Functional Angiotensin II Receptors in Cultured Vascular Smooth Muscle Cells

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ABSTRACT To study cellular mechanisms influencing vascular reactivity, vascular smooth muscle cells (VSMC) were obtained by enzymatic dissociation of the rat mesenteric artery, a highly reactive, resistance-type blood vessel, and established in primary culture. Cellular binding sites for the vasoconstrictor hormone angiotensin II (AII) were identified and characterized using the radioligand ^{125}I-angiotensin II. Freshly isolated VSMC, and VSMC maintained in primary culture for up to 3 wk, exhibited rapid, saturable, and specific ^{125}I-AII binding similar to that seen with homogenates of the intact rat mesenteric artery. In 7-d primary cultures, Scatchard analysis indicated a single class of high-affinity binding sites with an equilibrium dissociation constant (K_d) of 2.8 ± 0.2 nM and a total binding capacity of 81.5 ± 5.0 fmol/mg protein (equivalent to 4.5×10^4 sites per cell). Angiotensin analogues and antagonists inhibited ^{125}I-AII binding to cultured VSMC in a potency series similar to that observed for the vascular AII receptor in vivo. Nanomolar concentrations of native AII elicited a rapid, reversible, contractile response, in a variable proportion of cells, that was inhibited by pretreatment with the competitive antagonist Sar_1,Ile_8-AII. Transmission electron microscopy showed an apparent loss of thick (12–18 nm Diam) myofilaments and increased synthetic activity, but these manifestations of phenotypic modulation were not correlated with loss of ^{125}I-AII binding sites or hormonal responsiveness. Primary cultures of enzymatically dissociated rat mesenteric artery VSMC thus may provide a useful in vitro system to study cellular mechanisms involved in receptor activation-response coupling, receptor regulation, and the maintenance of differentiation in vascular smooth muscle.

The interaction of vasoactive hormones, such as the octapeptide, angiotensin II (AII), with vascular smooth muscle cells (VSMC) has important implications for normal vascular physiology (1) and the pathophysiology of hypertension (2). Although pharmacologic studies in whole animals, isolated vascular strips, and blood vessel homogenates have identified receptors for a number of vasoactive substances, the cellular localization of these receptors, biochemical correlates of their activation, and factors regulating their expression have not been well defined, in part due to the structural complexity of vascular tissues. Selective isolation and culture of the cellular components of blood vessels provides a potentially useful approach to this problem (3–12).

Large fibroelastic arteries, such as the aorta, have been a preferred source of VSMC for culture (3, 7, 8) because the inner one-third of the medial layer of these vessels normally does not contain other cell types. However, once established in in vitro, these cells often undergo a modulation of phenotype that is characterized by the loss of myosin-containing, thick (12–18 nm Diam) filaments, decreased content of immunoreactive myosin, increased proliferative and biosynthetic activity, and decreased contractile responsiveness to vasoactive hormones (reviewed in reference 10). In general, the tendency for cultured VSMC to undergo such changes has limited their usefulness for in vitro studies of vasoactive hormone action (10, 12).

In this report, we describe biochemical, ultrastructural, and functional characteristics of VSMC cultured from the rat mesenteric artery, a highly reactive resistance-type blood vessel. We document the retention of specific high affinity AII binding sites in cells that have proliferated in response to serum growth factors and have undergone certain morphological changes consistent with phenotypic modulation (10). Furthermore, at least a portion of these cellular binding sites appear to be

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functional receptors, as evidenced by the stimulation of reversible contraction with physiological concentrations of AII. This in vitro model system may be especially useful for studying the cellular mechanisms of AII action in vascular smooth muscle, as well as for identifying factors required for the maintenance of functional differentiation in this cell type.

MATERIALS AND METHODS

Isolation and Primary Culture of Rat Mesenteric Artery Smooth Muscle Cells

VSMC were harvested from enzymatically dissociated rat mesenteric arteries by modifications of the method described by Ives et al. (8) for rabbit aorta. All procedures were carried out under aseptic conditions. Male C-D strain Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA), weighing 225–250 grams, which had been maintained on standard Purina laboratory chow and tap water ad lib., were sacrificed by cervical dislocation. The superior mesenteric artery with its major branches was excised, *en bloc*, from its origin at the aorta to the mesenteric border of the intestine, and placed in a petri dish containing ice-cold Hanks Balanced Salt Solution (HBSS, Ca^{++}, Mg^{++}-free; M. A. Bioproducts, Walkersville, MD) with 0.2 mM added Ca^{++}. Fat, adventitia, and venous structures were removed by blunt dissection, and the cleaned mesenteric artery arcades (usually two per preparation) were transferred into a 50-ml plastic tissue culture flask (Costar Data Packaging, Cambridge, MA), containing 4.0 ml of Enzyme Dissociation Mixture: HBSS (Ca^{++}, Mg^{++}-free formula) with 0.2 mM added Ca^{++}, 15 mM HEPES buffer (pH 7.2–7.3), 0.125 mg/ml elastase (Pancreatopeptidase, E.C. 3.4.4.7; Sigma Chemical Co., St. Louis, MO, Type III, 90 U/mg), 0.250 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., chromatographically prepared, Type 1-S), 1.0 mg/ml collagenase (*C. histolyticum*, CLS Type I, Worthington Biochemical Corp., Freehold, NJ, 135–180 U/mg) and 2.0 mg/ml crystallized bovine albumin (Pentex, Miles Laboratories Inc., Elkhardt, IN). After incubation at 37°C for 45 min in a gyratory shaker bath (Model G76, New Brunswick Scientific, New Brunswick, NJ) the tissue suspension was aspirated into a 10-ml plastic syringe, titurated ten times through a 12-gauge stainless steel cannula (Bard Parker), and passed through a 100-μm nylon mesh (Nitex, Tetka, Inc., Elmsford, NY) to separate dispersed cells from undigested vessel wall fragments and debris. The filtered suspension was centrifuged in a siliconized conical glass tube (200 *g*, 5 min) and the cell pellet resuspended in 10–15 ml of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (vol/vol) heat-inactivated calf serum, 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin (M. A. Bioproducts).

Each preparation (two mesenteries) usually yielded $5–6 \times 10^5$ cells with 80–90% viability by trypan blue exclusion. Based on DNA determinations of vessel wall homogenates and cell pellets (see below), this yield represents ~20% of total cell population of the excised mesenteric artery segments. This dispersed cell suspension was aliquoted into one 75-cm^2 or three 25-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) ($6–8 \times 10^3$ cells/cm^2), which were incubated at 37°C in a humidified 5% CO_2–95% air atmosphere. After 18–24 h, the cultures were washed once with HBSS to remove nonadherent cells and debris, and fed with fresh medium. Plating efficiency usually ranged from 30 to 50%. Medium was routinely exchanged at 48–72 h intervals thereafter. Primary cultures were examined daily using an inverted phase-contrast microscope (Nikon) at 100–200 times magnification. Confluent monolayers typically formed within 7 d; cell density at saturation (8–10 d) stabilized at $3–4 \times 10^4$/cm^2. Recent modifications of this cell harvesting procedure, including substitution of Minimum Essential Medium (0.2 mM Ca^{++}) for HBSS, an increased concentration of soybean trypsin inhibitor (0.375 mg/ml), extension of incubation time to 90 min, pre-sieving of the digest suspension on a 250-μm stainless steel screen, and removal of nonadherent cells after 3 h have resulted in an approximate twofold increase in viable cell yield, and a plating efficiency consistently >50%.

