Role of protein kinase C in renal vasoconstriction caused by angiotensin II

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SCHOLZ, HOLGER, AND ARMIN KURTZ. *Role of protein kinase C in renal vasoconstriction caused by angiotensin II.* Am. J. Physiol. 259 (Cell Physiol. 28): C421–C426, 1990.—In this study we have examined the subcellar pathways along which angiotensin II (ANG II) causes renal vasoconstriction. Using the isolated perfused rat kidney model, we found that renal vasoconstriction produced by ANG II (100 pM) was not altered by the calmodulin antagonists calmidazolium (1 μM) and *N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide (W-7, 10 μM) but was blunted by staurosporine (100 nM) and 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H-7, 50 μM), two structurally distinct putative protein kinase C inhibitors. The phorbol ester 4α-phorbol 12,13-didecanoate (1–100 nM) did not alter renal vascular resistance, whereas phorbol 12-myristate 13-acetate (PMA, 1–100 nM) caused potent and dose-dependent vasoconstriction that was prevented by staurosporine (100 nM) and H-7 (50 μM). The vasoconstrictory effects of ANG II and PMA were attenuated by the calcium channel blockers verapamil (5 μM) and nifedipine (5 μM) and were reversibly inhibited when cobaltous chloride (2 mM) was added to the perfusate. Taken together, our findings support the concept that the renal vasoconstrictory effect of ANG II is essentially mediated by protein kinase C activation, which either requires or enhances the entrance of extracellular calcium.

angiotensin II; kidney; calcium; calmodulin; calcium channels

ANGIOTENSIN II (ANG II) is known as a potent renal vasoconstrictor that acts on all parts of the arterial tree in the kidney (4). The subcellular pathways, however, along which ANG II exerts its effect on smooth muscle cells in the kidney are less understood. Some evidence suggests that the renal action of ANG II might have some special characteristics. For instance, it was shown recently that the constrictor effect of ANG II is blunted by organic calcium channel blockers in the preglomerular arteriole but not affected in the efferent arteriole (7, 12). This phenomenon raises the question about the mediation of the effect of ANG II on a cellular level.

In cultures of vascular smooth muscle cells, ANG II has been found to stimulate phospholipase C activity, thus producing inositol trisphosphate and diacylglycerol (2), which lead to calcium mobilization and activation of protein kinase C, respectively (3, 8). Moreover, ANG II enhances transmembrane calcium influx in its target cells (2, 22, 23, 38). Whether this effect is mediated by receptor-operated second messenger-activated or by voltage-gated calcium channels is still a matter of debate.

Calcium mobilization brought about by release from intracellular stores and by enhancement of transmembrane influx is thought to initiate smooth muscle cell contraction by activating myosin light chain kinase, an effect that is mediated by a calcium-calmodulin-dependent reaction (19, 32, 33). In fact, a number of studies have provided evidence that the constrictor effect of ANG II is attenuated by organic and inorganic calcium channel blockers, suggesting an important role of transmembrane calcium entry in the action of ANG II (11, 20, 26, 29).

The physiological role of protein kinase C activation in smooth muscle cell contraction is less understood. It has been found that activators of protein kinase C cause a calcium-dependent delayed contraction of several smooth muscle cell types (1, 32–34). A present concept is that protein kinase C activation is necessary for the sustained contraction induced by vasoconstrictor hormones (1, 32–34). Most of the findings, however, were obtained with preparations of isolated smooth muscle cells, and it was our interest therefore to test the importance of the beforementioned subcellular pathways for the behavior of vascular smooth cells in the intact kidney in response to ANG II.

Using the isolated rat kidney perfused at constant pressure as a model, we have therefore examined the effects of calmodulin, protein kinase C, and calcium channel inhibitors on the renal vasoconstriction induced by ANG II.

