

## PREPARATION OF FUNCTIONAL SMOOTH MUSCLE CELLS FROM THE RABBIT AORTA\*

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Studies on hormone induced contraction of vascular smooth muscle strips *in vitro* have yielded a large body of pharmacological and biochemical data (1). Unfortunately, vascular strips possess inherent limitations for answering certain questions concerning sites of hormone binding on their constituent smooth muscle cells. Diffusion barriers in the tissue may hinder the use of macromolecular receptor probes and extracellular matrix elements may cause nonspecific binding of radiolabeled receptor probes. In intact tissue, it is also impossible to determine differences in receptor density and contractile responses among individual smooth muscle cells. The use of isolated cell preparations may eliminate these problems.

Previous studies have utilized two approaches for obtaining isolated cells from vascular smooth muscle. First, cultures of such cells have been obtained by outgrowth from explants of large vessels (2-5) or from dissociated cells (5-7). While cells cultured for long term generally retain some of the structural and biochemical features of vascular smooth muscle cells *in situ*, they appear to be phenotypically altered in that their content of myosin is markedly reduced (7) and they are not responsive to contractile stimuli (3, 4). It is not yet known whether receptors for vasoactive agents are present on these cells. Second, freshly dispersed vascular smooth muscle cells have been prepared by enzymatic digestion. Peters et al. (8) used crude collagenase, elastase, and hyaluronidase to dissociate the rabbit aorta into single smooth muscle cells many of which were highly damaged, while Gimbrone and Cotran (6) used crude collagenase alone to generate a mixed population of endothelial and smooth muscle cells from human umbilical veins or arteries. Neither of these groups described contractile responses of their cells and we have found the procedure of Peters et al. to result in small numbers of viable but hormonally unresponsive cells. Several groups (9-11) have described dissociation procedures yielding contractile smooth muscle cells, but from nonvascular tissue.

Given the limitations of these various procedures, we undertook to devise a tissue dissociation procedure which would produce good yields of hormonally responsive vascular smooth muscle cells from rabbit aorta (12). While our studies were underway, Chamley et al. (7) reported on the use of enzymatic digestion to obtain smooth muscle cells from a variety of mammalian blood vessels. An unstated percentage of these cells

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were capable of contracting in response to agonists after 2–3 days in culture and contractile responses disappeared after 9 days in culture. Responses to agonists immediately after dissociation were not reported nor was an extensive examination of their pharmacologic properties undertaken.

We describe here a new dissociation procedure for rabbit aorta which results in a good yield of dispersed smooth muscle cells capable of responding to agonists which cause contraction of aortic strips. In culture, these cells retain contractile activity for at least 5 days.

## Materials and Methods

### Materials

The medium (Krebs-Ringer Hepes solution [KRH]<sup>1</sup>) in which tissue dissociation and short-term incubations were carried out consisted of 105 mM NaCl, 5 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 2.0, or 0.2 mM CaCl<sub>2</sub> (depending on the stage of dissociation), 25 mM Hepes, 14 mM glucose, a complete amino acid supplement (13), 0.1% soybean trypsin inhibitor (STI), and 10 µg/ml phenol red. The medium was adjusted to pH 7.4 and equilibrated with air.

Cells were cultured in medium 199 with 10% fetal calf serum, and penicillin-streptomycin-fungizone solution (Flow Laboratories, Inc., Rockville, Md.). 35-mm Tissue culture dishes were from BioQuest, BBL & Falcon Products, Cockeysville, Md.

