Role of protein kinase C in inhibition of renin release caused by vasoconstrictors

ARMIN KURTZ, JOSEF PFEILSCHIFTER, ANGELIKA HUTTER, CHRISTIAN BÜHRLE, RAINER NOBILING, ROLAND TAUGNER, EBERHARD HACKENTHAL, AND CHRISTIAN BAUER
Physiologisches Institut, Universität Zürich-Irchel, Switzerland; Institut für Physiologie, Universität Regensburg, and I. Physiologisches Institut and Pharmakologisches Institut, Universität Heidelberg, Federal Republic of Germany

KURTZ, ARMIN, JOSEF PFEILSCHIFTER, ANGELIKA HUTTER, CHRISTIAN BÜHRLE, RAINER NOBILING, ROLAND TAUGNER, EBERHARD HACKENTHAL, AND CHRISTIAN BAUER. *Role of protein kinase C in inhibition of renin release caused by vasoconstrictors.* Am. J. Physiol. 250 (Cell Physiol. 19): C563–C571, 1986.—It was the aim of the present study to get insight into some of the intracellular mechanisms by which the vasoconstrictor hormones angiotensin II (ANG II), arginine vasopressin (AVP), and norepinephrine (NE) inhibit renin release from renal juxtaglomerular cells. To this end a primary cell culture from rat renal cortex was established that consisted of 50% juxtaglomerular cells. The cultured juxtaglomerular cells contained prominent renin granules closely resembling those in the intact kidney and responded to a number of stimuli of renin release. By using these cultures, we found that ANG II (10^{-7} M), AVP (10^{-6} M), and NE (10^{-5} M) inhibited renin release and increased the calcium permeability of the plasma membrane of the cultured cells. Both the effects on renin release and on calcium permeability could be diminished or even be abolished by the calcium channel blocker verapamil (Vp) (10^{-5} M). ANG II, AVP, and NE led to an increased formation of diacylglycerol (DAG), a well-known stimulator of protein kinase C (PKC). Moreover, a direct stimulation of PKC by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10^{-8}–10^{-6} M) also inhibited renin release and increased the calcium permeability of the cell membrane. Similar to ANG II, AVP, and NE, the effects of TPA on calcium permeability and renin release could be diminished by Vp. In conclusion, these results point toward a common mechanism by which vasoconstrictors inhibit renin release from renal juxtaglomerular cells: ANG II, AVP, and NE activate a phospholipase C, which generates DAG. A DAG-activated PKC then leads to an increased calcium permeability of the cellular plasma membrane, thereby causing a rise in the intracellular calcium concentration, which in turn inhibits renin release.

juxtaglomerular cells; angiotensin II; arginine vasopressin; norepinephrine; calcium

VASOCONSTRICTORS angiotensin II (ANG II), arginine vasopressin (AVP), and certain α-adrenergic agonists like norepinephrine (NE) are known to inhibit renin release in the kidney (cf. Ref. 17). This effect of vasoconstrictive hormones on renin release can be considered as a physiological negative feedback loop in the regulation of blood pressure (8). Concerning the mechanism by which vasoconstrictors inhibit renin release, it has been found that ANG II and AVP do so independently of their effects on renal hemodynamics (for reviews see Refs. 8, 17). Furthermore, it has been shown that ANG II and AVP are also active in inhibiting renin release from kidney slices and nonfiltering kidneys (24, 36). These results suggest that both vasoconstrictors inhibit renin release by a direct effect on the juxtaglomerular cells. Moreover, the inhibitory effect of ANG II and AVP on renin release was demonstrated to be dependent on extracellular calcium (30, 38, 39) and functioning calcium channels (30). Because an increase in the intracellular concentration of calcium is thought to inhibit renin secretion (31), it was speculated from these results that ANG II and AVP act on juxtaglomerular cells by enhancing the calcium influx, with a subsequent rise in intracellular calcium concentration ($[Ca]_i$) (12). So far, however, it has not been proven that vasoconstrictors indeed enhance calcium influx into juxtaglomerular cells, nor has evidence been provided on which mechanism facilitates the stimulation of calcium influx by vasoconstrictors.

In view of the physiological implications connected with the inhibition of renin release by vasoconstrictors, we were interested to find out whether or not a general intracellular mechanism exists by which vasoconstrictors like ANG II, AVP, and NE inhibit renin release from renal juxtaglomerular cells. By using a culture system rich in juxtaglomerular cells, we found that all three vasoconstrictors led to a subsequent stimulation of phospholipase C and protein kinase C. The increased activity of protein kinase C enhances the calcium permeability of the cell membrane, with a consecutive inhibition of renin release.

Part of this material has been presented at the 60th meeting of the German Physiological Society (18).

MATERIALS AND METHODS

Isolation of Kidneys

The kidneys were taken from juvenile male Sprague-Dawley rats (body wt 80–120 g) that had free access to

normal rat chow (Altromin) and water. Before extirpation, the kidneys were perfused in situ with 20 ml of buffer 1 (see below for composition) at a flow rate of 10 ml/min according to the method described by Curthoys and Bellemann (7). After perfusion the kidneys were removed and decapsulated under sterile conditions.

Preparation of Single Cell Suspension

The renal medulla was removed with a scalpel, and the cortex was minced into pieces of about $1 \times 1 \times 1$ mm^3. Cortical pieces from two kidneys were incubated in 50 ml of buffer 1 (without sodium citrate) supplemented with 0.25% trypsin (Sigma Chemical) and 0.1% collagenase from clostridium histolyticum (Boehringer, Mannheim, FRG) for 120 min at 37°C. During incubation, the solution was gently stirred with a magnetic bar and gassed with 5% CO_2 in air (pH 7.4). After incubation the suspension was poured through a nylon sieve (Verseidag, Kempen, FRG) with a pore size of 22 μm. Material passing the sieve was pelleted by centrifugation in a bench top centrifuge at 50 *g* for 10 min and washed two times with culture medium (see below for composition).