MATERIALS AND METHODS

Male SIV strain rats (250–350 g body wt) with free access to tap water and commercial pellet chow until the day of experiment were used throughout. According to the technique described by Schurek and Alt (36), isolated kidney perfusion was performed as follows: anesthesia of the animals was obtained by intraperitoneal injection of 150 mg/kg of 5-ethyl-5-(1-methylpropyl)-2-thio-barbituric acid (Inactin, Byk Gulden, Constance, FRG). The preparation was performed during artificial respiration with 25% O_2 maintained by a Harvard respirator (75 strokes/min, stroke volume 2 ml). The animals were substituted for volume loss by intermittent injections of small volumes (~0.5 ml each) of physiological saline into a catheter inserted in the jugular vein. After abdominal midline incision, the right kidney was exposed and placed in a thermostated metal chamber that was fixed at a micropuncture table. After intravenous heparin injection (2 U/g body wt, Liquemin, Roche, Basel, Switzerland), the arterial vessels branching off the abdominal aorta

were ligated. A double-barreled cannula was inserted in the aorta and placed directly at the origin of the right renal artery. Renal ischemia was avoided by starting the perfusion in situ with an initial perfusion flow rate of 8 ml/min. Thereafter, the kidney was isolated from the animal, and ~10 min later, constant perfusion pressure of 100 mmHg was established. Perfusion pressure was monitored through the inner part of the cannula (Statham transducer P10 EZ) and kept constant by means of a feedback-regulated peristaltic pump. The basic perfusion medium (200–220 ml) consisted of a Krebs-Henseleit solution containing all physiological amino acids in concentrations between 0.2 and 2.0 mM, as well as (in mM) 8.7 glucose, 0.3 pyruvate, 2.0 L-lactate, 1.0 α-ketoglutarate, 1.0 L-malate, and 6.0 urea. Thyroid hormone (T_3, 150 ng/100 ml), vasopressin 8-lysine (1 mU/100 ml), and 2 g/100 ml of bovine serum albumin were also added to the perfusate. Ampicillin (3 mg/100 ml) and flucloxacillin (3 mg/100 ml) were used as antibiotics. Hematocrit was adjusted to 10 ± 2% by the addition of three times freshly washed human erythrocytes. During the preperfusion period, before the erythrocytes were added, an in-line filter (8-μm pore size) was used to remove small particles from the perfusate. The perfusate medium was continuously dialyzed against a 20-fold volume of protein-free saline. Adequate oxygen supply to the kidney was maintained by equilibrating the dialysate with a gas mixture containing 95% oxygen-5% carbon dioxide. Perfusion flow rate was obtained from the revolutions of the peristaltic pump, which was calibrated before and after each experiment. Flow rate and perfusion pressure were monitored by a potentiometric recorder (Kipp & Zonen, Delft, The Netherlands). Stock solutions of the tested drugs (see below) were dissolved in freshly prepared perfusion medium and directly infused into the arterial limb of the perfusion circuit via a micromagnetic stirrer chamber. Flow proportional infusion rate of 2% of perfusion flow was performed by means of a peristaltic pump (2132 Microperpex, LKB, Bromma, Sweden).

Agents. Pyruvate was from Boehringer, Mannheim, FRG. Ampicillin and flucloxacillin came from Beecham, Bern, Switzerland. Glutamate, urea, and α-ketoglutarate were obtained from Merck, Darmstadt, FRG. L-Malic acid and L-lactate as sodium salts were provided by Serva, Heidelberg, FRG. Thyroid hormone was from Henning, Berlin, and L-amino acids were from Braun/Melsungen, FRG (Aminoplasmal free of carbon hydrate). Vasopressin 8-lysine was supplied by Sandoz, Basel, Switzerland. Bovine serum albumin, ANG II, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (PDD), nifedipine, *N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide (W-7), and 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H-7) were purchased from Sigma International. Calmidazolium and staurosporine were from Calbiochem. Verapamil was obtained from Knoll, Ludwigshafen, FRG.

Presentation of results. Graphs shown are precise redrawings from original traces. To indicate that redrawings were made, the data points from the original traces are also shown in Figs. 1–9. Numbers (n) in brackets given in RESULTS indicate the number of different kidneys with which a certain experiment was performed.

RESULTS

Basal flow rate through the isolated perfused rat kidney (IPRK) under our experimental conditions was 11.8 ± 1.9 (SE) ml·min^{-1}·g kidney^{-1} (n = 10).