For comparative studies, adventitial tissues that routinely were stripped from the rat mesenteric artery arcade before dissociation, or undigested tissue fragments removed by the 100-μm nylon mesh, were plated as explants in plastic culture dishes and their cellular outgrowths cultured. Bovine smooth muscle cells were cultured from explants of the inner one-third of the medial layer of calf thoracic aorta, using standard techniques (3). Smooth muscle cells also were isolated from calf thoracic aorta by the enzymatic dissociation procedure described above. Each of these cells was grown in DMEM with 10% calf serum and the additives indicated above.

^{125}I-AII Binding Assay

Monoiodinated ^{125}I-AII (sp act 900–1,500 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Radiochemical purity, verified by thin-layer chromatography, was >98%. The lyophilized ligand was redissolved in 50 mM phosphate buffer, pH 7.4, and stored in aliquots at −20°C for up to 3 wk. Aliquots were used only once after thawing. The 1–7 heptapeptide and 3–8 hexapaptide analogues of AII were gifts of Drs. E. Haber and J. Burton; all other peptides were obtained from Peninsula Laboratories, Inc. (San Carlos, CA). The angiotensin-I-converting enzyme inhibitor, SQ-14,225 (2-D-methyl-3-mercaptopropanoyl-L-proline) was a gift of Dr. Z. P. Horovitz. All other chemicals were obtained from Sigma Chemical.

For binding assays, 6- to 8-day-old primary cultures derived from 2–6 rat mesenteric arteries were harvested and pooled ($1.2–3.6 \times 10^6$ cells, final yields) as follows. Each culture flask was washed three times with HBSS at 37°C, and incubated on ice with 2 ml of 0.2% EDTA in isotonic saline for 5 min. The cells were then gently detached with a rubber policeman, centrifuged at 200*g* for 3 min at 4°C, and washed twice in ice-cold Assay Buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.2 at 22°C). The final cell pellet was resuspended in Assay Buffer (4°C) at a concentration of 2×10^6 cells/ml and assayed immediately. Cell viability after this procedure was 85–90% by trypan blue exclusion; nonenzymatic harvesting was found to be essential for preservation of specific ^{125}I-AII binding sites. Protein concentrations were determined on sonicates of cell suspensions by the method of Lowry et al. (14). The DNA contents of dissected mesenteric artery preparations, freshly isolated cells, and cells harvested from 7-d primary cultures were determined on formalin-treated homogenates, following the method of Hinegardner (15).

Binding assays were performed by incubating 50 μl of cell suspension (10^5 cells) with 50 μl of Assay Buffer containing 0.25% bovine serum albumin (BSA) and various concentrations of ^{125}I-AII in 12 × 75-mm polystyrene tubes (Falcon Plastics, 2052). Standard assay conditions, unless otherwise noted, were 30-min incubation at 22°C. At the end of the incubation period, 3 ml of ice-cold saline were added to each assay tube, and bound and free radioactivity were separated by rapid filtration through a Whatman GF/C filter (prewetted with Assay Buffer + 0.25% BSA). The assay tubes and filter wells were washed three times with additional 3-ml portions of ice-cold saline. The filters were dried in room air and the trapped radioactivity was counted in an automated gamma counter (Searle Radiographics, Inc., Des Plaines, IL) with an efficiency of 70%. Specific binding was defined as: (total binding) minus (binding in the presence of 1 μM unlabeled AII). Filter blanks, determined from identical incubations without cells, averaged 0.40 ± 0.06% (mean ± SD) of total counts initially present.

Degradation of ^{125}I-AII during the binding assays was assayed by thin-layer chromatography. Cell suspensions (2×10^6 cells/ml) were incubated with ^{125}I-AII (1 nM) at 22°C for 30 min then centrifuged at 800 *g* for 5 min at 4°C. The supernatant was saved on ice; the cell pellet was washed once in ice-cold Assay Buffer, resuspended in ice-cold 0.05 M acetic acid (in 150 mM sodium chloride), and incubated on ice for 30 min after recentrifugation, the acid eluate (neutralized with 1.0 M NaOH) was co-chromatographed, along with the original supernatant (incubation medium) and authentic ^{125}I-AII, on thin-layer cellulose plates (Eastman 6065), and developed with tert-butyl alcohol: 3% NH_3 (105:35) as solvent. Developed chromatograms were cut into 0.25-cm strips and counted for radioactivity.

Electron Microscopy

Freshly isolated mesenteric artery cell pellets, and cells cultured for intervals of 1 d to 10 wk in plastic petri dishes or flasks, were fixed with 4.0% glutaraldehyde–0.1 M cacodylate buffer containing 0.5% $CaCl_2$ (pH 7.4) at 4°C, postfixed in 2% OsO_4, stained with uranyl acetate or tannic acid *en bloc*, dehydrated, and embedded for transmission electron microscopy as previously described (5). Thin Epon sections, cut parallel or perpendicular to the plane of the cell layer, were stained with uranyl acetate and lead citrate, and examined in a Philips EM201 electron microscope. Several cells from different cultures were examined at each time interval studied.

Evaluation of Cellular Contraction

The responsiveness of individual cultured cells to vasoactive substances was evaluated with a Nikon compound phase-contrast microscope with a photographic attachment. For this purpose, cells grown on 25-mm Diam #2 glass cover slips were superfused, in modified Rose chambers, with oxygenated (95% O_2–5% CO_2) Krebs-Ringer's-Bicarbonate buffer, pH 7.4, containing 2 mM $CaCl_2$, or Minimum Essential Medium (Eagle, Suspension formula; M. A. Bioproducts) with 5% calf serum, at a rate of 0.25 ml/min. The microscope stage, chamber, and perfusion medium were maintained at 37°C with heated air. Sequential photomicrographs were taken at 15-s to 1-min intervals, at 100–400 times magnification, before, during, and after infusion of vasoactive agonists and antagonists. A positive contractile response was judged by the following criteria: rapid onset of cell shortening after addition of agonist, reversibility upon washout of agonist, and/or inhibition by appropriate pharmacologic antagonist.