Cell Culture

The isolated cells were seeded in 25-cm^2 tissue flasks or in 7-cm^2 tissue dishes (Greiner, FRG) containing 5 and 1 ml culture medium, respectively. Both flasks and dishes were incubated at 37°C in a humidified atmosphere containing 20% O_2-5% Co_2-75% N_2 using O_2- and CO_2-controlled incubators (Heraeus, Hanau, FRG). If not otherwise indicated, culture media were changed every 2nd day. For determination of the cell number, cells were detached with 0.25% trypsin in calcium- and magnesium-free, Hanks' balanced salt solution (HBSS) containing 0.5 mM EDTA. Trypsin was inactivated by addition of culture medium. The cell number was determined using a Coulter counter.

Preparation of Cell Extracts for Determination of Intracellular Renin Activity

The cell extract was prepared as described by Rightsel et al. (35). In brief, cells were detached from the culture flasks with 0.25% trypsin and 0.5 mM EDTA, washed once with serum-containing medium then twice with serum-free medium, suspended in isotonic saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, and 5 μg/ml leupeptin, and then sonicated at 50 W for 15 s using a Labsonic 1510 (Braun, Melsungen, FRG).

Determination of Renin Release

Renin release in presence of agents was studied on the 2nd day of culture outside of the incubator as follows. The culture dishes were placed on a heating block at 37°C. After withdrawal of the culture medium, the cells were quickly washed two times with prewarmed buffer 2 (see below), and the dishes were filled with 2 ml of this buffer. Fifteen minutes later the agents to be tested were added in a volume of 20 μl. Immediately before, as well as 10, 20, and 30 min after, addition of the respective agent, 200-μl samples of the cell-conditioned buffer 2 were taken for determination of renin activity. The samples were subsequently centrifuged, and the supernatant was frozen and stored at −80°C until assay. The rate of renin release was calculated from the increase in renin activity in the supernatants using a linear regression program.

Renin Activity Assay

Aliquots of cell extracts and cell-conditioned buffer were incubated for 1.5 h at 37°C using plasma of bilaterally nephrectomized male rats as renin substrate. The reaction was carried out in 0.2 M sodium maleate buffer (pH 6.0) containing 5 mM PMSF, 10 mM EDTA, and 0.1% gentamycin. These conditions were shown to be selective for renin activity (35). The reaction was stopped by placing the test tubes in an ice bath (2°C), and the generated angiotensin I was determined by radioimmunoassay (Isotopen-Dienst West, Dreieich, FRG) according to Haber et al. (14).

Renin Demonstration by Tetramethylrhodamine Isothiocyanate (TRITC) Immunofluorescence

Immunofluorescence staining for renin was done in cultures on days 1, 2, 3, and 5 after seeding. The cultures were fixed in a solution of 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS: 137 mM NaCl, 2.68 mM KCl, 8.3 mM Na_2HPO_2, 1 mM $CaCl_2$, 0.45 mM $MgCl_2$). Two minutes of rinsing in DPBS were followed by a 2-min incubation in 96% ethanol at −20°C and by a further 2-min rinsing in DPBS at room temperature. After 5 min in a solution of 10% normal swine serum (NSS) and 0.1% bovine serum albumin (BSA) in DPBS, the cultures were incubated with rabbit antiserum against rat renin diluted 1:200 in a solution of 10% NSS in DPBS. After 2 min of rinsing in DPBS, an incubation followed with TRITC-conjugated goat anti-rabbit immunoglobulin G (1:500, Sigma Chemical) for 30 min. Finally, the samples were rinsed for 2 min in DPBS and mounted with glycerol containing 10% DPBS and 0.25% NaI for fluorescence microscopy. The specificity of the immunoreaction was tested with usual control procedures. All slides were examined with a Planao 1:25/0.65 objective using a Photomicroscope III (Zeiss) equipped with a III RS epi-illuminator with the following filter combination: BP 510-560/FT 580/LP 590; HPO 50 excitation lamp.

For immunocytochemical demonstration and localization with the protein A-gold (PAG) technique, pellets of 10^6 cells were fixed in 1% glutaraldehyde and embedded in Lowicryl. Ultrathin sections were immunostained for renin as described by Taugner et al. (37). Rabbit antiserum against rat renin used in these studies was a generous gift of Dr. T. Inagami.

Immunofluorescence Staining for Intermediate Filament Desmin

This procedure was done exactly as described by Osborn and Weber (29). Affinity-purified antibody against

desmin was generously provided by Dr. Mary Osborn (Göttingen).

Phosphoinositide Metabolism

After 1 day of culture, medium was removed and the cells were incubated for 24 h in phosphate-poor minimal essential medium (MEM, 50 μM P_i) containing 100 μCi/ml $^{32}P_i$ or in MEM containing 5 μCi/ml [^{3}H]glycerol. After this prelabeling period the medium was replaced by fresh medium either without (control) or with agents. Incubations were terminated by addition of ice-cold methanol-chloroform, and lipid extraction was done as described (32). The extracted ^{32}P-labeled phospholipids were separated on thin-layer plates impregnated with potassium oxalate and EDTA with a solvent system consisting of chloroform-methanol-4 N NH_4OH (45/35/10, by vol). Neutral lipids were separated using *n*-heptane-diethyl ether-acetic acid (75/25/4, by vol) as described (32). Phosholipids and neutral lipids were localized by comigration of standards followed by iodine staining and ^{32}P-labeled lipids additionally by autoradiography. The spots were scraped from the chromatographs and counted by liquid scintillation in water (Cerenkov counting) for ^{32}P-labeled lipids. [^{3}H]labeled lipids were analysed with a thin-layer chromatography (TLC)-linear analyzer LB 2821 from Berthold, München, FRG. The detection efficiency for ^{3}H was ~1%.