ANG II (100 pM) elicited a prompt, reversible, and reproducible reduction of perfusate flow rate in the IPRK perfused at 100 mmHg (Fig. 1). The delay of action of ANG II in our system was <20 s (15 ± 2 s, n = 10). Initially, ANG II reduced flow rate by 9 ± 2 ml·min^{-2}·g kidney^{-1} (n = 10). After 5 min on average, flow rate was decreased to 40 ± 5% of the control value in presence of ANG II (100 pM, n = 10). Addition of calmidazolium (1 μM), a calmodulin antagonist (14), to the perfusate had no effect on basal flow rate (Fig. 2). The constrictory effect of ANG II was also unaltered by calmidazolium (Fig. 2, n = 4). W-7 (10 μM), another putative calmodulin antagonist (17), did not affect the response to ANG II (data not shown, n = 3). The protein kinase C inhibitor staurosporine (100 nM, Ref. 39) caused a slowly developing increase of renal flow rate that was dependent on the concentration used (Fig. 3, n = 3). At a concentration of 30 nM, staurosporine increased basal perfusion flow by 5 ± 1% (n = 3). Staurosporine applied at 100 nM led to an increase of basal renal flow rate by 13 ± 3% of control (n = 3). Moreover, staurosporine, inhibited the

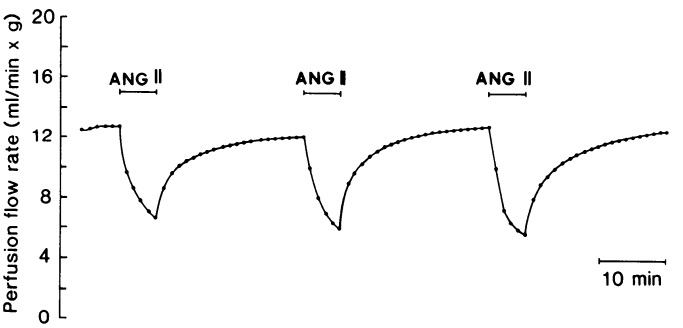

FIG. 1. Effect of angiotensin II (ANG II) on perfusate flow rate of isolated rat kidneys perfused at constant pressure (100 mmHg). Basal perfusion flow was 11.8 ± 1.9 ml·min^{-1}·g kidney^{-1} (±SE, n = 10). ANG II (100 pM) reversibly reduced renal perfusion flow rate to 40 ± 5% of control (n = 10). Note that no desensitization of vasoconstrictory effect occurred when ANG II (100 pM) was applied repeatedly at 25-min intervals.

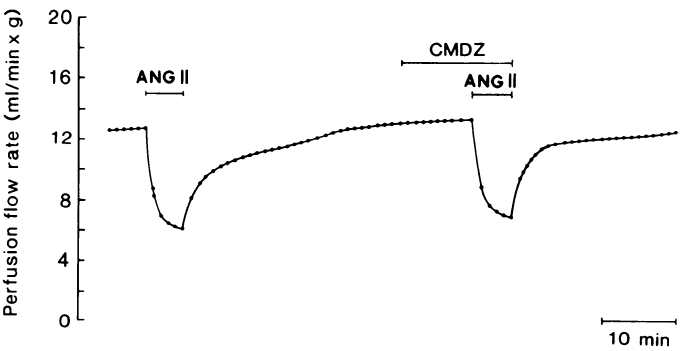

FIG. 2. Effect of calmidazolium (CMDZ) on renal flow rate and constrictory response to ANG II (100 pM). CMDZ (1 μM) affected neither basal flow rate nor vasoconstrictor effect of ANG II (n = 4).

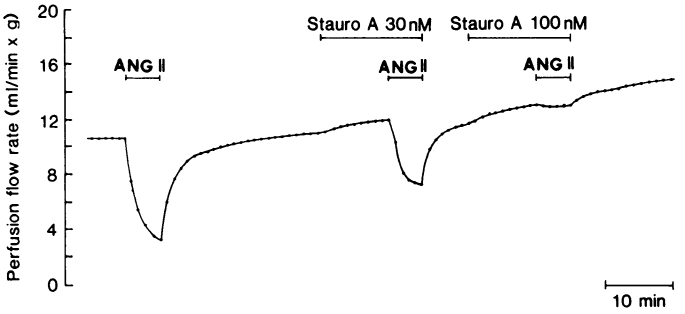

FIG. 3. Effect of staurosporine (stauro A) on basal perfusate flow rate and ANG II-induced renal vasoconstriction. Stauro A (30 and 100 nM) caused a slowly developing increase of renal flow rate of 5 ± 1 and 13 ± 3% of control, respectively (n = 3 each). Furthermore, a dose-dependent inhibition of the constrictory effect of ANG II (100 pM) was observed with a half-maximal effective dose estimated to be ~50 nM.

