes is mediated by an AT_1 receptor

Losartan, a specific AT_1 receptor antagonist, inhibited the Ang II-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner with an IC_{50} of about 3 nmol/liter ($N = 4$ to 10). Figure 7 shows an original recording of the effect of Ang II on $[Ca^{2+}]_i$ in the absence and presence of losartan (Fig. 7A) and the concentration response curve for the inhibitory effect of losartan (Fig. 7B).

DISCUSSION

Podocytes or glomerular visceral epithelial cells have been regarded as passive bystanders in the glomerular filtration process. However, recent studies point to an active role of podocytes

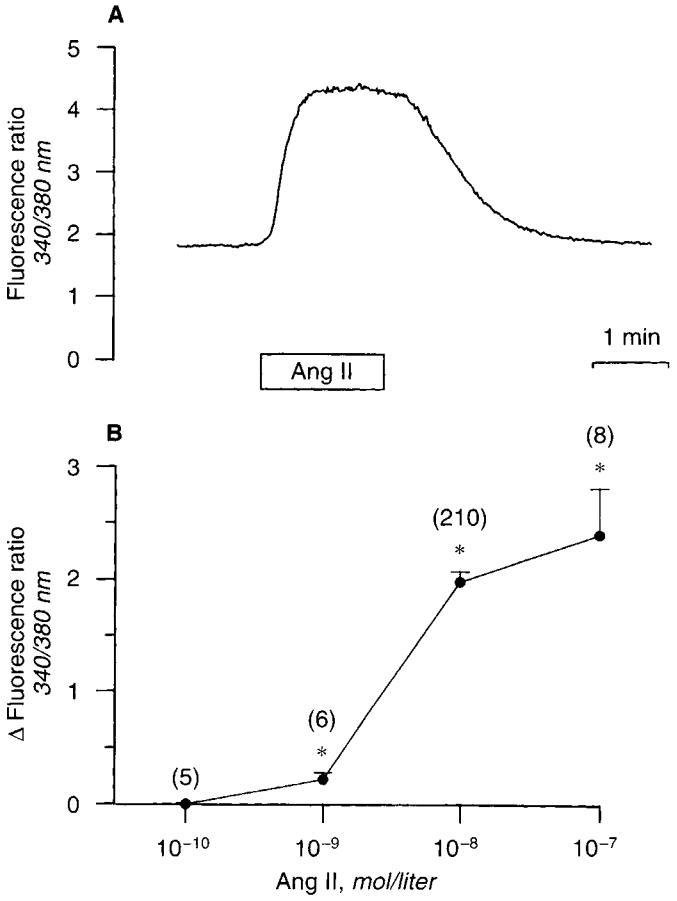

Fig. 3. Angiotensin II (Ang II) increased the cytosolic calcium activity ($[Ca^{2+}]_i$) in podocytes. (*A*) Typical microfluorescence recording of the effect of Ang II (10 nmol/liter) on $[Ca^{2+}]_i$ in a Fura-2-loaded podocyte. The fluorescence ratio with 340/380 nm excitation is shown as a function of time. Note that Ang II induced a monophasic and slow increase of $[Ca^{2+}]_i$. (*B*) Concentration response curve of Ang II-induced increase of $[Ca^{2+}]_i$. Numbers of observations are shown in parentheses. *Significant differences between control and peak values.

