Contraction of Cultured Rat Glomerular Cells of Apparent Mesangial Origin after Stimulation with Angiotensin II and Arginine Vasopressin

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ABSTRACT Studies to identify the physiological role of glomerular mesangial cells were undertaken using homogeneous cultures of rat glomerular cells of apparent mesangial origin (MS). Cultured MS cells were treated with arginine vasopressin (AVP), angiotensin II (AGII), prostaglandin E_2, and parathyroid hormone. AVP (0.1 nM) and AGII (1 nM) stimulated contraction of MS cells in vitro that was complete by 2 min at 37°C or 10 min at 23°C as observed by phase contrast and electron microscopy. Relaxation recurred 15 min after hormonal addition at 23°C. Similar experiments in cloned rat glomerular epithelial cells or "renin"-producing cells did not demonstrate a contractile response. The contraction of MS cells was independent of cyclic AMP (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP) production, even when cyclic nucleotides were measured as early as 30 s after hormonal stimulation. To demonstrate that contraction was a function of hormone-receptor interaction, binding of [^{3}H](8-lysine)vasopressin was studied. Specific binding for 1.6 and 5 nM hormone was both time- and dose-dependent. The estimated apparent affinity was 10 nM. In late MS cell passages (>16th) that no longer demonstrated hormone-stimulated contraction, no specific binding of [^{3}H](8-lysine)vasopressin was observed.

Incubations were modified to optimize the conditions for detecting the effect of hormones on cell cyclic nucleotide content. A supramaximal concentration of AVP (200 nM) increased the cAMP content of MS cells twofold in the presence of a phosphodiesterase inhibitor. Similar experiments with prostaglandin E_2 (1 μg/ml) led to a 1.5–6-fold increase in MS cell cAMP content, but no effect on contraction was observed. Neither hormone altered cGMP content. These data are further support for the independence of contraction and cyclic nucleotide production.

Our studies suggest that MS cells are the equivalent of smooth muscle cells in the glomerulus and that their contraction may be important in control of glomerular filtration.

INTRODUCTION

We have been able to isolate by a cloning technique, and maintain in tissue culture, three homogeneous cell types from rat glomeruli (1–3). One cell type has been identified as the glomerular epithelial cell (1). The second cell type is rich in cytoplasmic granules and contains "renin"-like material as measured by a radioimmunoassay technique, and the third cell type, mesangial-like (MS),[1] contains many bundles of microfilaments and is morphologically similar to a mesangial cell (2, 3). The exact function of mesangial cells is not clear, but it has been proposed that they function in the regulation of glomerular size and blood flow by contractility (4, 5). This process might be hormonally mediated because angiotensin II (AGII) (6), vasopressin (AVP) (7), parathyroid hormone (PTH) (8), and prostaglandin E_1 (PGE_1) (9), affect glomerular function by reducing the ultrafiltration coefficient (K_f). Because the K_f is the product of the surface area of the glomerular

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[1] *Abbreviations used in this paper:* AGII, angiotensin II; AVP, (8-arginine)vasopressin; cAMP, cGMP, cyclic AMP, GMP; K_D, estimated apparent affinity; K_f, ultrafiltration coefficient; LVP, (8-lysine)vasopressin; MIX, 1-methyl 3-isobutyl xanthine; MS, mesangial-like; PBS, Dulbecco's phosphate-buffered saline; PGE, prostaglandin E; PTH, parathyroid hormone.

capillary and its hydraulic permeability, a reduced K_f could be accomplished by contraction of mesangial cells. The above-mentioned hormones have a varied capacity to alter glomerular cyclic nucleotide content (10–13), but no direct correlation has been made with this hormonal response and reductions in K_f. Therefore, in order to define the physiological role of the glomerular mesangial cells, we tested three homogeneous glomerular cell types with AVP, PTH, PGE_2, and AGII, and found that AVP and AGII caused contraction of MS cells in vitro. This contraction corresponded with specific binding of [^{3}H](8-lysine) vasopressin (LVP) and was independent of cyclic nucleotide generation. Increased levels of cyclic AMP could be achieved in MS cells exposed to PGE_2 (1 μg/ml) and a supramaximal concentration of AVP (200 nM) in the presence of a phosphodiesterase inhibitor.