^{45}Ca Uptake

^{45}Ca uptake of the cultured cells in presence of agents was studied under the same experimental conditions as the renin secretion. The culture medium was substituted by the buffer two supplemented with 4 μCi/ml ^{45}Ca. The agents were added simultaneously. After 30, 60, or 120 s the buffer was withdrawn, and the cells were quickly washed with 10 × 1 ml ice-cold Krebs solution containing 10 mM $CaCl_2$. The cells were lysed by the addition of 1 ml 1 N NaOH, and the radioactivity was counted with a β-scintillation counter.

Buffers, Solutions, and Agents

Buffer 1. Hank's balanced salt solution (HBSS) was supplemented with 1 g glucose, 12.11 g sucrose, 2.2 g $NaHCO_3$, 2.6 mM glutamine, 0.84 g Na-citrate, and 10 mg/l BSA.

Buffer 2. This buffer consisted of 132 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM sodium acetate, 2 mM NaH_2PO_2, 10 mM glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, Flow), pH 7.2.

Culture medium. The culture medium consisted of RPMI-1640 (Boehringer), 25 mM HEPES (Flow), 0.66 U/l insulin from bovine pancreas (Sigma Chemical), 100 μg/ml streptomycin, 100 U/ml pencillin, and 10% fetal bovine serum (Boehringer).

Agents. All agents were purchased from Sigma Chemical. Stock solutions of ANG II (10^{-3} M) and AVP (10^{-3} M) were prepared in buffer 2 and stored at −80°C. Solutions of NE and verapamil (Vp) were prepared freshly before the experiments. A stock solution of 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 10^{-3} M) was prepared in dimethyl sulfoxide and stored at −20°C.

RESULTS

Cell Cultures

The yield of isolated cells was nearly linearly dependent on the incubation time during enzymatic dissociation. After an incubation period of 120 min the material retained by the 22-μm sieve consisted of connective tissue, individual tubules, and glomeruli. Isolated cells and cell debris passed the sieve. Cell debris was removed by repeated washing (see MATERIALS AND METHODS). The final yield was $6.0 \pm 0.1 \times 10^6$ cells per kidney (n = 8).

We found that ~5% of the seeded cells attached to the culture flask. The doubling time of the cells was ~3 days during the log phase of growth. The maximal cell density observed in 15 independent cell cultures was $\sim 5 \times 10^5$/cm^2. During the 1st wk of culture, round single cells and clonal cells growing contact inhibited were predominant. With the beginning of the 2nd wk of culture, the latter cells detached from the culture flask, and spindle-shaped, overlapping cells grew rapidly. Determination of the distribution of the intermediate filament desmin showed that ~90% of these spindle-shaped cells displayed a strong immunofluorescence for desmin.

Figure 1 shows the mean cellular renin activity, i.e., the averaged renin activity/10^6 adherent cells, during the time of culture. It is evident that the mean cellular renin activity increased with the commencement of culture, had a maximum in the 1st 5 days of culture, and decreased thereafter. In contrast to the mean cellular renin activity, the total intracellular renin activity decreased exponentially with the onset of culture. The daily decrease of renin activity amounted to ~17% of the total cellular renin activity. Significant intracellular renin activity, however, could be found until the end of the 3rd wk of culture. Passages of the primary cultures contained only small amounts of renin activity when compared with the maternal cells (max 5 ng ANG I·h^{-1}·10^6 cells^{-1}).

Light microscopic observations until the 5th day of culture showed two types of cells (Fig. 2): round, nearly spherical cells with diameters of 10–25 μm (type A, Fig. 2) and flat, spindle-shaped or polygonal cells of widely varying size (type B, Fig. 2). Frequently, the type A cells appeared to grow in close proximity to or on top of the type B cells. TRITC immunofluorescence specific for renin was observed in 80% (n = 200) of the type A cells but only 5% of the type B cells (n = 153). The ratio between cell type A and B, however, varied with the age of the cultures. The abundance of renin-containing cells was high (40–50%) during the 1st 2 days after seeding and decreased to 6% after the 5th day. The immunocytochemical localization of renin with the PAG technique at the ultrastructured level clearly demonstrated that the

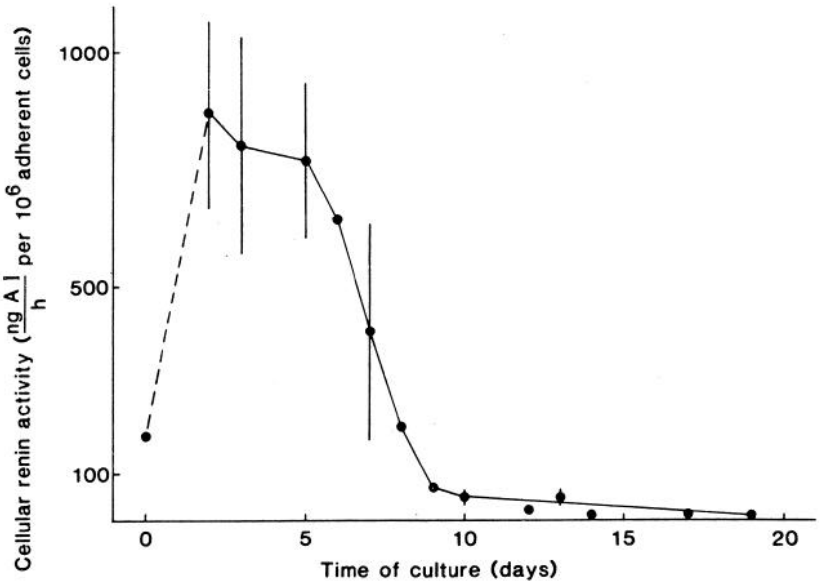

FIG. 1. Dependence of mean cellular renin activity on time of culture. Values are means ± SE of 4 independent cell culture lines. A I, angiotensin I.