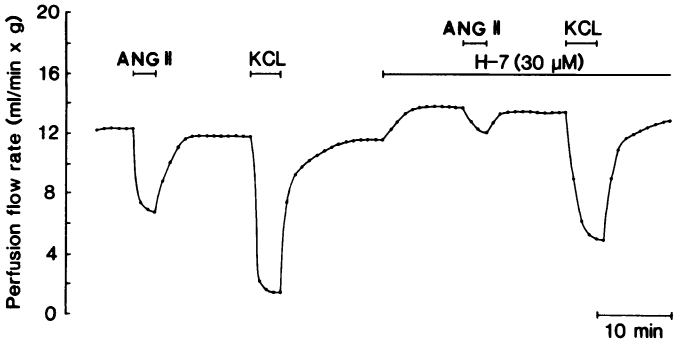

FIG. 4. Effect of 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H-7) on basal renal flow rate and constrictory response to ANG II and KCl. H-7 (30 μM) reversibly increased basal perfusion flow by 19 ± 3% of control (n = 3) and markedly attenuated ANG II-induced (100 pM) renal vasoconstriction (n = 3). In contrast, constrictor effect of KCl (30 mM) was only slightly affected by H-7 applied at concentrations of 10–50 μM (n = 3).

constrictory effect of ANG II in a dose-dependent fashion (Fig. 3). From three log (flow rate)-log (concentration) relationships, the concentration of staurosporine required to inhibit the effect of ANG II half maximally was estimated to be ~50 nM.

H-7, another putative protein kinase C inhibitor that structurally differs from staurosporine (16), also caused a dose-dependent and reversible increase of basal perfusate flow rate by 19 ± 3% of control when applied at a concentration of 30 μM (Fig. 4, n = 3). H-7, moreover, inhibited the constrictory response to ANG II (100 pM) in a concentration-dependent fashion with a half-maximal effective dose (ED_{50}) value estimated to be ~30 μM (Fig. 4).

Like with ANG II, the administration of KCl led to a prompt reduction of renal perfusion flow rate that was dose dependent and fully reversible. At a concentration of 30 mM, KCl decreased basal flow rate to 8 ± 1% of control (Fig. 4, n = 5). In contrast to ANG II, KCl-induced deceleration of flow rate was only slightly decreased after a 10-min preincubation with H-7 applied at concentrations of 10–50 μM (Fig. 4).

PMA, an activator of protein kinase C (28), caused a protracted, strong, and persisting reduction of flow rate. The deceleration of flow rate induced by PMA was concentration dependent (Fig. 5). PMA applied at concentrations of 10 and 100 nM decelerated flow rates by

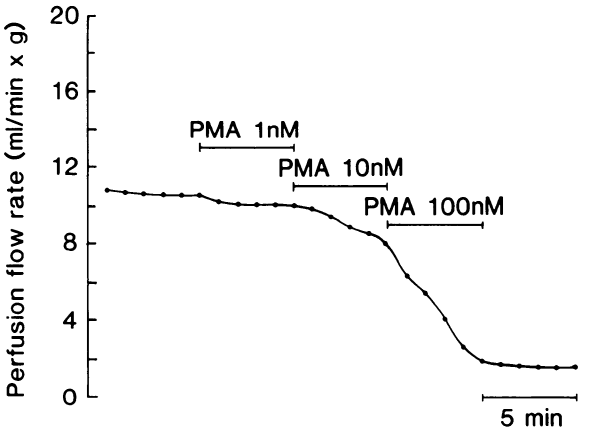

FIG. 5. Dose-dependent decrease of renal perfusate flow in response to phorbol 12-myristate 13-acetate (PMA). PMA (10 and 100 nM)-induced deceleration of flow rates was 0.5 ± 0.2 and 1.5 ± 0.1 ml·min^{-2}·g kidney^{-1}, respectively (n = 3). PMA, at a concentration of 100 nM, reduced flow rates to 15 ± 5% of control values (n = 3). Note that strong vasoconstrictory effect persisted after termination of PMA application.