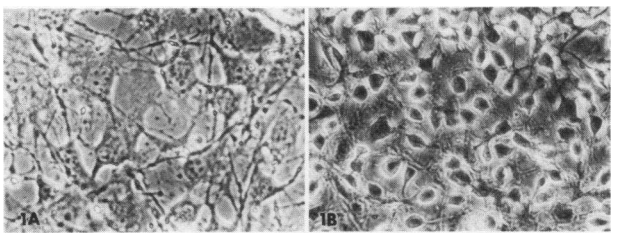

FIGURE 1 (A) Phase-contrast photomicrograph of cloned MS cells maintained in culture overnight in Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution with 10% fetal calf serum to maximally relax the cells. Notice that the cells are well spread with long processes. (B) Phase-contrast photomicrograph of cloned MS cells treated with 1 nM AGII in Hanks' balanced salt solution with Ca^{++} and Mg^{++}. Note the rounded, refractile appearance of these contracted cells. Similar observations were made in the presence of AVP. ×800.

METHODS

Isolation and culture of glomerular cells. The procedure employed for isolating homogeneous populations of glomerular cells (i.e., epithelial, renin cells, and MS cells) has been described in great detail in a previous report (1). Briefly, primary cultures from outgrowths of whole glomeruli and dissociated glomerular cells pooled from five rats were plated for cloning in RPMI 1640 medium supplemented with 20% fetal calf serum (Microbiological Associates, Walkersville, Md.) diluted in half with conditioned medium (from Swiss 3T3 cells in log phase growth) containing 0.66 U/ml of insulin. Cloned cell types were tested for fibroblast contamination by their ability to grow in RPMI 1640 containing 20% dialyzed fetal calf serum and D-valine substituted for L-valine (14), a condition in which fibroblasts do not grow. Cloned MS cells were carried in RPMI/conditioned medium. Detailed ultrastructural examination and hormone-sensitive cyclic nucleotide responses were evaluated in primary cultures and in passages 7, 8, 12, 14, and 16 and no significant changes were noted. Experiments were conducted in 3.3- or 10-cm tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.).

Contraction experiments. 18 h before the contraction experiments, the three cell types to be tested had their growth medium changed to Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum. This condition was found to maximally relax the MS cells (Fig. 1A) similar to observations in smooth muscle cells (15). Cells were incubated in Hanks' balanced salt solution with Ca^{++} and Mg^{++} with or without: (*a*) AVP, 1 μM–0.1 nM; (*b*) AGII, 1 μM–0.1 nM; (*c*) PTH, 1 μg/ml; and (*d*) PGE_2, 1 μg/ml. To facilitate photographic analysis of the contraction event, most experiments were performed at 23°C. At 37°C contraction took place within 2 min, whereas at 23°C it took 5–10 min. All three cell types were tested for their ability to respond to decreasing concentrations of hormone in separate dishes using phase-contrast microscopy. No quantitative differences in contractions were observed above threshold hormone concentrations. For electron microscopic examination of contracted and control cells, cultures were fixed in 2% glutaraldehyde in Hanks' balanced salt solution with Ca^{++} and Mg^{++}, pH 7.4, for 1 h at room temperature and processed by the usual techniques.