80 U/mg Purified elastase, chromatographically purified collagenase from *Clostridium histolyticum* (450 U/mg), STI, and chromatographically purified DNase I (2,800 U/mg) were obtained from Worthington Biochemical Corp., Freehold, N. J. For more recent experiments we used equivalent amounts of purified collagenase prepared in our laboratory (14).

l-norepinephrine, acetylcholine chloride, carbamylcholine chloride, phenoxybenzamine, and atropine sulfate were from Sigma Chemical Co., St. Louis, Mo. [Asp<sup>1</sup>,Ile<sup>5</sup>]-angiotensin II (human) was synthesized by Dr. R. E. Galardy. [Sar<sup>1</sup>,Ala<sup>8</sup>]-angiotensin II was kindly supplied by Eaton Laboratories, Division of Norwich Pharmaceuticals, Norwich, N. Y. Washout of angiotensin from culture dishes during the contraction experiments was measured by the addition of 2 ml 10<sup>-6</sup> M [<sup>3</sup>H]labeled angiotensin II (AT II) (SA = 3.6 × 10<sup>-1</sup> Ci/mmol) obtained from New England Nuclear, Boston, Mass. Counts before and after washing were used to determine the reduction in hormone concentration obtained with a given number of wash cycles.

*Light and Electron Microscopic Procedures.* Suspensions of dispersed cells and slices of intact tissue (0.5 mm<sup>2</sup>) were fixed in 2% formaldehyde and 2% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) for 2 h at 4°C. The aldehyde fixed cell suspension was centrifuged in a Beckman model 152 microfuge (Beckman Instruments, Inc., Fullerton, Calif.). Tissue and cell pellets were postfixed in 1% OsO<sub>4</sub> in 0.1 M Na cacodylate, pH 7.4 for 30 min at 4°C, washed in cold isotonic saline, and stained in block with 0.5% Mg uranyl acetate in isotonic saline for 2 h at 25°C. The material was dehydrated in ethanol and propylene oxide and embedded in Epon (Polysciences, Inc., Warrington, Pa.) (15). Thin sections were doubly stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101 electron microscope. 0.5-µm Epon sections were stained with 1% toluidine blue in 1% Na borate (15) and examined with a Zeiss Photomicroscope II (Carl Zeiss, Inc., New York). Living cells were examined with phase or differential interference contrast optics in the same microscope, except for cultured cells which were studied with a Zeiss inverted phase contrast microscope.

## Results

### Dissociation Procedure

**METHODOLOGY.** Adult male New Zealand white rabbits weighing ≈2 kg were killed by intravenous injections of 4 ml of 0.1% sodium pentobarbital. Thoracic aortas

<sup>1</sup> Abbreviations used in this paper: AT II, angiotensin II; Hepes, N-2-hydroxymethyl piperazine-N'-2-ethanesulfonic acid; KRH, Krebs-Ringer Hepes; STI, soybean trypsin inhibitor.

were removed from the arch to the celiac axis, opened lengthwise, and pinned with the intima facing up to Petri dishes with wax coated bottoms containing KRH solution (2.0 mM  $\text{Ca}^{2+}$ ) at 25°C. The intima was removed by light scraping with a scalpel blade and the media was dissected bluntly from underlying adventitia. The separated sheets of media were chopped into slices 0.5 mm per side with a McIlwain tissue chopper (Brinkmann Instruments, Inc., Westbury, N. Y.) and washed twice at 25°C in KRH with 0.2 mM  $\text{Ca}^{2+}$ . Slices from four aortas were pooled ( $\approx 1$  g tissue wet weight) and added to 10 ml KRH with  $[\text{Ca}^{2+}]$  of 0.2 mM and containing 8 mg collagenase, 12 mg elastase, 10 mg STI, and 0.2 mg DNase I in a 25-ml Erlenmeyer flask. This mixture was shaken linearly at 120 strokes per min in a 37°C bath (American Optical Corp., Scientific Instrument Div., Buffalo, N.Y.). The pH of the suspension, which was monitored by phenol red and pH electrode, fell in the early phase of dissociation. 10  $\mu\text{l}$  of 1 N NaOH was added every 15 min after the start of the dissociation to maintain a pH of 7.4. All solutions were gassed with air and all glassware was siliconized. The progress of dissociation was monitored by phase contrast microscopy. During the first 40 min, the tissue pieces became fluffier by eye but no free cells were visible. Between 40 and 50 min the cell packing became very loose at the edges of the tissue and some free cells were seen. At 55–65 min, when many free cells and small clumps of cells were visible, but  $\approx 20\%$  of the original tissue slices were still partly intact, the slurry was subjected to vigorous pipetting through a siliconized 7 inch Pasteur pipet (tip flamed to diameter of 0.5 mm) to break up remaining tissue slices and cell clumps without damaging single cells. The mixture was then filtered through 150  $\mu\text{m}$  Nytex gauze, Tetko, Inc., Elmsford, N.Y., to remove large tissue debris and centrifuged at 150  $g$  for 2 min in a Sorvall GLC-1 centrifuge (Du Pont Instruments, Newtown, Conn.) to separate single cells from the enzymes and stop the digestion. Dissociation times longer than 75 min were found to result in reduced yields of viable cells. The pelleted cells were washed twice with fresh, enzyme-free KRH medium containing 2.0 mM  $\text{Ca}^{2+}$  and resuspended in 2 ml of the same medium in a 10-ml Erlenmeyer flask at 37°C. Cells could be maintained in suspension for several hours by slow agitation in a 37°C bath and occasional pipetting with the flamed Pasteur pipet to break up aggregates.