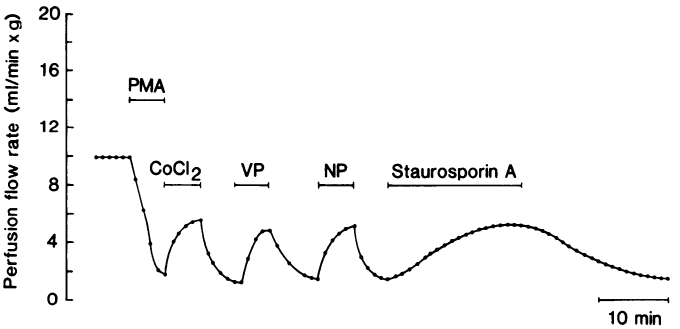

FIG. 6. Effect of cobaltous chloride, verapamil (VP), nifedipine (NP), and staurosporine (staurosporin A) on PMA-induced renal vasoconstriction. Cobalt (2 mM, n = 4), as well as calcium channel blockers VP (5 μM, n = 4) and NP (5 μM, n = 4), reversibly attenuated reduction of flow rate elicited by PMA (100 nM). In comparison, staurosporin A (100 nM) caused a more slowly developing and protracted reduction of vasoconstrictory effect of PMA (n = 3).

0.5 ± 0.2 (n = 3) and 1.5 ± 0.1 ml·min^{-2}·g kidney^{-1}, respectively (Fig. 5, n = 5). At a concentration of 100 nM, PMA decreased flow rates to 15 ± 5% of the control value (n = 5). With respect to the deceleration of flow rates, the concentration of PMA required to achieve half-maximal effect was estimated to 30 nM. Flow reduction by PMA (100 nM) was inhibited by a 10-min preincubation with staurosporine (100 nM, n = 4) and H-7 (50 μM, n = 3), respectively (data not shown). At a concentration of 100 nM, PMA caused a long-lasting reduction of blood flow that was partly reversed by staurosporine (Fig. 6). PDD, a phorbol ester inactive protein in protein kinase C stimulation (28), had no effect on the flow rate when applied in a concentration range from 1 to 100 nM (data not shown, n = 3).

Addition of cobaltous chloride (2 mM) to the perfusate significantly increased basal flow rate by 22 ± 2% (n = 4). Cobalt prevented the reduction of flow rate by ANG II (100 pM, n = 4), an effect that was fully reversible immediately after removal of cobalt (Fig. 7). Cobaltous chloride also blunted the effect of PMA (100 nM, n = 3, Fig. 8). Verapamil (5 μM) and nifedipine (5 μM) increased basal flow rates by 18 ± 6 and 21 ± 5%, respec-

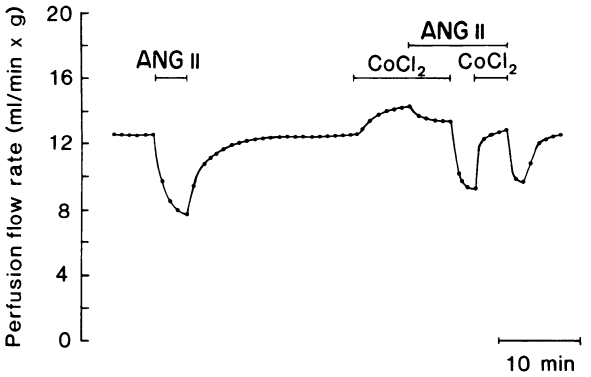

FIG. 7. Effect of cobaltous chloride on renal perfusate flow and ANG II-induced vasoconstriction. Cobalt (2 mM) caused a slight but significant increase of basal flow rate by 22 ± 2% of control (n = 4) and reversibly reduced vasoconstrictory effect of ANG II (100 nM, n = 4).