Assay of cyclic nucleotides. Monolayer cells were incubated under contraction conditions as described above or in Dulbecco's phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and 1 mM 1-methyl 3-isobutyl xanthine (MIX) with or without hormone at 37°C. Cells were prepared for cyclic nucleotide assay by an adaptation of the methods of Cooper et al. (16) and Corbin et al. (17). At the end of each incubation, aliquots of media were removed, boiled for 5 min, and centrifuged for 2 min at 7,000 *g*. Supernates were saved for assays. Cell dishes (3.3 cm) were placed on ice, media aspirated, and cells scraped with a Teflon policeman into 750 μl of sonication buffer (4°C) containing: 5 mM potassium phosphate, 2 mM EDTA, 0.5 mM MIX, and 150 mM KCl, pH 6.8. This suspension was sonicated for 3 s at 4°C, an aliquot was removed for protein determination (18), boiled for 5 min, and centrifuged for 2.5 min at 7,000 *g*. Elapsed time from scraping through sonication was ~30 s. Media and cell supernates were either assayed immediately or stored at −15°. No significant loss of cyclic nucleotides was observed with storage.

cAMP and cGMP were measured by radioimmunoassay adapted from the procedure of Steiner et al. (19) using New England Nuclear radioimmunoassay kits (New England Nuclear, Boston, Mass.). Specificity and sensitivity of the antibodies in preliminary experiments were found to be within the manufacturer's specifications. Acetylation of standards and samples yielded a lower limit of sensitivity of 1 fmol for both nucleotides.

Validation of this rapid technique of cell preparation for cyclic nucleotide assay was conducted as follows: (*a*) incubations were terminated by the addition of ice-cold 10% trichloroacetic acid to cells in 3.3-cm dishes. Cells were scraped from dishes on ice and homogenized with a Teflon pestle (12 strokes). Homogenates were divided into two aliquots and tracer amounts of [^{3}H]cAMP (~5,000 cpm, 0.3 pmol/10 μl) were added to respective aliquots to monitor recovery. Proteins were precipitated with centrifugation, supernate acidified, and trichloroacetic acid extracted with ethyl ether as previously described (13). Aliquots of extracts were assayed for cyclic nucleotides as described above. After calculation of recovery and subtracting tracer nucleotide content, values obtained in both control and hormone-stimulated cells (12 determinations) were 95±5% of those values using the more rapid cell preparation; (*b*) exogenous cAMP or cGMP added to cells with sonication buffer before scraping were quantitatively recovered; (*c*) linearity of the cyclic nucleotide assays was obtained with dilution; and (*d*) hydrolysis of cyclic nucleotides was obtained with cyclic nucleotide phosphodiesterase.

In addition, the specificity of the cyclic nucleotide assays was tested. A 100-fold excess of cGMP or 1,000-fold excess of ATP did not interfere with cAMP determinations; similar

additions of cAMP and ATP did not alter the cGMP assay. The constituents of the incubation media (with or without hormone) or the sonication buffer did not interfere with the cyclic nucleotide assays at 100-fold usual assay concentrations.

Cell number was determined in several dishes in each experiment. When cyclic nucleotide data were expressed per mean cell number or per milligram protein of each dish, similar results were obtained. Therefore, cyclic nucleotides are expressed as pmoles per time per 10^6 cells±SEM. There was ~200 μg of protein per 10^6 cells. The Student's *t* test for unpaired data was used to evaluate statistical significance.

[³H]LVP binding to MS cells. Growth medium was removed from MS cells grown in six 10-cm dishes (24×10^6 cells). The cells were washed twice in PBS, then placed on ice and scraped into 5 mM Tris-HCl, pH 7.4, and 1 mM EDTA (5 ml/dish). After homogenization of pooled cells with a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.) (15 strokes), cells were centrifuged at 10,000 *g* for 15 min at 4°C. The pellet was resuspended in the above-described buffer by vortexing. The cell extracts were incubated for various time periods with either 1.6 or 5 nM [³H]LVP in the presence or absence of 2 μM unlabeled AVP in an adenylate cyclase assay medium as previously described (20). Bound and free hormone were separated by filtration through Millipore filters (Millipore Corp., Bedford, Mass.) (21). The difference between total binding and nonspecific binding, measured in the absence or presence of AVP, respectively, was plotted as a function of time. Nonspecific binding increased linearly with time and, after a 20-min incubation, represented 41 and 37% of total binding measured at 5 and 1.6 nM labeled peptide, respectively. Each sample was assayed in duplicate.