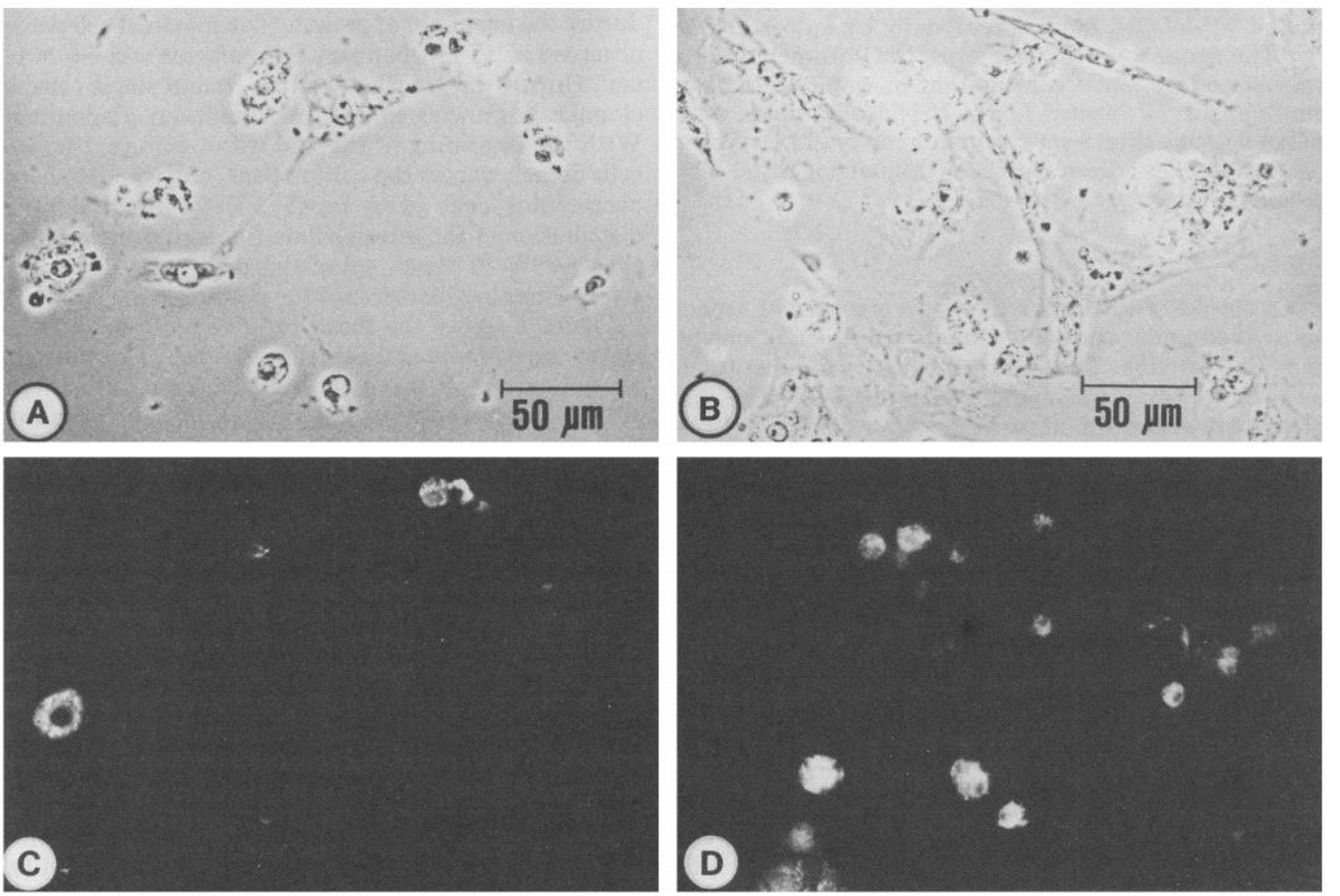

FIG. 2. Phase-contrast photomicrographs of sample of cultured cells 2 (*A*) and 3 (*B*) days after seeding. Corresponding renin immunofluorescence is displayed in (*C*) and (*D*). Only round (type A) cells exhibit immunofluorescence.

specific labeling is confined to the intact secretory granules of the cells (Fig. 3).

Effects of ANG II, AVP, and NE on Cultured Cells

Figure 4 and Table 1 summarize the effects of ANG II (10^{-7} M), AVP (10^{-6} M), and NE (10^{-5} M) on renin release and calcium uptake of the cultured cells. It is evident from this figure and table that all three hormones inhibited renin release and enhanced the calcium influx into the cells. In this respect, ANG II and AVP were found to be more potent compared with NE. Vp alone, on the other hand, significantly stimulated renin release and inhibited calcium influx. In the presence of Vp (10^{-5}