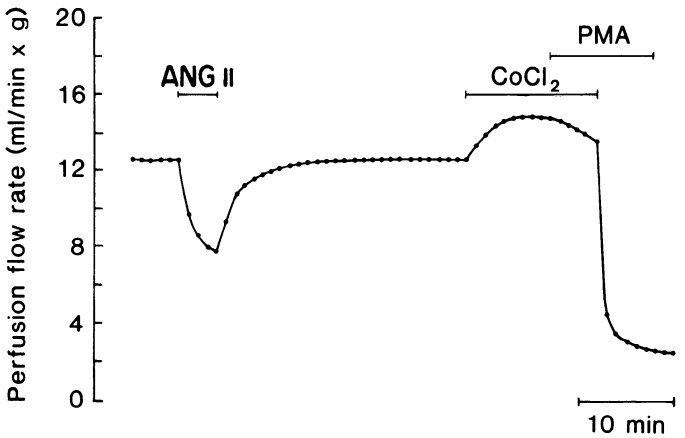

FIG. 8. Effect of cobaltous chloride on basal perfusate flow rate and PMA-induced renal vasoconstriction. Cobalt (2 mM) significantly increased basal perfusion flow rate and blunted constrictory response to PMA (100 nM, n = 3). Note that after removal of cobalt PMA elicited prompt and sustained vasoconstriction. As control, constrictory effect of ANG II (100 pM) is shown.

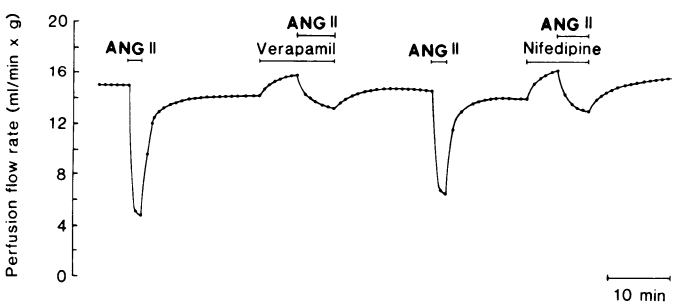

FIG. 9. Effect of calcium entry blockers verapamil and nifedipine on renal flow rate and ANG II-induced renal vasoconstriction. Verapamil (5 μM) and nifedipine (5 μM) significantly increased basal flow rates by 18 ± 6 and 21 ± 5%, respectively (n = 3 each), and reversibly attenuated reduction of perfusate flow caused by ANG II (100 pM).

tively (n = 4 for each drug). Both drugs attenuated the reduction of flow rate caused by ANG II (Fig. 9). In the presence of verapamil (5 μM) or nifedipine (5 μM), ANG II (100 pM) decreased renal perfusate flow only to 86 ± 3% of control (n = 3 each) as compared with 40 ± 5% when ANG II (100 pM) was applied alone (see above). Like cobaltous chloride, verapamil and nifedipine reversibly attenuated also the drop of flow rate induced by PMA by ~50% (47 ± 3%, Fig. 6, n = 3).

DISCUSSION

Our findings show and thus extend previous findings that ANG II is a potent vasoconstrictor in the rat kidney (4). Doubtless ANG II exerts a direct effect on vascular smooth muscle cells in the kidney (12). Recent experimental evidence, moreover, shows that ANG II also affects the sodium transport in the proximal tubule (25). As a consequence, ANG II may influence the tubuloglomerular feedback (TGF) by its tubular effect, thus acting indirectly on renal vascular resistance. Therefore the direct action of ANG II on renal vascular smooth muscle cells may be superimposed by its tubular effect. In our experimental system, we have no evidence for a functioning TGF. For instance, average sodium reabsorption is <90% of the filtered load (35). If present, TGF responses should be maximally activated under these conditions, because the sodium chloride load at the macula densa is expected to be strongly increased. Therefore, we suppose that the effects of ANG II as seen in this study are predominantly due to direct actions of ANG II on vascular smooth muscle cells in the kidney.