*Yield of Cells.* Yields were determined by comparing DNA content of the isolated cell pellets and starting material. Samples were homogenized and sonicated, then precipitated and washed with cold 0.5 M perchloric acid. DNA was extracted by heating to 80°C for 20 min in 0.5 M perchloric acid and assayed by the method of Burton (16). DNA in aortic media slices was  $\approx 900 \mu\text{g}$  per g wet weight. The cell pellet resulting from dissociation of 1 g of tissue contained an average of 200  $\mu\text{g}$  DNA and  $2.3 \times 10^7$  cells by hemocytometer counts. This represents a yield of  $\approx 25\%$  of the starting DNA. Greater than 95% of these cells excluded trypan blue and approximately 80–90% adhered to tissue culture dishes.

*Role of Individual Agents in the Dissociation Protocol*

**CALCIUM IONS.** The  $[\text{Ca}^{2+}]$  to which aortic strips were exposed during dissociation appeared to be a factor in determining whether cells could contract immediately after dissociation. When 2 mM  $\text{Ca}^{2+}$  was used, good yields of viable cells were obtained but the cells appeared crenated and often only a small percentage responded to hormonal stimulation. Dissociations performed in medium containing less than 0.1 mM  $\text{Ca}^{2+}$

produced low yields of nonviable or noncontractile cells. The optimal  $[Ca^{2+}]$  required to obtain maximal yields of hormonally responsive cells during tissue dissociation was 0.2 mM. Restoration of  $[Ca^{2+}]$  to 2.0 mM immediately after dissociation was required to maintain cell structure and function thereafter.

**ENZYME CONCENTRATIONS.** Peters et al. (8) used 300 U/ml crude collagenase, 40 U/ml elastase, and 800 U/ml hyaluronidase to dissociate the rabbit aorta into single cells. In our procedure the presence of hyaluronidase did not alter the yield, the time needed for dissociation, or response of the cells to hormones after dissociation. We found that 300–400 U/ml collagenase was optimal for tissue dissociation in agreement with Peters et al. (8). However, the contaminant enzymes found in crude collagenase (primarily clostripain) may damage cells since dissociations performed with it resulted in cells which were unresponsive to hormones. Therefore, purified enzyme was used in this study. We also found that increasing the concentration of elastase from 40 U/ml to 100 U/ml decreased the time needed for dissociation and increased the yield of functional cells.

1 mg/ml STI was used in our dissociation procedure to inhibit tryptic or chymotryptic activities (17) potentially contaminating the enzyme preparations. We have found our collagenase to be essentially free of such activities (14); however, serine protease contamination has been demonstrated in elastase preparations similar to the one which we are using (18, 19). In five experiments in which STI was not used less than 5% of the dissociated cells contracted in response to hormones.