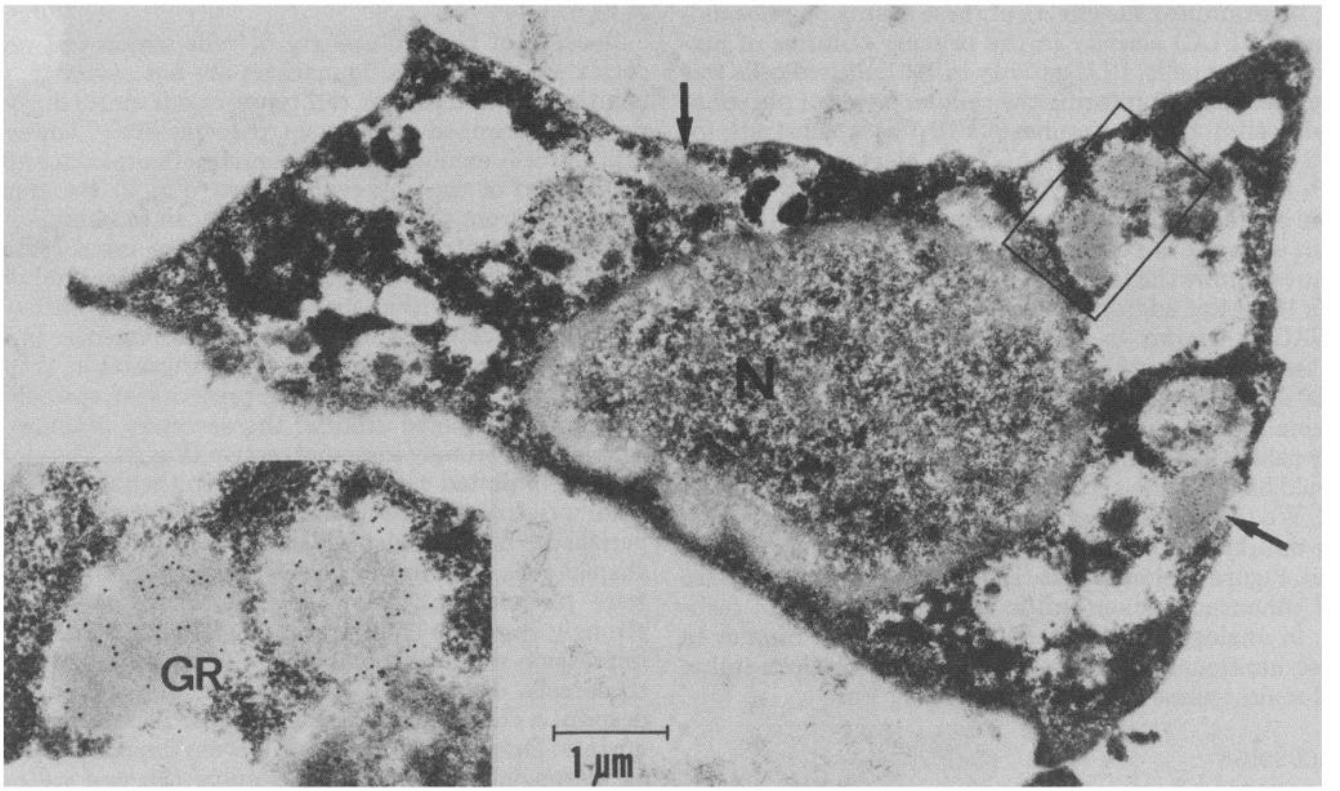

FIG. 3. Immunocytochemical localization of renin with protein A-gold technique in cultured cell 2 days after seeding. Some secretory granules (GR) are homogeneously electron dense and clearly labeled with gold particles (*arrows* and *inset*). Other granules have been subjected to partial or even complete loss of their content, possibly during sample processing. N, nucleus.

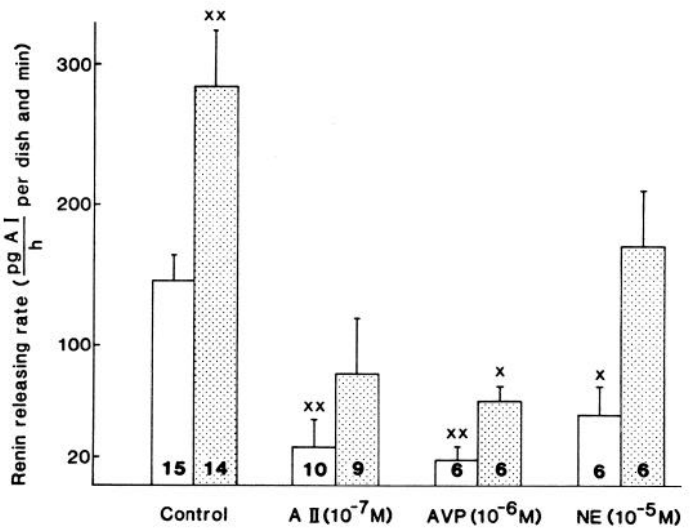

FIG. 4. Effects of angiotensin II (A II), arginine vasopressin (AVP), and norepinephrine (NE) on renin release from cultured cells in the absence (*open bars*) and presence (*stippled bars*) of verapamil (10^{-5} M). Values are means ± SE. Figures at bottom of columns indicate number of independent experiments. A I, angiotensin I. ^{x} Significantly different from control, $P < 0.10$, Student's unpaired t test. ^{xx} $P < 0.05$.

M), NE failed to inhibit renin release and to enhance calcium influx. Calcium influx in the presence of ANG II and AVP was also diminished by Vp but was still higher compared with the control values. The inhibition of renin release was also diminished by Vp, but the renin-releasing rate did not return to control values in the presence of Vp in these cases.