Hormone stock solutions. Synthetic lysine vasopressin (100 IU/mg), synthetic arginine vasopressin (350 IU/mg) and synthetic angiotensin II were obtained from Sigma Chemical Co., St. Louis, Mo. and stored at −20°C. Prostaglandin E_2 (Upjohn Co., Kalamazoo, Mich.) was stored at −20°C in ethanol (1 mg/ml). Highly purified bovine PTH (~3,000 U/mg, gift of Dr. Henry T. Keutmann, Endocrine Unit, Massachusetts General Hospital, Boston, Mass.) was stored as a lyophilized powder. [³H](8-lysine) vasopressin (8.5 Ci/mmol) was obtained from Dr. Margat, Commissariat à l-Energie Atomique, Saclay, France, and prepared as previously described (21). The tritiated peptide was purified by affinity chromatography on neurophysin-bound Sepharose (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, N. J.) and the purified product stored in liquid nitrogen. The peptide had similar biological and binding activity as previously described (21).

RESULTS

MS cell contraction

MS cells maintained in Ca^{++}- and Mg^{++}-free incubation medium for 18 h are depicted in Figs. 1A and 2. These cells are large and flat with spindly processes in culture and are seen to contain numerous bundles of microfilaments that run parallel to the plasma membrane similar to that observed in mesangial cells *in situ* (22). In addition, MS cells contain surface-dense patches (Fig. 2) that have been described in smooth muscle (23).

MS cells contracted when AGII (1 μM–1 nM) or AVP (1 μM–0.1 nM) were added, with virtually every cell responding (Figs. 1B, 3). MS cells did not contract in the presence of PTH (1 μg/ml) or PGE_2 (1 μg/ml) and the remaining two cell types (epithelial and renin cells) did not contract when exposed to maximal concentrations of any of the four hormones. Contraction was studied in a minimum of three separate culture dishes for each hormone concentration tested at each cell passage. Three consecutive passages (14, 15, and 16; ~4 mo in culture) of an isolate of MS cells contracted; however, after the 16th passage, the cells lost their ability to contract which corresponded with the loss of vasopressin receptors (see below).

At 37°C the response was achieved by 2 min. Contraction took between 5 to 10 min at room temperature and by 15 min the MS cells resumed their normal morphology. Ultrastructurally, the most striking features of the contracted MS cells were: (*a*) their rounded appearance, making them seem larger than controls at similar magnifications (Fig. 3); (*b*) the nuclear deformations (i.e., folds) similar to those described by Majno et al. (24) for contracted endothelium (Fig. 3B); and (*c*) the loss and/or disorientation of microfilaments and dense patches similar to that described for smooth muscle cells from the stomach of *Bufo marinus* (23).

Binding of [³H]LVP

Fig. 4 illustrates the specific binding of [³H]LVP as a function of time for 1.6 and 5 nM hormone. Hormonal binding is time- and dose-dependent, with maximum binding reached by 10 min. In comparison, nonspecific binding increased linearly with time (data not shown). Utilizing the maximum amount of hormone bound at 1.6 and 5 nM, an estimated apparent affinity, K_D, for [³H]LVP was 10 nM, similar to that found in rat kidney medullary membranes (25) and intact cultured kidney cells (26).

In cell passages that lost their contractile response, there was no specific binding of [³H]LVP.