**Tissue Culture of Dispersed Aortic Cells.** Immediately after dissociation, the smooth muscle cells were washed and resuspended at  $2 \times 10^5$  cells/ml in medium 199 buffered with 25 mM Hepes, pH 7.4, containing penicillin-streptomycin-fungizone. 0.5 ml of this suspension was plated onto 35-mm Falcon tissue culture dishes, with 80–90% attachment of the cells within 2 h. Fetal calf serum was not used for the first 2 h as it significantly reduced the number of cells that attached to the dishes. Thereafter, 10% FCS was used since significantly greater numbers of the attached cells spread out on the dishes in its presence than in its absence. All media were changed every 24 h.

**Contractile Response of Acutely Dispersed Aortic Cells.** The contraction of acutely dispersed aortic smooth muscle cells was observed by phase and Nomarski differential interference contrast microscopy. Several drops of a cell suspension containing  $2-3 \times 10^6$  cells per ml were placed in a channel 3 mm wide formed between two no. 0 cover slips on a glass microscope slide which was maintained at 37°C with a thermostated air curtain. A third no. 0 cover slip was placed over the channel. Cells adhered to the glass and could be washed with fresh medium without changing their position on the slide. The medium was rapidly changed by placing 50–100  $\mu$ l at one end of the channel and a slip of filter paper at the other end. The filter paper was removed after 10–20 s to avoid drying of the channel.

When the bathing medium was replaced with medium containing a sufficient concentration of an agonist, cells shortened to 75–85% of their original length (Figs. 1–3) within 10–15 s. This length change was accompanied by the appearance of membranous evaginations on the cell surface which changed position and size continuously over a period of 5–10 min. When agonist-free medium was reintroduced, these evaginations slowly disappeared and the cells returned partially to their original length and could then be restimulated. Up to four such contraction-relaxation cycles

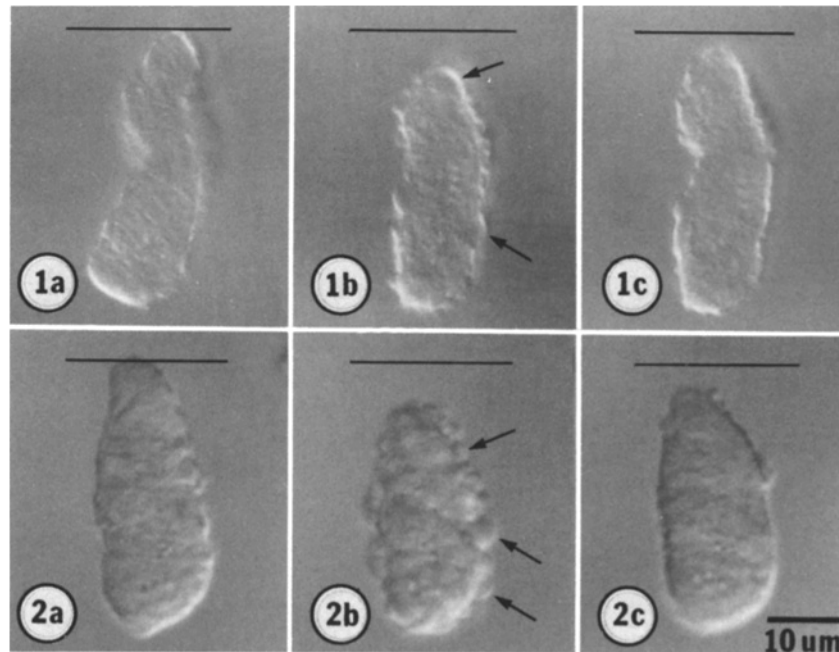
TABLE I  
*Responses of Isolated Aortic Smooth Muscle Cells to Vasoactive Agents Immediately after  
 Dissociation*