TABLE 1. *Effects of ANG II, AVP, and NE on ^{45}Ca influx into cultured cells in the absence and presence of Vp*

Condition	^{45}Ca Uptake, cpm/dish		
	30 s	60 s	120 s
Control	940±95	1,516±94	2,588±188
Control + Vp	733±61	1,137±110	1,734±127
ANG II	1,338±84	2,179±85	3,702±193
ANG II + Vp	1,081±88	1,759±113	3,020±208
AVP	1,466±71	2,728±118	4,659±195
AVP + Vp	1,259±62	1,794±70	3,106±148
NE	1,043±65	1,681±105	2,958±83
NE + Vp	808±85	1,380±59	1,915±156

Values are means ± SE of 10 experiments. ANG II, angiotensin II (10^{-7} M); AVP, arginine vasopressin (10^{-6} M); NE, norepinephrine (10^{-5} M); Vp, verapamil (10^{-5} M).

Because the effects of these vasoconstrictors on both renin release and calcium, influx could be diminished or even abolished by the calcium channel blocker Vp, it seems very likely that ANG II, AVP, and NE exerted their effects by an augmentation of the calcium permeability of the cellular plasma membrane. We therefore investigated by which mechanism ANG II, AVP, and NE lead to an enhanced calcium permeability. Because ANG II, AVP, and NE have been found to be potent activators of phospholipase C in various tissues (for review see Ref.

25), we examined the effects of these agents on phospholipase C (PLC) activity in the primary cultures of juxtaglomerular cells. PLC activity in the cultured cells was determined by monitoring the cellular levels of phosphatidylinositol 4,5-bisphosphate (PIP_2) as a substrate for PLC and diacylglycerol (DAG), the cleavage product of PIP_2 (4). We found that ANG II, AVP, and NE led to a rapid breakdown of PIP_2 with a maximum after 15 s and to an increase in DAG with a maximum also after 15 s. Figure 5 shows the level of PIP_2 and DAG in the cultured cells 15 s after addition of ANG II, AVP, and NE.

DAG is known to be a potent activator of protein kinase C (PKC) (28). If the activation of PKC by DAG were a mechanism by which vasoconstrictors enhance calcium influx and inhibit renin release of juxtaglomerular cells, then a DAG-independent activation of PKC should have effects similar to those of ANG II, AVP, and NE. We therefore investigated the effects of TPA, which is a well-known stimulator of PKC (6), on the cultured cells. Figure 6 shows that TPA inhibited renin release and enhanced calcium influx in a dose-dependent fashion, in analogy with ANG II, AVP, and NE. Similar to these hormones, the effects of TPA on calcium influx and renin release could be inhibited by Vp.

DISCUSSION

It was the aim of the present study to find out by which sequence of events vasoconstrictive hormones may inhibit renin release in juxtaglomerular cells. To this end we first established a cell culture suitable for studies of renin secretion at the cellular level. We have shown that by means of simple cell disaggregation methods, including kidney perfusion with chelators and enzymatic treatment of minced cortical pieces, primary cultures of renal cortical cells could be obtained that contained high activities. Because the assay of renin activity was performed under conditions known to be selective for renin (35) and ~50% of the cultured cells strongly reacted with specific antibodies against renin, we infer that the angiotensin-generating activity in the cell cultures is due to renin itself and not to other proteases such as cathepsin D (9, 15).

Because of the multiplicity of cells within the renal cortex for which specific markers are not available, the identity of the different cell types in our cultures could not be determined definitively. It seems likely, however, that the cells exhibiting strong immunofluorescence after application of the anti-renin serum (Fig. 2) are granulated cells from the afferent arteriole, since immunohistochemical control experiments with rat renal cortical slices showed that under comparable conditions only the granulated cells within the pars medialis of the afferent arteriole displayed a similar immunofluorescence. In addition, an immunocytochemical investigation at the ultrastructural level revealed the presence of epithelioid cells in the pelleted culture, the secretory granules of which were strongly immunoreactive (Fig. 3). The clonal contact-inhibited cells might be of epithelial origin, an assumption confirmed by the observation that these cells perished during the 1st wk of culture (7). The spindle-shaped cells, growing in an overlapping manner, which were predominant from the 2nd wk of culture on, strongly resemble cultured mesangial cells. This visual impression was confirmed by the finding that ~90% of these cells displayed a strong immunofluorescence for desmin, a characteristic component of intermediate filaments. Desmin-containing cells were identified in situ as mesangial cells in the glomerulus (2), and cultured mesangial cells were reported also to contain desmin (19).

The dependence of the averaged renin activity/10^6 cells on the age of culture was similar for all cell cultures investigated. The averaged renin activity increased after seeding of the cells, reached a maximum during the 1st wk of culture, and decreased exponentially thereafter (Fig. 1). At its maximum, the averaged cellular renin content was higher by a factor of ~30,000 than the renin activity of the cloned juxtaglomerular cells of Rightsel and co-workers (35). In contrast to the averaged cellular renin activity the total cell-bound renin activity per culture dish decreased exponentially from the onset of the culture. Hence we may speculate that the increase of the averaged cellular renin activity during the first days