Hormonal modulation of cyclic-nucleotides

Contraction conditions. In conditions where hormonally-stimulated contraction was observed (23°C) the modulation of cyclic nucleotides by these same substances was studied. Basal cAMP content was 12.2 ±1.7 pmol/10 min per 10^6 cells ($n = 12$) and cyclic GMP was 115.5±2.5 fmol/2 min per 10^6 cells ($n = 9$). Data were derived from at least three separate culture dishes in each of three cell passages. These values are the sum of cellular and medium cyclic nucleotides (10-min incubation medium contains <15% of the cell cAMP content, 2-min incubation medium contains <5% of the cell cGMP content). There was no significant increase or decrease in cyclic nucleotide content after 30-s to 10-min incubations with the following substances: AGII, 1 μM; AVP, 200 nM; LVP, 200 nM; PTH, 1 μg/ml; or PGE_2, 1 μg/ml.

MIX incubation conditions. Incubations were modified to optimize the conditions for detecting an

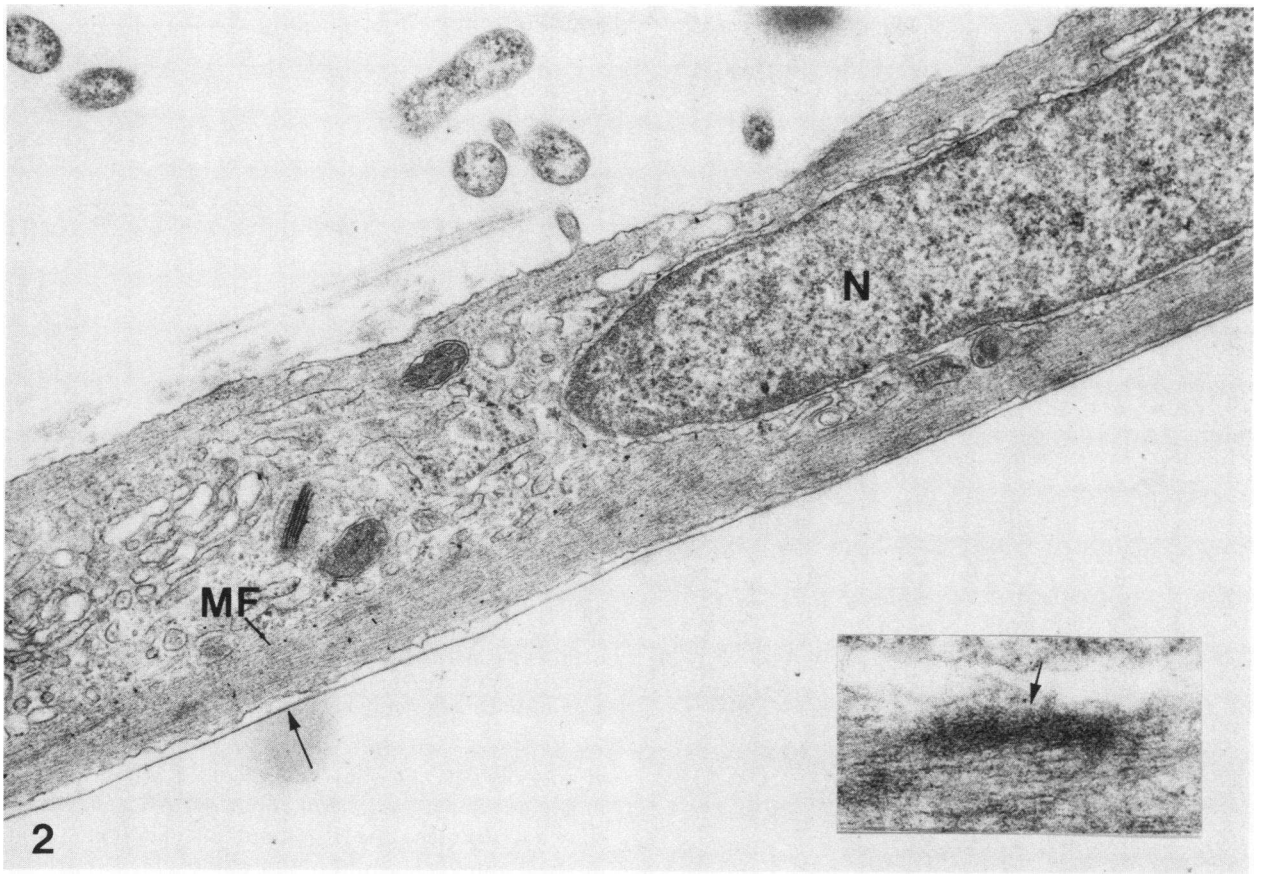

FIGURE 2 Transmission electron micrograph of a MS cell maintained in tissue culture overnight in Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution with 10% fetal calf serum. Note the numerous bundles of microfilaments (MF) and the oval nuclear profile (N). Arrow indicates substrate to which the cells are attached. ×39,000. Inset: portion of a cultured MS cell displaying a surface dense patch (arrow). ×48,500.

effect of hormones on cell cAMP content. Table I illustrates the results of incubating MS cells with maximal concentrations of hormone in the presence of the phosphodiesterase inhibitor, MIX. Basal cAMP levels (minus MIX) were only one-fifth those observed in contraction