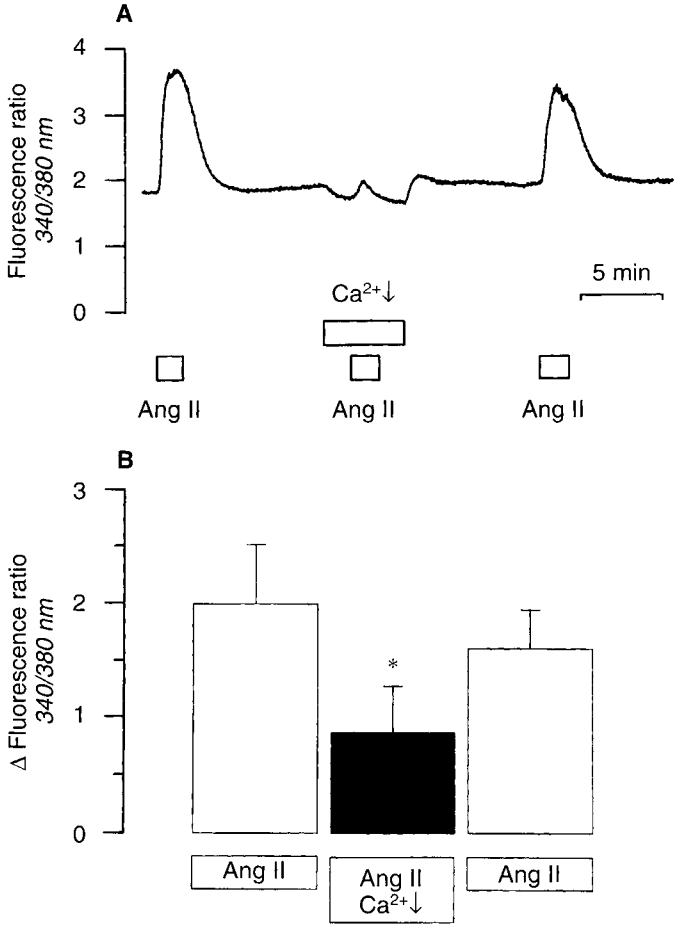

Fig. 4. Influence of a reduced extracellular Ca^{2+} on the angiotensin II (Ang II)-mediated $[Ca^{2+}]_i$ increase. (*A*) Original recording showing the effect of Ang II (10 nmol/liter) in the presence of low extracellular Ca^{2+} (10 μmol/liter). (*B*) Summary of the experiments. *Significant differences between control and peak values.

in glomerular function and disease [20]. It has been speculated that the contractile foot process of the podocyte might not only have a static function, but also, by contraction or dilation, regulate the glomerular filtration rate (GFR) by changing filtration surface area and thereby K_f [9]. In addition, the podocyte is the target cell of several glomerular diseases. Membraneous nephropathy, minimal change disease and focal segmental sclerosis have all been related to primary or secondary podocyte injury. The details of the regulation of biological functions of podocytes and their contribution to glomerular injury are poorly understood [8]. Most of the knowledge concerning the biology of podocytes is based on results obtained from cultured glomerular epithelial cells. However, in the past the origin and characteristics of cultured glomerular epithelial cells may have been looked at too simplistically. It has been assumed that the podocyte, which is a highly differentiated polygonally shaped cell *in vivo*, shows a cobblestone-like appearance under culture conditions [12, 21]. Very recently it has been reported that two different phenotypes of podocytes are present in cell culture: an undifferentiated phenotype with a cobblestone-like appearance and a more differentiated phenotype with an arborized morphology [13]. Both phenotypes stain positively for Wilm's tumor antigen, whereas only the arborized cell type expresses pp44, a specific marker for podocytes' foot processes *in vivo* [14]. There is a great body of evidence that the cells examined in this study were of visceral origin. They had a characteristic arborized appearance, were often binucleated and had a very low proliferative capacity. Cells with identical morphological characteristics have been identified by Nørgaard for the first time and it has been suggested that these cells are derived from the visceral epithelium [22]. Not only the morphological appearance, but also the immunological characteristics indicate that the cells studied here were of visceral origin: they stained positively for pp44, WT-1, desmin and vimentin, but did not stain for cytokeratin and factor VIII. Thus, the cells express the immunological properties of podocytes *in vivo* [13].