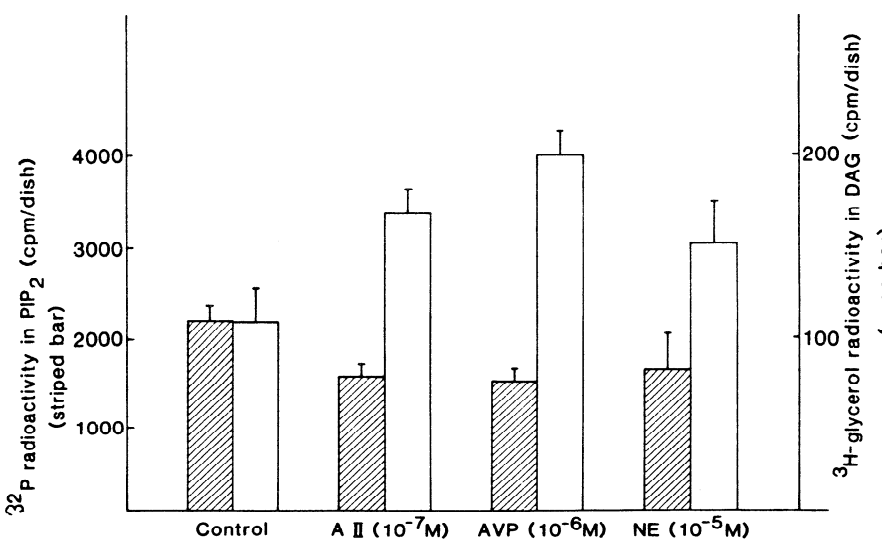

FIG. 5. Effects of angiotensin II (A II), arginine vasopressin (AVP), and norepinephrine (NE) on ^{32}P-label in phosphatidylinositol 4,5-bisphosphate (PIP_2, *striped bars*) and [^{3}H]glycerol label in diacylglycerol (DAG, *open bars*) in cultured cells prelabeled with ^{32}P and [^{3}H]glycerol. Values are means ± SE (n = 10).

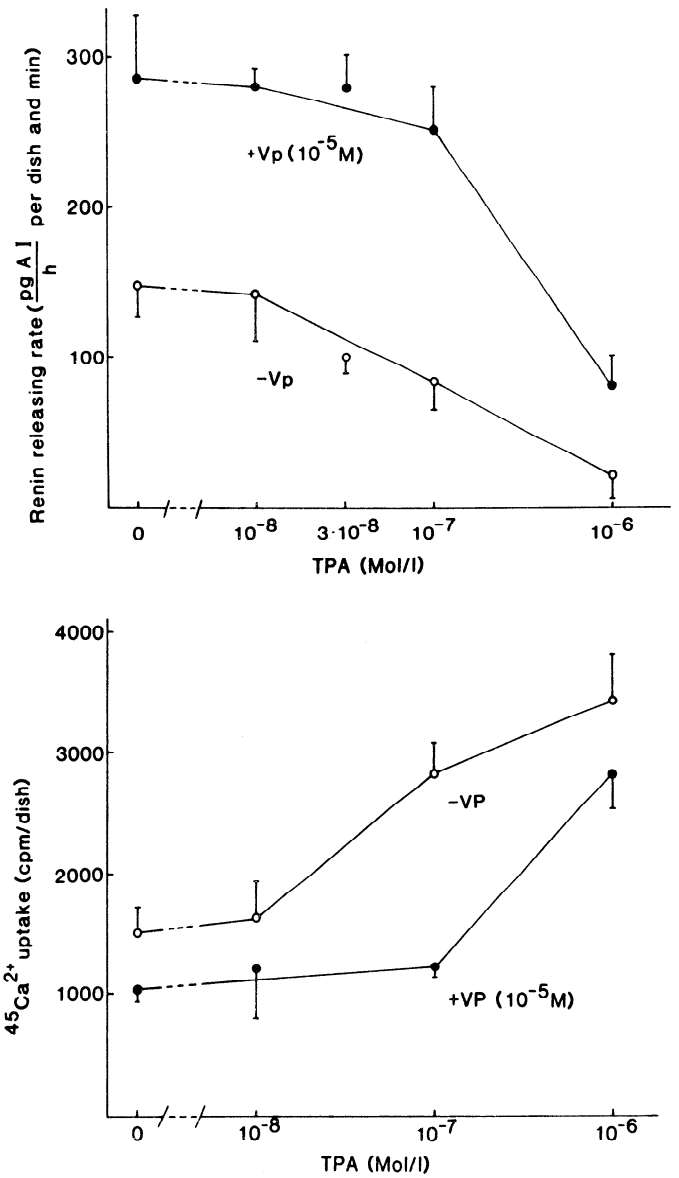

FIG. 6. Effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on renin release (*upper graph*) and ^{45}Ca uptake (*lower graph*) of cultured cells in the presence and absence of verapamil (Vp, 10^{-5} M). Each experimental point is mean ± SE of 10 experiments. A I, angiotensin I.

could have resulted from a selective attachment of renin-containing cells rather than from a transitory increase of renin synthesis. The daily rate of renin secretion was fairly proportional to the cellular renin content and amounted to 10–20% of the cell-bound renin activity. Calculations revealed that the cumulative secretion of renin quantitatively corresponded to the cumulative loss of cell-bound renin activity. These findings and the results of the immunofluorescence experiments indicate that the immunoreactive cells quantitatively release their renin content into the culture medium.

From the results of Galen et al. (13), and Rightsel et al. (35), who worked with human tumoral juxtaglomerular cells and cloned rat juxtaglomerular cells, respectively, we may further conclude that epithelioid cells lose the ability to store renin within granules and that they mainly release an inactive form of renin when kept under cell culture conditions. The granular cells described in this paper were found to contain prominent renin granules, at least during the 1st wk of culture. Furthermore, using acidification (22) and trypsin activation (26), we found that ~80% of the renin released belonged to the active form (results not shown). Taken together, our results suggest that the cultured cells described in this paper are quite similar to epithelioid cells in situ, as far as renin storage and renin release is concerned. This inference is also corroborated by the results of the experients in which renin secretion was stimulated by pharmacological agents (20). We conclude from these considerations that the cultured juxtaglomerular cells might be a suitable model to study the effects of vasoconstrictors on juxtaglomerular cells.