RESULTS

Isolation and Primary Culture of Vascular Smooth Muscle Cells

Enzymatic dissociation of dissected rat mesenteric arteries yielded a population of viable cells highly enriched in smooth muscle. Examination of the final cell pellet by electron microscopy (Fig. 1) showed elongated and ovoid cells that exhibited various structural features characteristic of smooth muscle (16). The cytoplasm of the majority of intact cells was filled with bundles of myofilaments, interspersed with fusiform condensations or dense bodies, while other organelles such as mito-

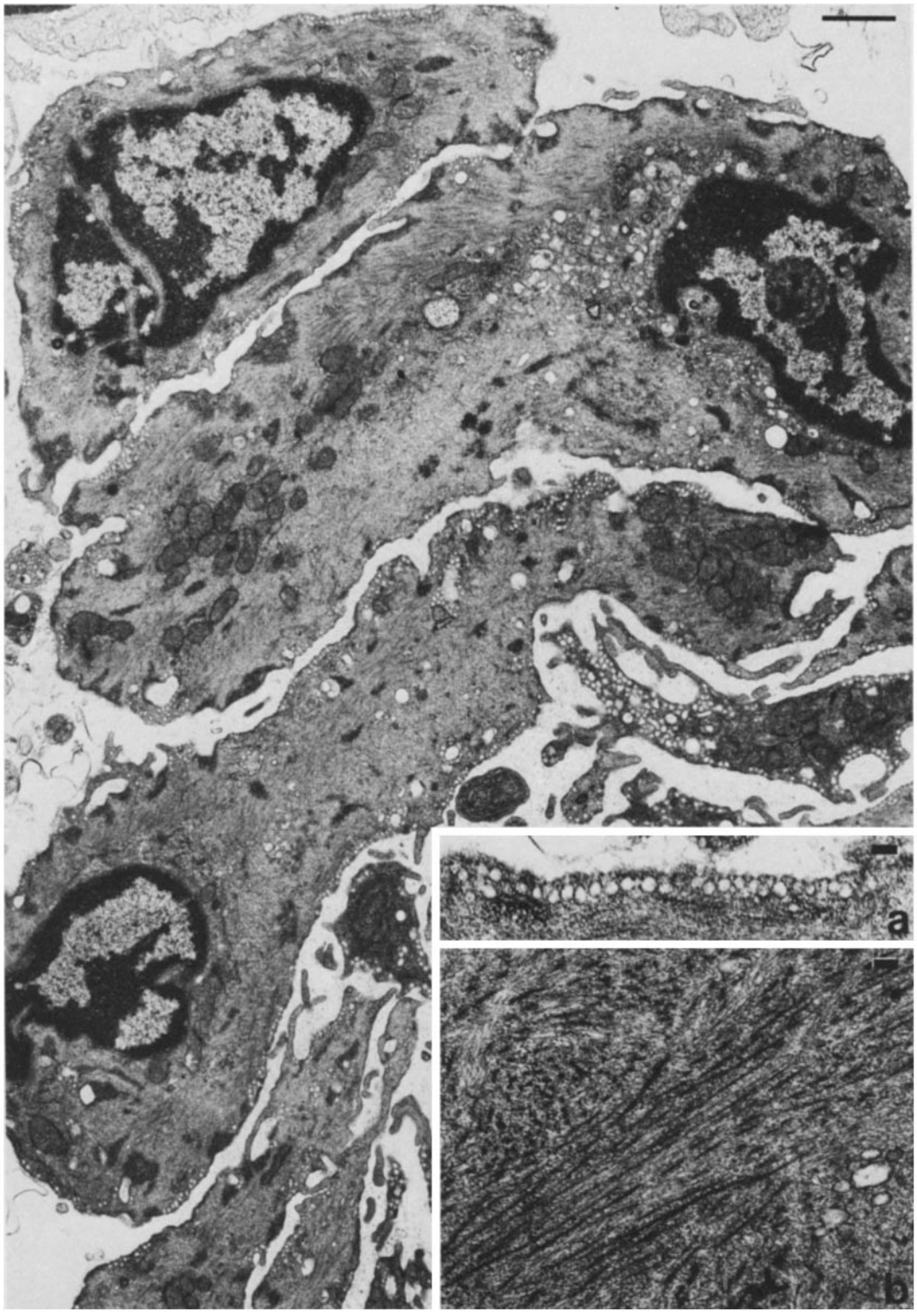

FIGURE 1 Electron micrograph of a section through a cell pellet obtained by enzymatic dissociation of rat mesenteric artery. Portions of several smooth muscle cells are seen. × 11,800. bar, 1 μm. (*a*) Row of plasmalemmal vesicles (intracellular caveolae) at cell surface × 40,000. bar, 0.1 μm (*b*) Myofilament bundles, visible in longitudinal and cross-section, contain prominent thick (12–18 nm) filaments surrounded by thin (5–8 nm) filaments × 37,500. bar, 0.1 μm.

chondria, rough endoplasmic reticulum and Golgi complexes were located near the nuclear poles. Longitudinal rows of plasmalemmal vesicles were visible along the cell surface (Fig. 1, inset *a*), but a distinct basal lamina was absent, presumably having been removed by the enzymatic dissociation procedure. At higher magnification (Fig. 1, inset *b*), myofilament bundles were seen to consist of arrays of thick (12–18 nm Diam) myosin-containing filaments, which were surrounded by numerous thin (5–8 nm Diam), actin and tropomyosin-containing filaments (10, 16).

Several hours after plating, any erythrocytes, cellular debris, and amorphous connective tissue fragments present in the resuspended cell pellet were readily rinsed from the culture dish. By 48–72 h, attached cells were well spread on the plastic substratum and had assumed an elongated shape (Fig. 2*a*). Mitotic activity usually was first noted at 56–60 h, and by 3–4 d extensive proliferation was occurring (apparent population doubling time, 24–36 h). After 7–8 d, most cultures had reached confluence, whereupon a topographical pattern of parallel alignment with areas of overlapping was apparent (Fig. 2*b*). Cell size appeared increased by phase-contrast microscopy, and the protein: DNA ratio in 7-d primary cultures (133 μg protein/μg DNA) was 2.5-fold greater than in freshly dissociated cells (53 μg protein/μg DNA). Postconfluent primary cultures that were left undisturbed for periods up to 8–10 weeks developed nodular protrusions, similar to those described previously in human vascular smooth muscle cultures (5). Although small islands of polygonal cells (Fig. 2*c*) occasionally were noted early in sparse primary cultures, these contaminants (presumably endothelial cells) did not proliferate. Dense outgrowths of fibroblast-like cells (Fig. 2*d*) were obtained from the tissue residue removed by sieving on 100-μm nylon mesh; however, similar spindle-shaped cells were not detectable in primary smooth muscle cell cultures at any stage.