conditions. MIX increased basal cAMP content approximately twofold; both AVP (200 nM) and PGE_2 (1 μg/ml) significantly increased cellular cAMP levels, and AVP increased medium cAMP as well. Under these conditions, however, the effect of PGE_2 was quite variable. A sixfold increase in cell cAMP and a twofold increase in medium cAMP were observed in some cell passages. Preincubation with indomethacin (100 μM) for 24 h did not change the response of cells to either AVP or PGE_2 despite evidence for PGE_2 production by MS cells.[2] The increase in cAMP production in response to AVP was found in three separate MS clones, each carried for a minimum of ten passages. The other cloned glomerular cell types (i.e., epithelial and renin cells) did not respond to AVP.

There was no hormonal stimulation of cell cGMP production in MS cells in the presence of MIX after 30-s to 10-min incubations.

DISCUSSION

The physiological function(s) of glomerular mesangial cells remains unknown. Several studies have led to the hypothesis that hormonal modulation of a mesangial cell may regulate glomerular filtration; (*a*) glomeruli have been shown to contract spontaneously in vitro (27) and in the presence of AGII (28); mesangial cells have been implicated in both of these processes because they contain microfilaments and myosin (22, 29), and by autoradiography they localize [^{3}H]AGII (30). However, visualization of their contraction has never been observed; (*b*) the in vivo physiological studies by Brenner and his associates (8) using PTH, AVP (7), and PGE_1 (9), and by Blantz et al. (6) using AGII have demonstrated

[2] Kreisberg, J. I., R. Zusman, and D. A. Ausiello. Unpublished observations.