Agent	Concentration	Response	Experiments*
AT II	$10^{-8}$ M	+	23
Norepinephrine	$10^{-6}$ M	+	20
Carbamylcholine	$10^{-5}$ M	±	$\frac{2}{8}$
1-Sar-8-Ala AT II	$10^{-7}$ M	-	4
Phenoxybenzamine	$10^{-5}$ M	-	3
Atropine	$10^{-4}$ M	-	3
AT II + 1-Sar-8-Ala AT II	$10^{-8}$ M $10^{-7}$ M	-	4
Norepinephrine + phenoxybenzamine	$10^{-6}$ M $10^{-5}$ M	-	3
Carbamylcholine + atropine	$10^{-5}$ M $10^{-4}$ M	-	3

\* Number of experiments giving stated result. All agents were not tested in all experiments.

could usually be performed. Morphologic changes were similar for all agonists tested and appeared to be similar (at the light microscope level) to those described by Fay and Delise (10) in isolated frog stomach smooth muscle cells. Using the conditions of dissociation described, most cell preparations contained contractile cells. In the 25 preparations which were used for this study an average of 10–20% of the cells in the population responded to agonists but occasionally this reached 50% based on counts of 100 cells photographed in several experiments. The qualitative responses of the cells to the various agents studied are summarized in Table I. Precise quantitation of the number of cells responding to various concentrations of agonists has not yet been carried out.

Cells exposed to  $10^{-8}$  M or higher concentrations of Asp<sup>1</sup>, Ile<sup>5</sup> AT II responded as described above (Fig. 1) in most preparations. In  $\approx 5$  experiments contraction was observed with  $10^{-9}$  M AT II. Approximately 10 min after administration of AT II at  $10^{-8}$  M, and in the continued presence of hormone, the cells slowly relaxed spontaneously and were unresponsive to a second administration of AT II at the same or higher concentration. If, however, the cells were washed continuously for 10 min with fresh medium, another cycle of contraction and relaxation could be induced by AT II. Tachyphylaxis was not observed with the other agonists tested and appeared to be unique to AT II. [Sar<sup>1</sup>, Ala<sup>8</sup>] AT II, when administered in concentrations 10-fold higher than the dose of AT II, blocked its contractile activity reversibly. Neither  $10^{-5}$  M atropine nor  $10^{-6}$  M phenoxybenzamine had any effect on the response to AT II at all doses used.



FIGS. 1 and 2. Isolated aortic smooth muscle cells contracting in response to AT II and norepinephrine. 1a, cell before stimulation; 1b, same cell after exposure to  $10^{-7}$  M AT II for 10 s; 1c, after 10 min wash with hormone-free medium; 2a, second cell before stimulation; 2b, same cell after exposure to  $10^{-5}$  M norepinephrine for 10 s; 2c, after 10 min wash with hormone-free medium. Bottom of the cells have been lined up in photographs and line at the top of each is reference to indicate extent of contraction. Note membranous evaginations (arrows) in the contracting cells (1b, 2b). Nomarski optics,  $\times 1,000$ .

$10^{-6}$  M or higher doses of norepinephrine were also found to cause contraction of the dispersed aortic smooth muscle cells (Fig. 2). Cells relaxed slowly only after washout of the agonist.  $10^{-5}$  M phenoxybenzamine blocked the response to  $10^{-6}$  M norepinephrine, but neither [Sar<sup>1</sup>, Ala<sup>8</sup>] AT II nor atropine had any effect on this response.

Response of the cells to  $10^{-5}$  M acetylcholine or carbamylcholine was variable. On several occasions small numbers of cells contracted in response to these agents. No contraction was ever observed when  $10^{-4}$  M atropine was added with the cholinergic drug.

In a limited series of experiments where the same cell field could be observed over extended times, we frequently observed contraction of the same cells in response to both norepinephrine and AT II, suggesting the presence of two different agonist receptors on the same cell. Conversely, in several cases cells in the same field responded preferentially to norepinephrine but not to AT II (Fig. 3), suggesting that there may be some heterogeneity in the cell population in relation to the hormone receptors which they carry or that AT II-stimulated contraction is more easily destroyed by the cell dissociation conditions.