Ultrastructural Changes in Primary Smooth Muscle Cultures

Smooth muscle cells underwent extensive ultrastructural changes in primary culture. Within 24 h, there was a marked decrease in organized myofilament bundles, and thick (12–18 nm Diam) filaments were no longer apparent. In particular, the absence of thick filaments at 24 h was documented in several individual smooth muscle cells that had been observed

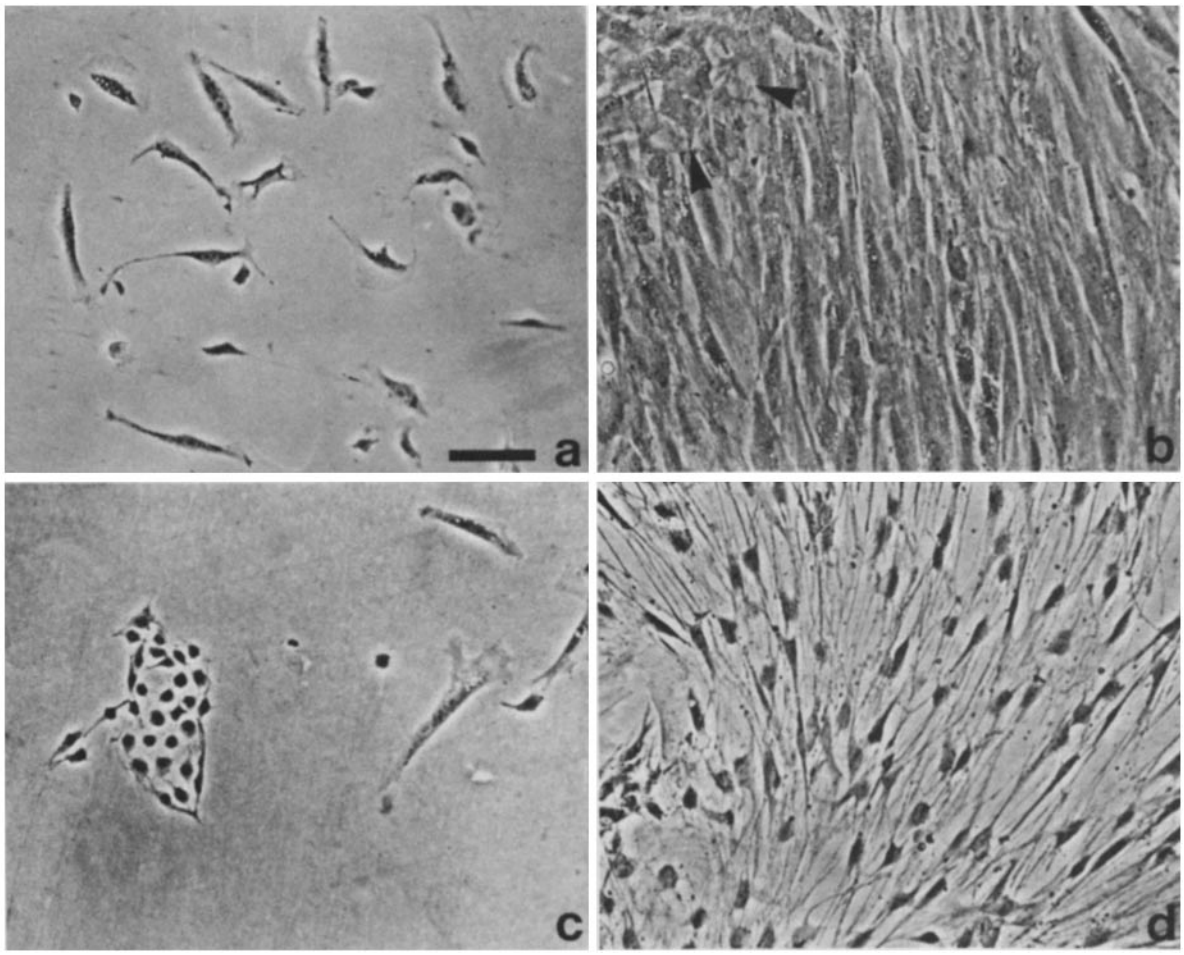

FIGURE 2 Phase-contrast photomicrographs of enzymatically dissociated rat mesenteric artery cells in primary culture × 350; bar, 30 μm. (*a*) Primary culture of smooth-muscle-rich final cell pellet at 3 d. Most cells are elongated and contain an ovoid nucleus with 1–3 nucleoli and perinuclear collections of phase-dense granules. (*b*) Confluent primary culture at 7 d shows parallel alignment of cells with developing hillock (arrows) of intertwined, overlapping cells, a growth pattern characteristic of vascular smooth muscle cultures. Cell size and cytoplasmic/nuclear ratio appear increased compared with freshly isolated and 3-d cultured cells. (*c*) Island of small polygonal cells (presumably endothelium) in a sparse primary culture at 3 d. These focal contaminants failed to proliferate in culture. (*d*) Outgrowth of spindle-shaped fibroblast-like cells from explant of undigested tissue residue trapped on 100-μm nylon mesh (see Materials and Methods). Such cells were not observed in primary smooth muscle cultures.

to undergo a contractile response (see below) upon superfusion with AII. By 72 h (Fig. 3 *a*), most cells contained autophagic vacuoles, abundant free ribosomes and polysomes, but only isolated bundles of thin (5–8 nm Diam) and intermediate (100 nm Diam) filaments. As cultures grew to confluent densities (days 6–8), increasing numbers of thin filaments became organized in longitudinal fashion; however, thick filaments not observed. In postconfluent primary cultures, examine intervals from 2 to 10 wk, accumulation of fibrillar and ar phous extracellular matrix components was evident, and cytoplasm of most cells was filled with broad bundles longitudinally organized filaments (Fig. 4 *a*). At higher m