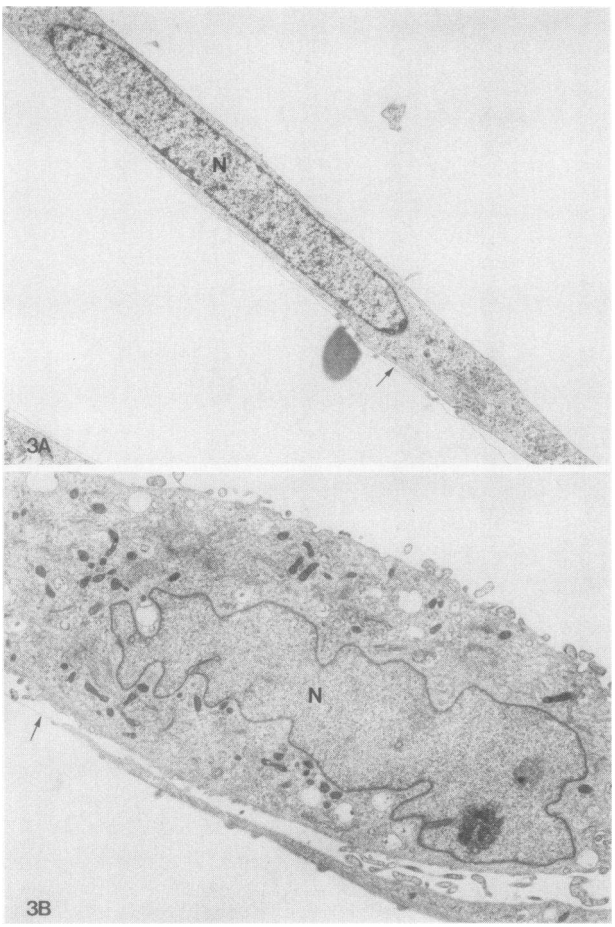

FIGURE 3 (A) Transmission electron micrograph of a MS cell maintained overnight in Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution with 10% fetal calf serum. Note the oval-shaped nucleus (N). (B) Transmission electron micrograph of a MS cell exposed to 1 nM AGII in Hanks' balanced salt solution with Ca^{++} and Mg^{++}. Note that although this cell appears larger than the cell in Fig. 3A, it is the same magnification. The larger appearance is due to the rounded, contracted state of the cell. Also, note the accordian-shaped nucleus (N) with the numerous folds quite characteristic of contracted cells. Similar observations were made in the presence of AVP. Arrows indicate the surface to which cells are attached. ×9,600.

that these substances can decrease the ultrafiltration coefficient, K_f. A decreased capillary surface area, a component of K_f, could be accomplished via contracting mesangial cells (6–9). AVP has been demonstrated to be the most potent contractile substance on rat blood vessels, with equivalent responses elicited by AGII at higher concentrations (15); (*c*) in an attempt to find the modulators of hormone action in glomeruli, studies in isolated glomeruli have shown that PGE_2 stimulated adenylate cyclase (12), whereas PTH stimulated the production of both cAMP and cGMP (10–13). AVP increased the activity of adenylate cyclase in isolated rabbit glomeruli (11), but 10 μM AVP had no effect in isolated rat

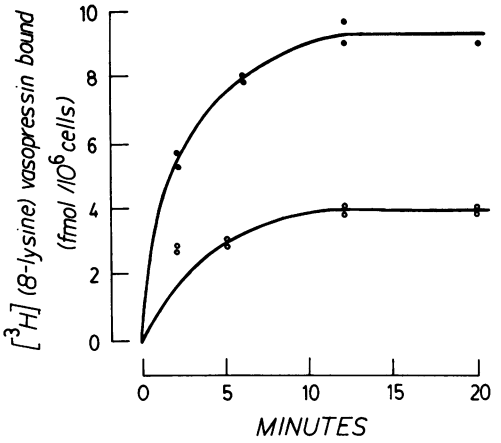

FIGURE 4 Time-course of [^{3}H]LVP binding to a broken MS-cell preparation. Six culture dishes (24 × 10^6 cells) of monolayer cells were washed with PBS and scrapped at 4°C into 5 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, and homogenized. The pooled homogenates were centrifuged at 10,000 *g* for 15 min and the pellet resuspended in homogenizing buffer. Sample aliquots were incubated for various periods of time with either 5 nM (●) or 1.6 nM (○) [^{3}H]LVP in the absence or presence of 2 μM AVP. Specific binding, determined as described in Methods, is plotted vs. time. Data of duplicate assays are illustrated at each time point.

glomeruli (10). The effect of AVP on cGMP production in glomeruli has not been reported. AGII has been shown to decrease cAMP content in isolated glomeruli in some studies (13) but not in others (11, 12); it does not alter glomeruli cGMP content (13). The glomerular cell type(s) responsive to those hormones has not been identified. Our studies were therefore designed to determine whether a cloned MS cell responds to hormones by contracting, and whether this contraction is modulated by cyclic nucleotides.