We found that ANG II, AVP, and NE inhibited renin release from these cells and that their effects could be diminished by the calcium channel blocker Vp (Fig. 4). With regard to ANG II and AVP, our findings confirm experiments that were carried out with isolated perfused kidneys and renal cortical slices (30, 38, 39). Furthermore, we found that NE also inhibited renin release in our culture preparations. Although it is well documented that certain α-adrenergic agonists indeed inhibit renal renin release (cf. Ref. 17), the effect of NE on renin release is subject to controversy. Experimental evidence indicates that NE at low concentrations stimulates renin release and at concentrations higher than 10^{-6} M inhibits renin release (5, 11, 21, 27). This dual effect of NE on renin release can be explained by the conception that NE at low concentrations stimulates renin release via activation of β-receptors, whereas inhibition of renin release at higher concentrations is mediated via α-receptors (1, 17). Our finding that NE 10^{-5} M inhibited renin release from the cultured juxtaglomerular cells is in harmony with this idea. Moreover, we obtained evidence that ANG II, AVP, and NE stimulated the calcium influx into juxtaglomerular cells and that the rate of renin release was inversely correlated with the calcium influx (Fig. 7). Although the calcium influx into the cultured cells has to be interpreted carefully, because juxtaglomerular cells only accounted for one-half of the total cell number in the cultures, our findings corroborate the results obtained by Fray et al. (12) and Park et al. (30). These authors concluded that ANG II and AVP inhibit renin release by enhancing the calcium influx into juxtaglomerular cells.

Because the calcium influx into cells is considered to be limited by the availability of open channels in the plasma membrane, the question arises of whether or not vasoconstrictors increase the calcium permeability in juxtaglomerular cells by a common mechanism. ANG II, AVP, and NE have been shown to activate PLC in various tissues (25). Therefore we tested whether an enhancement of the calcium permeability could be mediated via a stimulation of PLC. We found that ANG II, AVP, and NE all led to a breakdown of PIP_2 in our culture system (Fig. 5). PIP_2 is a substrate for PLC, and one of the cleavage products of PIP_2 is DAG (4), which was found to increase on stimulation with ANG II, AVP,

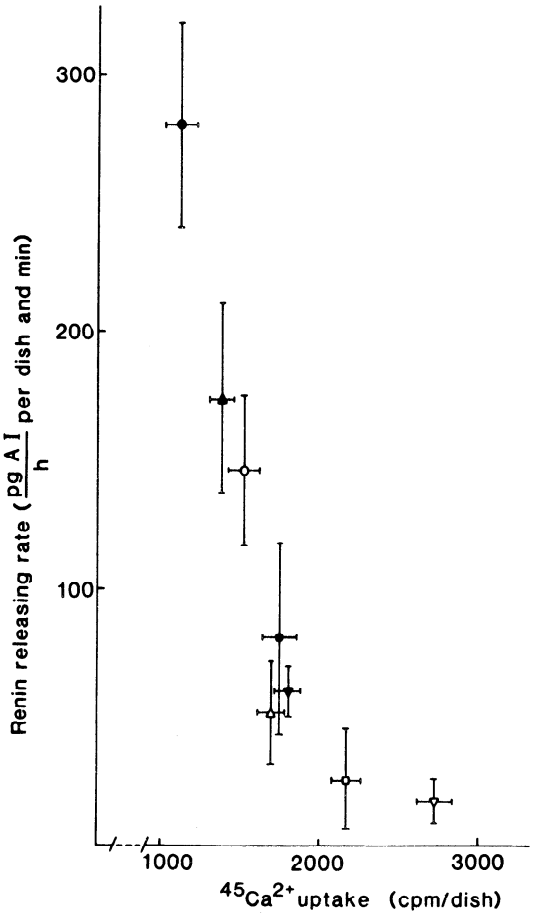

FIG. 7. Correlation between calcium influx during 1st min and rate of renin release of cultured cells evoked by vasoconstrictors in the presence (*filled symbols*) and absence (*open symbols*) of verapamil (10^{-5} M). Data were taken from Fig. 4. A I, angiotensin I. ○,● Control; △,▲ norepinephrine (10^{-5} M); ▽,▼ arginine vasopressin (10^{-6} M); □,■ angiotensin II (10^{-7} M).

and NE. DAG is known to be a potent activator of PKC (28). There is growing evidence that stimulation of PKC opens calcium channels (10), and for certain cell types it has been demonstrated that an activation of PKC may mediate the cellular effects of activators of PLC (4, 28). If the influence of ANG II, AVP, and NE on renin release and calcium permeability is in fact due to a DAG-dependent stimulation of PKC, one should expect similar effects by directly stimulating PKC. Indeed we found that an activation of PKC by the phorbol ester TPA inhibited renin release and stimulated calcium influx (Fig. 6). In view of this finding it seems unlikely that the vasoconstrictive hormones could have stimulated PKC via an increased calcium influx. Exactly which family of calcium channels is being acted on by PKC remains to be investigated for the renin-containing cells. In hepatocytes, at least, it has been shown that ANG II, AVP, and NE open a common pool of calcium channels (23). In summarizing our results we want to propose the following concept for the mechanism of action by which vasoconstrictors inhibit renin release from renal juxtaglomerular cells: vasoconstrictors stimulate a phospholipase C, thereby generating DAG. DAG activates PKC, which in turn opens calcium channels by an as yet unknown mechanism. This results in an enhanced calcium influx into the juxtaglomerular cells and an increased intracellular calcium concentration, which is considered to inhibit renin release (cf. Ref. 12). The general validity of this line of thought is further strengthened by our recent finding (33) that platelet-activating factor, a vasoconstrictive substance (16) produced in the kidney (34), has effects on renin release, calcium influx, and PLC activation in our culture system similar to those of ANG II, AVP, and NE.

Our present work is now concentrated on the enrichment of the juxtaglomerular cells in culture and the elucidation of the mechanism by which PKC opens calcium channels in the plasma membrane.

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