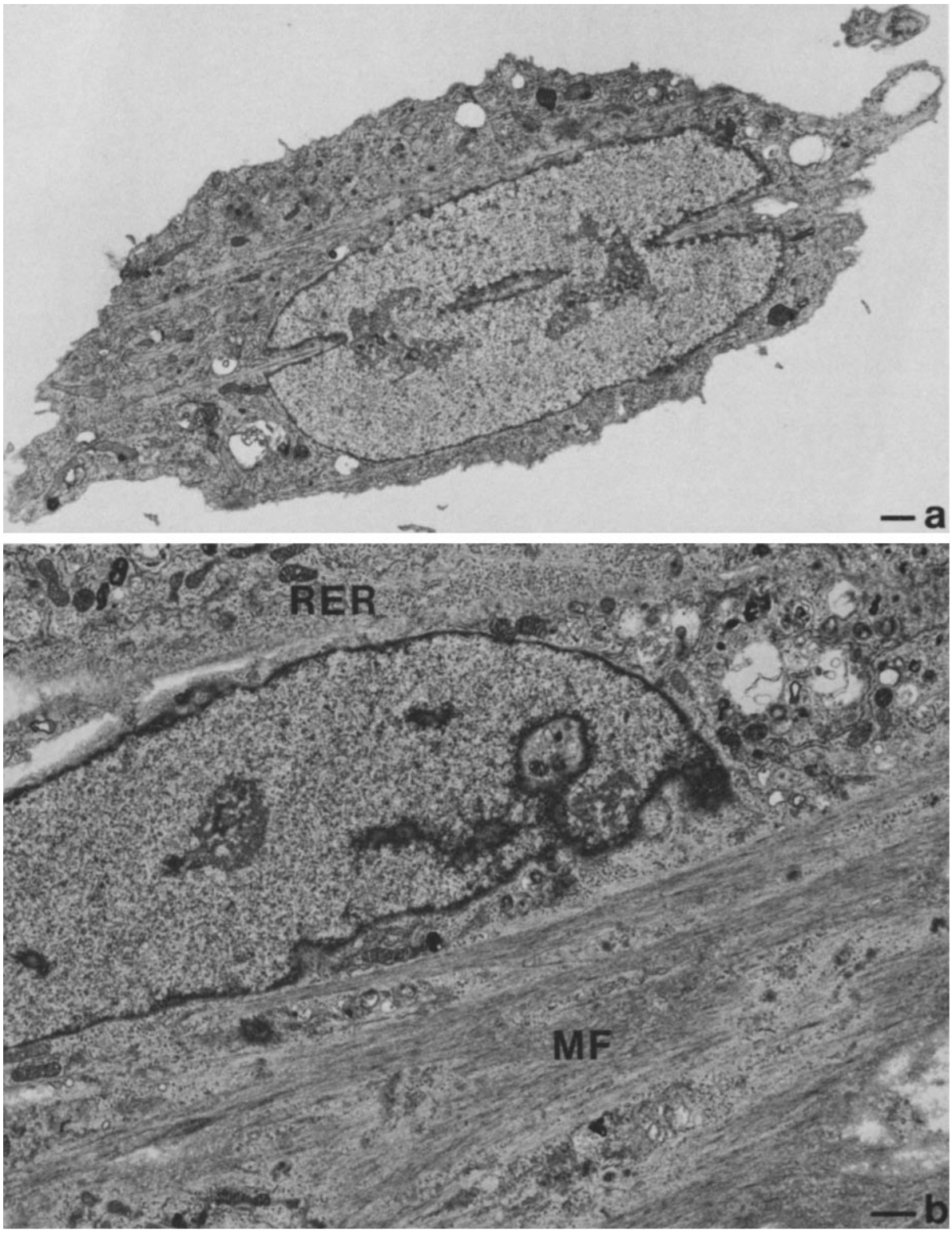

FIGURE 3 Electron micrographs of rat mesenteric artery smooth muscle cells in primary culture. Cells were fixed *in situ* and sections taken parallel to the surface of the culture dish. Bars, 1 μm. (*a*) Cells in 3-d subconfluent culture. Note loss of myofilament bundles. × 5500. (*b*) Portions of 2 cells in 8-d confluent culture. Myofilaments have reorganized in longitudinal bundles (*MF*), and polysomes and rough endoplasmic reticulum (*RER*) are present. × 7300.

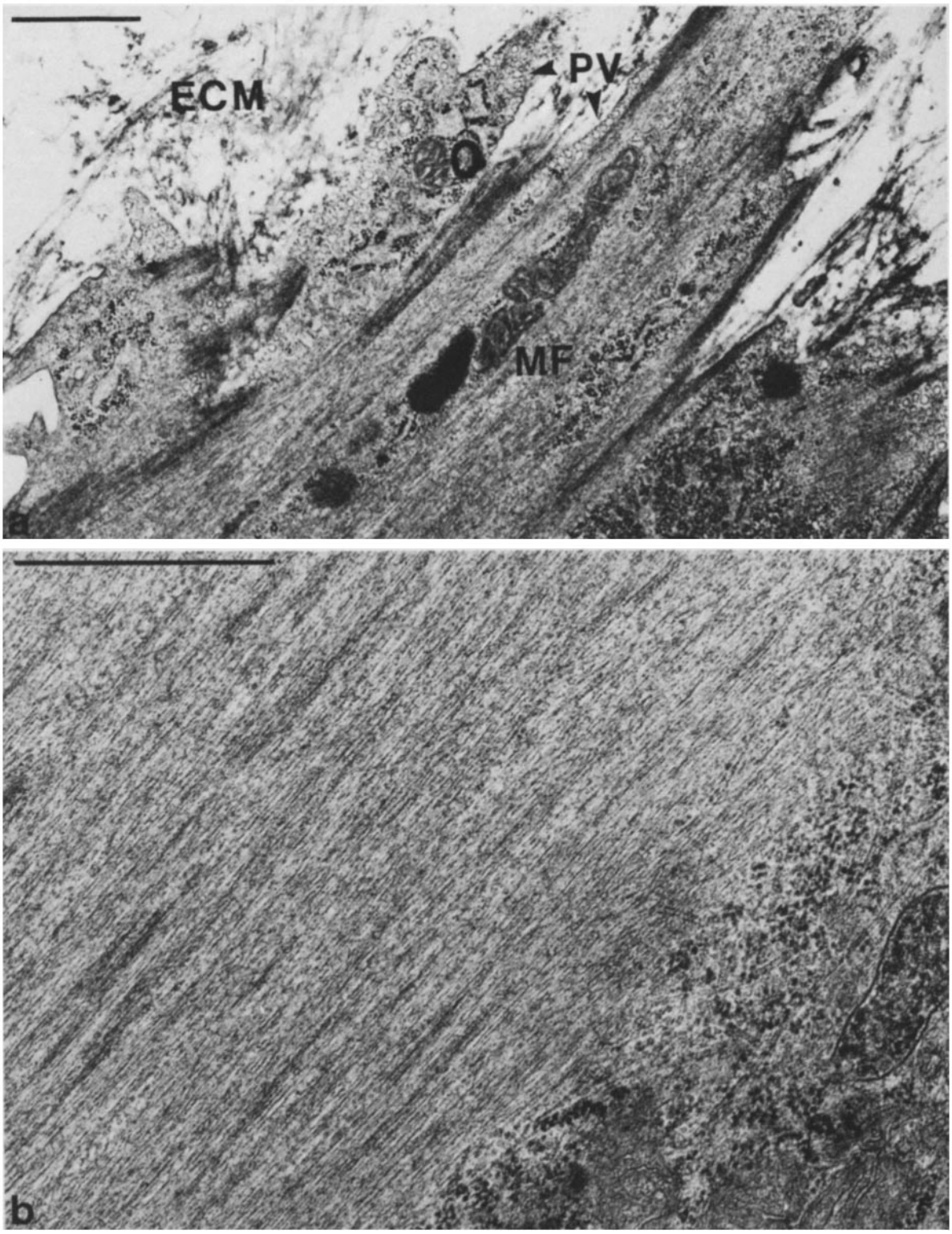

FIGURE 4 Electron micrographs of rat mesenteric artery smooth muscle cultures. Bars, 1 μm. (*a*) Section through peripheral area of smooth muscle cell in eight-week-old primary culture. Note fibrillar and amorphous extracellular matrix (*ECM*), rows of plasmalemmal vesicles (*PV*), and longitudinal bundles of myofilaments (*MF*). × 21,500. (*b*) Detail of myofilaments in smooth muscle cell in postconfluent primary culture. Bundles of thin (5–8 nm) filaments are interspersed with fusiform dense bodies; no thick (12–18 nm) filaments are seen. × 43,000.

nification (Fig. 4*b*), these myofilament bundles were seen to consist predominantly of 5- to 8-nm filaments, interspersed with electron-dense fusiform condensations; no 12- to 18-nm Diam filaments were observed in numerous cells examined at each time point.