TABLE I
Maximal Effect of Hormones on cAMP Content in MS Cells

	Cell cAMP	Media cAMP
	pmol/20 min/10^6 cells	
Control (minus MIX)	2.4±1.5	—
Control (plus MIX)	5.2±0.1	1.5±0.01
AVP, 200 nM	13.0±0.3*	2.1±0.03*
PGE_2, 1 μg/ml	8.8±0.3*	1.1±0.1
PTH, 1 μg/ml	6.2±1.0	1.5±0.01

Confluent cell monolayers (3.3-cm dishes) were washed with PBS and incubated for 20 min at 37°C with PBS containing 0.2% bovine serum albumin, 1 mM MIX (where added), with or without hormone. Media and cells were prepared for measurement of cAMP content as described in Methods. Data are expressed as mean±SE of duplicate determinations on each of four culture dishes. All hormonal incubations were conducted in the presence of 1 mM MIX.
* $P < 0.01$ vs. control.

Although there is paucity of markers for mesangial cells, the following statements can be made about the isolated cell type used in this study; (*a*) it is morphologically similar to a mesangial cell in that it contains many bundles of microfilaments (Fig. 2); (*b*) <5% of isolated glomeruli contain vascular poles (1); light microscopic observations of each isolate of whole glomeruli revealed that these vascular poles did not attach to culture flasks, making it unlikely that the MS cell is derived from vascular smooth muscle; (*c*) it is not a fibroblast because it grows in D-valine-substituted media (14); and (*d*) it is not an endothelial cell because it does not contain factor VIII. The latter has been demonstrated to be present in rat glomerular endothelium *in situ* using a purified antibody to rat factor VIII.[3]

We were able to demonstrate in MS cells a contractile response to 1 nM AGII and 0.1 nM AVP. No response was elicited with either PTH or PGE_2. Of interest is the close correlation of these results with the studies of contraction in smooth muscle cells. The difference in response to AGII and AVP in MS cells is consistent with the threshold concentrations of each hormone necessary to stimulate isometric contraction in rat aortic strips (15). In addition, the concentration of LVP necessary to achieve half-maximal contraction of the canine femoral artery was 10 nM (31), similar to the apparent K_D for binding of [^{3}H]LVP in MS cells.

There is abundant evidence that increases in cAMP play no role as mediators of the contractile response of smooth muscle cells (32–34). It therefore seems likely that the increase in cAMP achieved in MS cells in the presence of a phosphodiesterase inhibitor with 200 nM AVP bears no relationship to the contractile response observed with 0.1 nM AVP. The lack of a contractile response to PGE_2, despite a stimulation of cAMP production, is further support for this conclusion.

The role of hormone-induced decreases in cAMP production and/or increases in cGMP production as mediators of smooth muscle contraction is more controversial. Diamond (33), in recent review of this topic, concluded that increased cGMP (or decreased cAMP) production was not responsible for smooth muscle contraction under a variety of conditions. Where increases in cGMP production did accompany contractions, it appeared to be secondary to increases in intracellular ionized calcium. Because we were unable to demonstrate hormonal decreases in cAMP or increases in cGMP as soon as 30 s after hormonal addition, we cannot implicate cyclic nucleotides in the contractile response of MS cells. It is conceivable that changes in cyclic nucleotide levels in subcellular compartments, undetermined by our measurement of total tissue content, may play a role in the contraction response.

The effect of the potent vasodilator PGE_1, or PTH and cAMP on K_f in vivo (7–9), might be an alteration of glomerular permeability rather than a decrease in glomerular capillary surface area as proposed by Brenner and associates (9), and we would, therefore, not anticipate these agents to cause a contractile response in MS cells. We cannot rule out the possibility that the absence of an effect of PTH on MS cells is the result of a loss of hormonal sensitivity during culture.

In conclusion, we have demonstrated for the first time the contraction of glomerular mesangial cells in vitro in response to AGII and AVP. These hormones have been shown to elicit contraction of smooth muscle cells at concentrations similar to those necessary to produce this response in MS cells. Therefore, our studies suggest that MS cells are the equivalent of smooth muscle cells and that their contraction is important in control of glomerular filtration.

[3] Kreisberg, J. Unpublished observations.

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