The calmodulin antagonists W-7 and the more specific calmidazolium were without any effect on ANG II-induced renal vasoconstriction. At the same concentrations, both drugs blunted erythropoietin formation in response to hypoxia in the same kidney model (35). Thus the concentrations of the calmodulin antagonists used were sufficient to exert cellular effects, and, in consequence, our findings suggest that either the calmodulin species in renal vascular smooth muscle is not susceptible to calmidazolium and W-7 or that a calmodulin-mediated reaction is not the dominant subcellular pathway for renal vasoconstriction induced by ANG II. On the other hand, staurosporine, a putative protein kinase C inhibitor, blunted the constrictory response to ANG II with an ED_{50} of ~50 nM. This value argues in favor that the effect of staurosporine was mediated by protein kinase C inhibition rather than by inhibition of other enzymes. Moreover, H-7, a structurally different putative inhibitor of protein kinase C also inhibited ANG II-induced renal vasoconstriction in a dose-dependent fashion with an estimated ED_{50} of ~30 μM. The specificity of the effect of H-7 was supported by the finding that the constrictory response to KCl was only slightly affected by H-7 applied in a concentration range of 10–50 μM, whereas ANG II-induced vasoconstriction was markedly attenuated. Conversely, direct stimulation of protein kinase C by active phorbol esters such as PMA caused a dose-dependent constriction, whereas phorbol esters that do not activate protein kinase C such as PDD had no effect on renal vascular resistance. A constrictory effect of phorbol esters has also been observed in other smooth muscle cell preparations (6, 30). Unlike with these findings, however, the constrictory effect of PMA in the kidney occurred with only a brief period of delay (<1 min), which could reflect a realistic time requirement for the activation of protein kinase C by phorbol esters (28).

Because it is known that ANG II stimulates protein kinase C activity in vascular smooth muscle cells, our results suggest an important role of protein kinase C activity in renal vasoconstriction induced by ANG II.

This conclusion is in good accordance with previous observations made on preparations of tracheal smooth muscles or on cultures from rat aorta (6, 30).

Although calmodulin antagonists did not alter the constrictory effect of ANG II, our findings provide evidence that ANG II requires the entrance of calcium from the extracellular space to produce renal vasoconstriction. This estimation is based on the observations that the calcium antagonists cobalt, verapamil, and nifedipine attenuated or even blunted the constrictory effect of ANG II in the kidney. Because the effects of these agents were quickly reversible, it is likely that they exerted their effects by impeding transmembrane calcium entry rather than by unspecifically blocking receptors or enzymes. This conclusion fits with a number of studies emphasizing the dependence of the renal vasoconstrictor action of ANG II on calcium entry (11, 20, 26, 29).

A more interesting finding in our study was that the vasoconstrictor effect of PMA was also inhibited by calcium antagonists. These results indicate that the constrictory effect of PMA in the kidney is, like that of ANG II, highly dependent on calcium entry. A dependency of PMA-induced vasoconstriction on calcium entry is also in good accordance with recent results obtained by Schrier and co-workers (6) in cultures of rat aortic smooth muscle cells and with a concept developed by Rasmussen et al. (32–34) that a continuous transmembrane Ca^{2+}-cycling system is involved in the smooth muscle contractile effect of protein kinase C activators (27).

One could speculate that protein kinase C activation enhances calcium influx into vascular smooth muscle cells and that this process is essentially involved in the constrictor action of ANG II. This view would be supported by the findings that PMA enhances ^{45}Ca uptake in cultures of rat renal mesangial cells (31) and rat renal juxtaglomerular cells (24), an effect that is inhibited by the calcium channel blocker verapamil. Phorbol esters have also been shown to enhance calcium uptake by rat aortic smooth muscle cells (10, 13, 15, 21).

Alternatively, it cannot be ruled out at present that renal vascular smooth muscle cells in situ have a high (basal) calcium permeability, being the prerequisite for rather than the result of protein kinase C action on contraction. If true, then, this argument should also hold for the action of ANG II. It would thus oppose the general opinion that ANG II actively enhances transmembrane calcium influx either by activating second messenger-operated calcium channels, as in renal juxtaglomerular cells (22), or by activation of voltage-gated calcium channels, resulting from the depolarizing effect of ANG II on vascular smooth muscle cells (5).

In either case, our findings suggest that the vasoconstrictor effect of ANG II in the kidney is essentially mediated by protein kinase C activation. Whether the supply with calcium, by enhancing calcium entry, is the dominant effect by which protein kinase C mediates the constrictory effect of ANG II remains to be clarified. Experimental findings on skinned vascular smooth muscle cells also indicate a direct effect of protein kinase C on the contraction process (9). Recently, it has been shown that activation of protein kinase C by tumor-promoting phorbol esters led to phosphorylation of myosin light chains in vascular and tracheal smooth muscle preparations (18, 37). Thus it seems likely that protein kinase C activation could exert at least two effects that act in concert on contraction of smooth muscle cells in the kidney.

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