Binding of ^{125}I-AII to Cultured Vascular Smooth Muscle Cells

Freshly isolated rat mesenteric artery smooth muscle cells and cells maintained in primary culture for 4 to 20 d exhibited

specific ^{125}I-AII binding (Table I, Fig. 5). In 7-day-old primary cultures, cellular binding of ^{125}I-AII was saturable at nanomolar concentrations of ligand (Fig. 5). Specific binding was >98% of total binding. Scatchard analysis (inset, Fig. 5) indicated a single class of high-affinity binding sites, with an apparent equilibrium dissociation constant (K_d) of 2.8 ± 0.2 (SD) nM, and a total binding capacity of 81.5 ± 5.0 fmol/mg protein. This binding capacity in 7-d cultures corresponded to a receptor density of ≃45,000 sites per cell. Although ^{125}I-AII binding in cultured cells was increased (≃twofold) as compared with freshly isolated cells, the number of binding sites per cell appeared to remain relatively constant from 4 to 20 d (Table I).

Binding of ^{125}I-AII in 7-d-old primary cultures was rapid, with a half-time for association at 22°C of 2.5 min (Fig. 6). After the addition of a large excess of unlabeled AII, an initial rapid dissociation of bound ^{125}I-AII was followed by a slower phase of dissociation, which reached ~55% of the original value at 30 min. This result is not entirely attributable to endocytosis of hormone, as >85% of specifically bound ^{125}I-AII (after 40-min incubation at 22°C) was releasable from the cell surface by brief proteolytic treatment (228 U/ml TPCK-trypsin, Worthington Biochemical Corp., 5 min, 37°C, pH 7.2). Fitting of the early phase kinetic data depicted in Fig. 6 to the second order and pseudofirst order rate equations yielded values for the association and dissociation rate constants of 3.8 × 10^5 M^{-1} sec^{-1} and 9.8 × 10^{-4} sec^{-1}, respectively. The K_d calculated from these rate constants) (k_{-1}/k_1) was 2.6 nM, in good agreement with that obtained from steady-state Scatchard analysis.

The specificity of the binding sites for AII compared with its analogues and antagonists is illustrated in Fig. 7. The AII antagonists (Sar_1, Ile_8)- and (Sar_1, Ala_8)-AII were equipotent with the native hormone in competing for binding, while ($desAsp_1$)-AII (AIII) was only ~15% as potent. The relative potency of AI (the immediate metabolic precursor of AII in vivo) was unchanged when incubations were performed in the presence of 10 μM concentrations of the AI converting enzyme inhibitor, SQ-14,225 (data not shown), indicating the lack of significant conversion of AI to AII under these assay conditions. The inactive metabolites of AII, 1–7 heptapeptide and 3–8 hexapaptide, were essentially without effect in competing for binding.

Thin-layer chromatographic analysis of radioactivity remaining free in the assay medium, after 30-min incubation at 22°C with cultured smooth muscle cells, showed considerable degradation of ^{125}I-AII (Fig. 8 *a*). However, >75% of the radioactivity remaining bound to the cells and subsequently eluted co-chromatographed with authentic ^{125}I-AII (Fig. 8 *b*).

For purposes of comparison, smooth muscle cells were cultured, in parallel, from the medial layer of calf thoracic aorta using a standard explant outgrowth method (3) and the enzymatic dissociation procedure described in this report. High-affinity ^{125}I-AII binding was rarely detectable in aortic explant outgrowth cells. In contrast, ^{125}I-AII binding consistently was present in primary cultures of VSMC enzymatically isolated from bovine aorta; however, the amount of binding observed with these cells usually was less (on a per cell basis) than that seen with rat mesenteric artery cells. Fibroblastlike cultures, obtained from the adventitial strippings of rat mesenteric arteries (see Materials and Methods), also showed <20% of the specific ^{125}I-AII binding obtained with 7-d primary cultures of

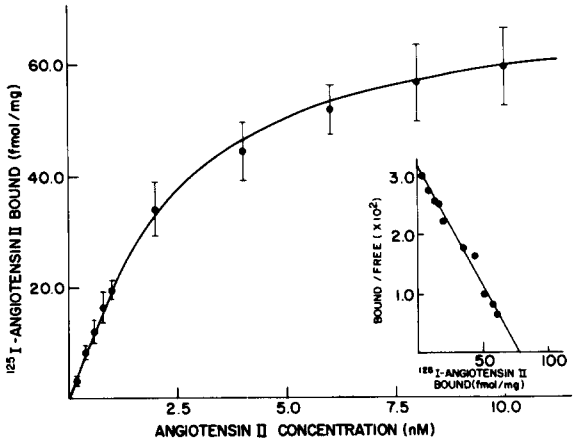

FIGURE 5 Saturation of ^{125}I-angiotensin II binding to 7-d-old cultured rat mesenteric artery smooth muscle cells. Increasing concentrations of radioligand were incubated with suspensions of 10^5 cells for 30 min at 22°C in a metabolic shaker bath. Each point represents mean specific binding (±1 S.D.) in five separate experiments. Inset: Scatchard analysis of ^{125}I-angiotensin II binding data. A single class of high-affinity binding sites is evident (K_d = 2.8 ± 0.2 nM); the total binding capacity (= 81.5 ± 5.0 fmol/mg) represents ~45,000 sites per cell.

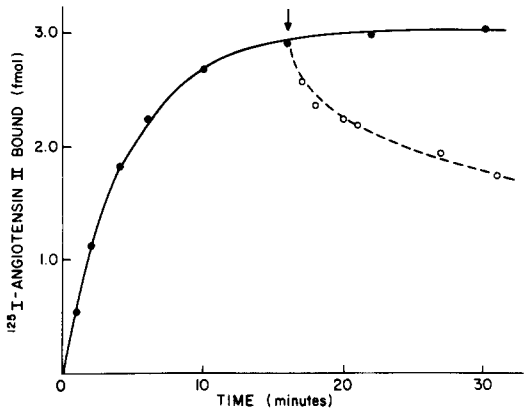

FIGURE 6 Time-course of binding of ^{125}I-angiotensin II to rat mesenteric artery smooth muscle cells. Radioligand (0.5 nM) was incubated with 10^5 cells for various periods of time at 22°C (solid circles). In certain incubations, unlabeled angiotensin II (1 μM final concentration, 5 μl) was added at time indicated (arrow) and the amount of ^{125}I-angiotensin II remaining bound was followed as a function of time (open circles). Each point represents the mean of four separate experiments.