It has been reported that angiotensin II binds to glomeruli, mainly to mesangial cells but not to podocytes [23, 24]. However, these data have been recently reinterpreted by Kriz et al [9]. They suggest a more widespread distribution of Ang II receptors, probably including podocytes. In addition, very recently we have examined the effects of Ang II on cellular functions of rat

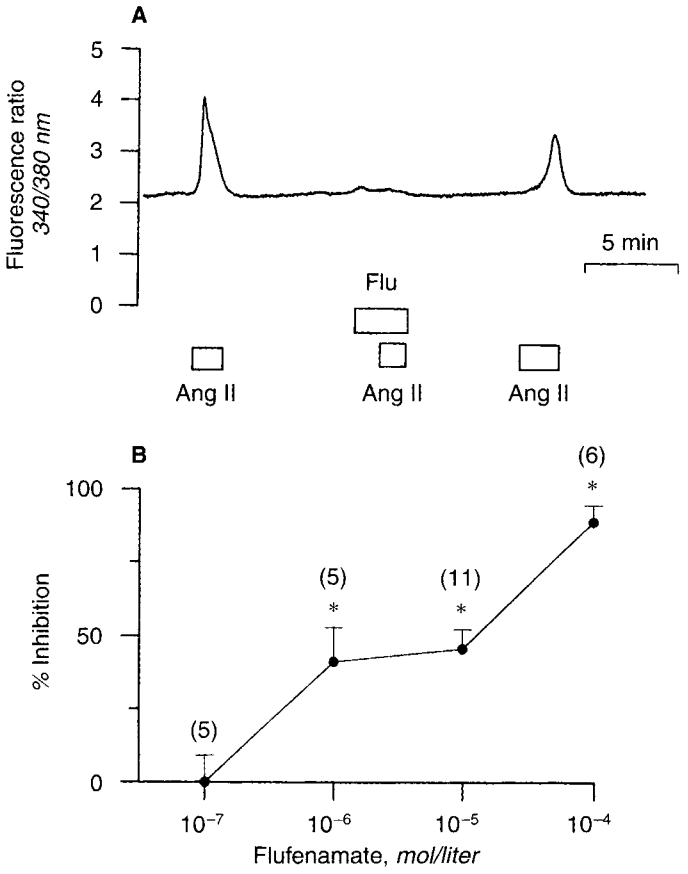

Fig. 5. Flufenamate (Flu) inhibited the angiotensin II (Ang II)-mediated $[Ca^{2+}]_i$ increase in podocytes. (*A*) Original recording showing the effect of Flu (100 μmol/liter) on Ang II (10 nmol/liter)-mediated $[Ca^{2+}]_i$ response. (*B*) Concentration response curve for the inhibitory effect of Flu. Numbers of observations are shown in parentheses. *Significant differences between the effect of Ang II in the presence or absence of Flu.

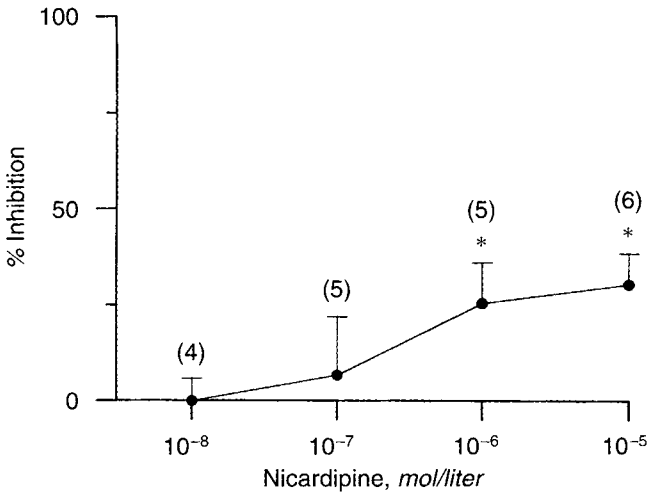

Fig. 6. Effect of nicardipine on the $[Ca^{2+}]_i$ response to angiotensin II (Ang II). Concentration response curve for the effect of nicardipine. *N* is shown in parentheses. *Significant differences between the effect of Ang II in the presence or absence of nicardipine.

podocytes in the intact freshly isolated glomerulus [25]. After stripping of the Bowman capsule, membrane voltage and ion currents of podocytes have been examined with the patch clamp technique. Angiotensin II led to a concentration-dependent depolarization of podocytes, indicating that Ang II might also influence podocyte function in the intact glomerulus [25].