TABLE I

Retention of ^{125}I-Angiotensin II Binding Sites in Cultured Rat Mesenteric Artery Smooth Muscle Cells

Days in culture	^{125}I-Angiotensin II bound	Angiotensin II receptor density
	fmol/ 10^6 cells	*sites/ cell*
0	10.0 ± 1.6	25,100
4	18.7 ± 2.5	46,900
6	18.9 ± 1.9	47,400
8	17.1 ± 2.3	42,900
20	14.8 ± 2.5	37,100

Aliquots of 10^5 cells were incubated with ^{125}I-AII (1 nM) in 100 μl of Assay Buffer for 30 min at 22°C and specific binding was determined as described in the text. Day "0" represents cells assayed immediately after the 45-min enzyme dissociation procedure; the remaining values are for cells maintained in primary culture for the indicated times. The binding data (mean ± SD, duplicate determinations from 3–5 separate experiments) have been extrapolated using the Scatchard analysis data shown in Fig. 5 to yield estimates of the corresponding AII receptor density per cell at each time-point.

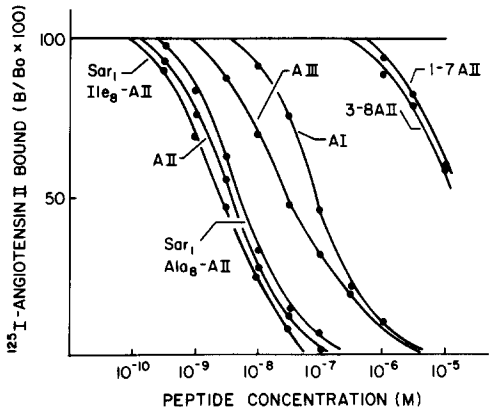

FIGURE 7 Specificity of ^{125}I-angiotensin II binding sites in cultured rat mesenteric artery smooth muscle cells. ^{125}I-angiotensin II (0.5 nM) was incubated with 10^5 cells for 30 min at 22°C and specific binding determined. B and B_0 refer to specific binding in the presence and absence of the indicated unlabeled angiotensin analogues and antagonists. Data points represent mean of four separate experiments.

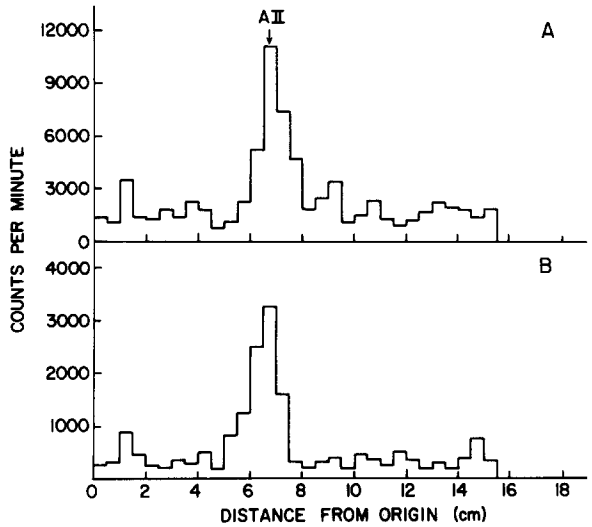

FIGURE 8 Thin-layer chromatography of radioactivity after incubation of ^{125}I-angiotensin II with rat mesenteric artery smooth muscle cells. 2 × 10^6 cells were incubated with ^{125}I-angiotensin II (1 nM) at 22°C for 30 min. (*A*) Unbound radioactivity remaining in medium after incubation; (*B*) Bound radioactivity acid-eluted from cells. ~76% of the radioactivity associated with cells appears to represent undegraded angiotensin II.

rat mesenteric VSMC. Some smooth muscle cells, presumably from small side branches of the mesenteric artery, were present in these fibroblastlike cultures.

Hormone-induced Cell Contraction

Enzymatically dissociated rat mesenteric artery VSMC in primary culture responded to AII with a reversible contractile response (Fig. 9). Nanomolar concentrations of AII (threshold dose, 0.3 nM) induced a progressive shortening in cell length that began within 30–45 s and reached a maximum after 1–5 min. When hormone-free buffer was reperfused, relaxation began within 3–5 min; subsequent challenge with hormone often elicited a second contraction. Contractile responses to AII were blocked by pretreatment with the specific antagonist (Sar$_1$, Ile$_8$)-AII (1 μM). The cells also contracted reversibly when exposed to 10^{-8} to 10^{-6} M concentrations of epinephrine, and this response was blocked by the alpha-adrenergic antagonist phentolamine (0.1 mM).

The percentage of contractile cells varied among individual primary cultures and at different times in the same culture. Responsiveness typically was maximal (≃30%) during the first 3 d in culture but was greatly reduced during the subsequent period of cell proliferation. Hormone-induced contractility again was evident after confluence (7–9 d), although, due to cell-cell overlap, the actual percentage of contracting cells was not readily discernible. Some primary cultures remained hormonally responsive after being maintained at postconfluent densities for as long as two months. Spontaneous (i.e., not stimulus-induced) contractions were not observed in primary rat mesenteric artery VSMC cultures.

DISCUSSION

The purpose of these studies was to develop an in vitro system to study the interaction of vasoactive hormones, such as AII, with target cells in muscular, resistance-type arteries. The enzymatic dissociation procedure described in this report yields viable smooth muscle cells that proliferate in primary culture, yet retain various biochemical and functional properties relevant to the study of vasoactive hormone action.

The ^{125}I-AII binding sites identified in cultured rat mesenteric artery VSMC are essentially similar to those of the AII receptors present in particulate fractions prepared from the same blood vessel in vivo (13). The affinity of these cellular binding sites (K_d, 2.8 nM by Scatchard analysis; 2.6 nM by kinetic analysis) is sufficiently high to permit interaction with AII at concentrations present in rat blood under various physiological conditions (17). This high affinity contrasts with the considerably lower affinities (K_d, 13–55 nM) reported for AII binding sites in the aorta (18–20). Association and dissociation rate constants estimated from kinetic analysis of ^{125}I-AII binding (Fig. 6) are consistent with the time-course of AII action in intact blood vessels. Scatchard analysis (Fig. 5, inset) indicates that the radioligand is interacting with a single class of high affinity sites without cooperativity, that is, according to the law of simple mass action. Pharmacologic studies have shown a similar mechanism for the interaction of AII with its receptors in blood vessels (21). The specificity of cellular binding sites for AII and its analogues (Fig. 7) is identical to that observed for vascular AII receptors in vivo and in isolated preparations of blood vessels (20, 22, 23). Moreover, the ability of cultured cells to contract in response to AII (Fig. 9), in the concentration range at which radioligand binding was observed, indicates that at least a portion of these binding sites are functional receptors.