So far, no data are available about cellular mechanisms and actions of Ang II, the transduction pathways and the characterization of Ang II receptors in differentiated podocytes *in vitro*. In long-term cultured glomerular epithelial cells with a cobblestone appearance an Ang II-induced and cAMP-dependent actin aggregation have been reported [26]. The Fura-2 method used in the present study allowed us to examine the effect of Ang II on $[Ca^{2+}]_i$ in single podocytes with an arborized morphology. The data show that Ang II produced a monophasic and slow increase of $[Ca^{2+}]_i$ to a maximal peak value in podocytes. The pattern of the $[Ca^{2+}]_i$ response to Ang II in podocytes differs from that reported in mesangial cells. In rat mesangial cells Ang II induces a biphasic $[Ca^{2+}]_i$ increase with a fast transient peak and a sustained plateau [27]. Furthermore, hydrogen peroxide (H_2O_2), which increases $[Ca^{2+}]_i$ in mesangial cells [28], did not induce a $[Ca^{2+}]_i$ increase in arborized podocytes even at high concentrations of 100 μmol/liter (Pavenstädt, unpublished observations). The data indicate that in comparison to mesangial cells podocytes possess different mechanisms for the regulation of $[Ca^{2+}]_i$.

The Ang II-mediated sustained $[Ca^{2+}]_i$ peak in podocytes was significantly reduced in a solution with an extracellular lowered Ca^{2+}. Only a small but significant increase of $[Ca^{2+}]_i$ by Ang II was observed under these experimental conditions. This indicates that Ang II-mediated increase of $[Ca^{2+}]_i$ is largely due to a Ca^{2+} influx from the extracellular space, but that Ang II is also able to release Ca^{2+} from intracellular stores. To determine whether the Ca^{2+} influx induced by Ang II was due to activation of L-type voltage-dependent Ca^{2+} channels, $[Ca^{2+}]_i$ increase by Ang II was examined in the absence and presence of nicardipine. Only high and not physiological concentrations of nicardipine slightly attenuated Ang II-induced $[Ca^{2+}]_i$ increase in podocytes. Thus, it seems unlikely that a L-type voltage-dependent Ca^{2+} channel is activated by Ang II in podocytes in culture. However, it should be considered that the receptor activity regulating the L-type Ca^{2+} channel may be down-regulated *in vitro* and therefore not elicit the same response as *in vivo*.

To examine whether a non-selective ion channel may be involved in the Ang II-mediated $[Ca^{2+}]_i$ response, experiments with flufenamate, a blocker of non-selective ion channels were performed [19]. In the presence of flufenamate a concentration-dependent and reversible inhibition of Ang II-mediated $[Ca^{2+}]_i$ increase was observed, suggesting that Ang II-mediated $[Ca^{2+}]_i$ increase in podocytes is due to an opening of non-selective ion channels.

Losartan has been demonstrated as a selective blocker of the AT_1 receptor in many organs and tissues [29]. The Ang II-stimulated rise of $[Ca^{2+}]_i$ was reversibly inhibited by losartan, indicating that the $[Ca^{2+}]_i$ response to Ang II was mediated by an AT_1 receptor.

In this study for the first time functional properties of cultured arborized podocytes have been examined. The results strongly indicate that Ang II enhances $[Ca^{2+}]_i$ an important second messenger of many cellular functions in cultured rat podocytes. It