Specific ^{125}I-AII binding was increased twofold in cells that had been cultured for 4 to 20 d, as compared with freshly isolated cells (Table I). This conceivably could reflect regeneration of receptor sites destroyed during the enzymatic dissociation procedure, as suggested by Ives et al. (8), or, alternatively, may be related to the cellular hypertrophy (increased cell size and presumably cell membrane surface area) observed in the cultured cells (Fig. 2*a*, *b*; Table I, legend). It is noteworthy that once cells were established in primary culture, the number of AII binding sites per cell remained relatively constant (Table I). Studies in progress in our laboratory, however, indicate that the expression of AII receptors in cultured VSMC is not a static process, and may be subject to hormonal regu-

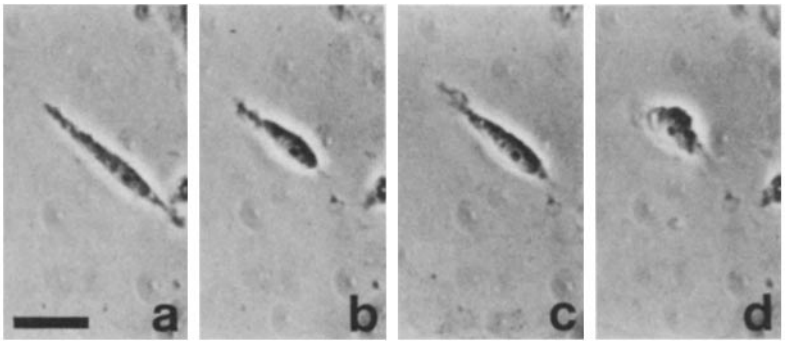

FIGURE 9 Sequential phase-contrast photomicrographs of contractile response of isolated rat mesenteric artery smooth muscle cell in primary culture. × 500. bar, 20 μm. (*a*) 15-min superfusion with control medium; (*b*) 1 min after infusion of 10 nM angiotensin II; cell has shortened to 65% of original length; (*c*) 10-min washout with control medium; note partial relaxation to 86% of original length; (*d*) 3 min after rechallenge with 10 nM angiotensin II; cell has shortened to 42% of original length.

lation. Exposure to elevated levels of angiotensin II in vitro induces time-dependent, reversible decreases in receptor number (down-regulation), that appear to reflect, in part, changes in the kinetics of receptor turn-over (24). This cultured VSMC system, isolated from neurogenic and systemic influences, provides the opportunity to directly correlate receptor status with biochemical and contractile responsiveness, and thus should facilitate the study of cellular mechanisms of angiotensin receptor regulation (25).

As seen by electron microscopy, the well organized myofilament bundles present *in situ* (not shown) and in enzymatically isolated rat mesenteric artery smooth muscle cells (Fig. 1) underwent extensive disruption within 24 h in culture. Thick (12- to 18-nm Diam, presumably myosin-containing) filaments were not detected in cultured cells examined at times ranging from 24 h to 10 wk, although longitudinally oriented bundles of thin (5- to 8-nm Diam, presumably actin-containing) filaments were prominent in postconfluent cultures (Figs. 3 and 4). It is especially noteworthy that thick filaments were not visualized using standard ultrastructural methods, even in cells that were undergoing a hormone-induced contractile response. Thus, the absence of thick filaments by electron microscopy did not correlate, in the present study, with loss of specific vasoactive hormone receptors (Table I) or hormone-induced contractility (Fig. 9). Experimental difficulties in demonstrating thick, myosin-containing filaments in vascular smooth muscle cells often have been attributed to suboptimal preparatory techniques or other methodological considerations (26–28).

In contrast to the apparent absence of myosin-containing filaments by electron microscopy, primary rat mesenteric artery VSMC do exhibit a specific immunofluorescent cytoskeletal pattern when stained with antibodies directed against smooth muscle myosin (D. Larson, K. Fujiwara, R. W. Alexander, M. A. Gimbrone, Jr., unpublished observations). As we have recently reported (29), myosin heavy-chain protein also is detectable in 7- to 10-d confluent primary VSMC cultures by immunoprecipitation and gel electrophoresis; furthermore, exposure of these cells to AII stimulates ($K_d \simeq 0.2$ nM) phosphorylation of a 20,000-dalton protein that exhibits properties of myosin light chain. This hormone-induced phosphorylation is inhibited by dibutyryl cyclic-AMP and the calmodulin inhibitor chlorpromazine. In addition, we previously have observed that these cultures produce prostaglandins in response to AII (30) and have a catecholamine- and prostaglandin-stimulated adenylate cyclase system (31). Thus, various biochemical mechanisms involved in vasoactive hormone receptor coupling appear to remain intact in these cultured VSMC, despite their proliferation, cellular hypertrophy, and altered ultrastructural appearance.

Phenotypically modulated VSMC, cultured from explants of large blood vessels such as the aorta, have been used extensively to study cell growth, extracellular matrix synthesis and lipid metabolism (10). Such cultures have been especially useful for characterization of platelet-derived (32) and other (33, 34) growth-stimulatory factors implicated in the pathogenesis of atherosclerosis. Morphological and biosynthetic similarities noted between aortic explant cells in culture and the proliferating "modified smooth muscle cells" present in atherosclerotic lesions (35, 36) have provided a rationale for this experimental approach. However, the consistent failure of explanted large vessel VSMC to show agonist-induced contraction has hampered their use in studies of the interactions of vasoactive hormones with smooth muscle cells (10). As seen in this study, high-affinity ^{125}I-AII binding sites were not consistently detectable in explant outgrowth cultures of bovine aortic media VSMC but were present in enzyme-dissociated primary cell cultures prepared from the same tissue. It appears that the explant techniques may select for a population of cells capable of migrating and proliferating but contributing little to vascular tone, or, alternatively, results in the dedifferentiation of cells with loss of specific hormone receptors. The usefulness of enzymatic dissociation for isolating hormonally responsive VSMC recently has been demonstrated by other investigators, working with various large arteries and veins (7, 8). The current study extends this approach to a highly reactive, muscular artery that is more representative of the peripheral resistance vasculature.

Finally, the demonstration of functional angiotensin receptors in cultured VSMC, which have undergone proliferation and marked morphological changes, implies that the transition from "contractile" to "phenotypically modulated" state (10) may involve several discrete processes and need not be a global phenomenon (37). The in vitro system described in this report should lend itself to the study of the various factors (e.g., mechanical forces, cellular interactions, extracellular matrix components, hormonal, and other trophic influences) necessary for the maintenance of normal structure and function in the vascular smooth muscle cell.

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