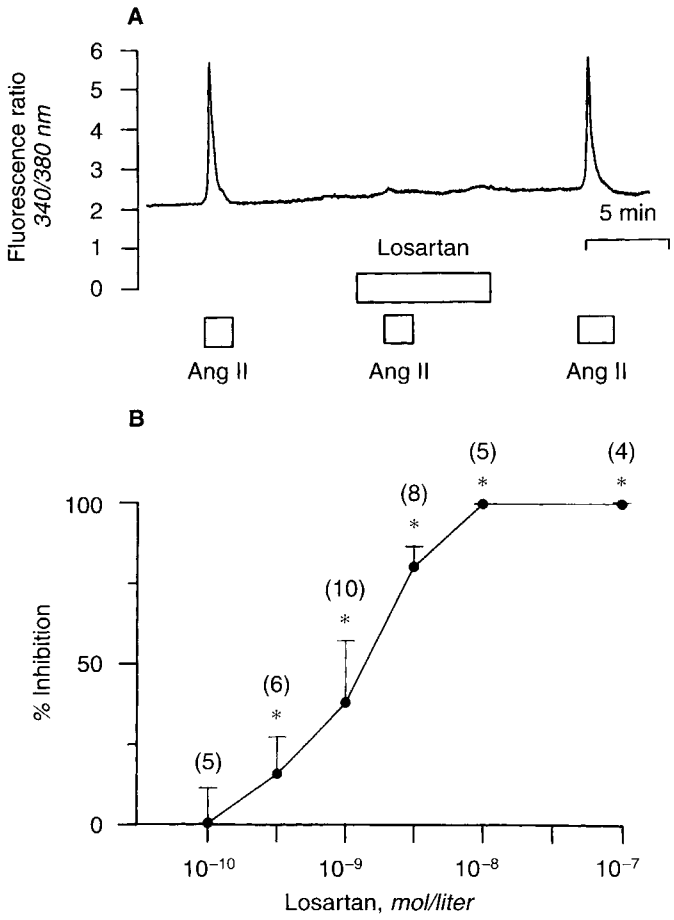

Fig. 7. The angiotensin II (Ang II)-mediated $[Ca^{2+}]_i$ increase in podocytes was mediated by an AT_1 receptor. (*A*) Original recording of the effect of the AT_1 receptor antagonist losartan on Ang II (10 nmol/liter)-mediated $[Ca^{2+}]_i$ response in a single podocyte. (*B*) Concentration response curve of the effect of losartan. Numbers of observations are shown in parentheses. *Significant differences between the effect of Ang II in the presence or absence of losartan.

is still a matter of debate whether Ang II-mediated reduction of K_f is a consequence of mesangial cell contraction [4, 9]. Our observations support the idea that podocytes may be involved in the glomerular actions of Ang II. However, an extrapolation of the present results to the *in vivo* situation should be made with some caution. It is well known that many cell types change their functional properties during cell culture. Although the immunological properties of the examined cells in culture fit very well to the immunological properties of podocytes *in vivo*, functional changes of the highly differentiated podocyte probably occur during cell culture [13, 30]. In this regard, the failure of podocytes in culture to contract in the presence to Ang II in this study should be interpreted cautiously. *In vitro* changes of cell morphology are a poor indicator of functionality and also depend on several cell culture conditions such as type of coating of culture dishes. At present we do not know whether podocytes in culture can be induced to build up functional intact foot processes and slit membranes at all. The signal transduction mechanisms induced by Ang II may not contract the podocyte's cell body, but may influence the contractile state of foot processes and thereby influence the width of the slit membranes. Further studies using imaging techniques with higher optical resolution and computer assisted analysis of the morphology of the podocyte in response to Ang II will be needed to clarify this issue.

In summary, angiotensin II increases $[Ca^{2+}]_i$ in rat podocytes in culture via an AT_1 receptor. The increase in $[Ca^{2+}]_i$ is mainly due to an Ang II-stimulated influx of Ca^{2+} from the extracellular space. The Ca^{2+} influx is probably not mediated by an L-type-Ca^{2+} channel, but is due to an opening of a non-selective, flufenamate-sensitive channel. An angiotensin II-mediated cell contraction could not be detected in podocytes in culture.

ACKNOWLEDGMENTS

Losartan was kindly donated by MSD Sharp & Dohme GMBH, Haar, Germany. This study was supported by DFG Pa 483/1-2 and Gr 480/11.

Reprint requests to Priv. Doz. Dr. H. Pavenstädt, Department of Medicine, Division of Nephrology, Hugstetterstr. 55, D-79106 Freiburg, Germany.

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