*Contractile Response of Cultured Cells.* The contractile response of aortic smooth muscle cells cultured 1–5 days after dissociation was studied in culture dishes at 37°C.

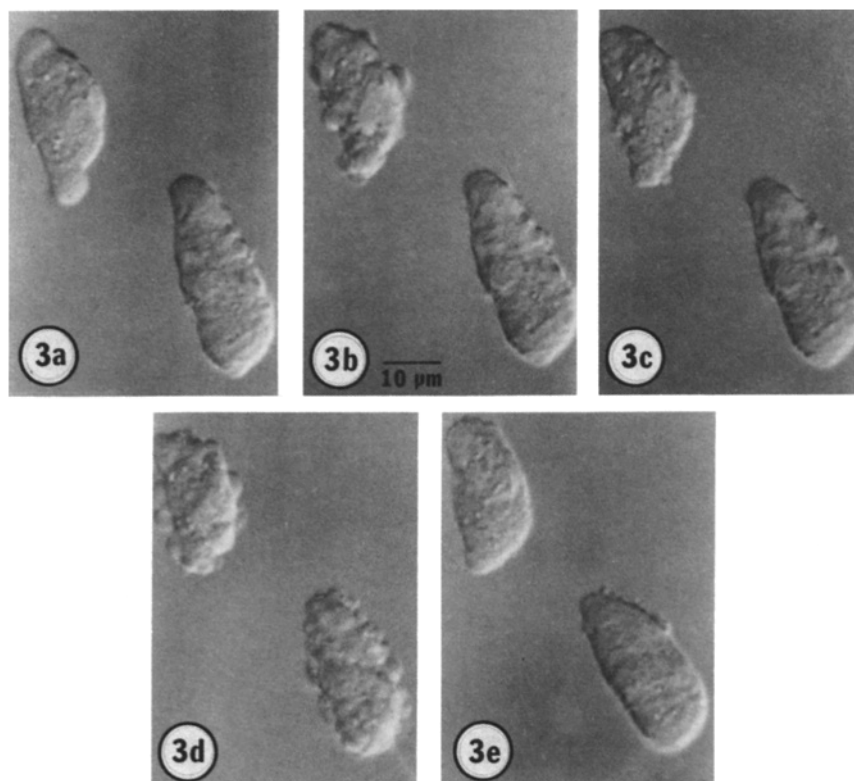


FIG. 3. Two isolated aortic smooth muscle cells contracting in response to norepinephrine and AT II demonstrating differential response to these agents. 3a, cells before stimulation; 3b, after exposure to  $10^{-7}$  M AT II for 10 s. Note that only the upper cell has responded; 3c, after 10 min washout of AT II; 3d, after exposure to  $10^{-5}$  M norepinephrine for 10 s. Both cells respond; 3e, after a 10-min wash with hormone free medium. Nomarski optics  $\times 750$ .

Changes in length, shape, and phase lucency were observed within 15 s after addition of agonists and these changes progressed until some of the more extended cells had shortened to 60% of their original length by 4 min after application of agonists. Other, less extended cells developed cytoplasmic evaginations reminiscent of those in the freshly prepared cells; still others retracted into a more spherical shape (Fig. 4). Not all cells exhibited the same sensitivity to agonists, nor was the rate of contraction the same in all cells. A small percentage failed to respond in each preparation.

The contractile response of cultured cells was proportional to the concentration of agonist added (e.g. in one experiment 35% of the cells contracted in response to  $10^{-8}$  M AT II, 45% contracted with  $10^{-7}$  M AT II and 90% contracted with  $10^{-6}$  M AT II). After 50 min and six exchanges of fresh medium (sufficient to reduce the AT II concentration 1,000-fold; see Materials and Methods) 95% of the contracted cells had relaxed to their resting shapes and 30% were capable of contracting again in response to  $10^{-5}$  M norepinephrine.

In four separate experiments, the proportion of cells contracting in response to agonists after 24 h of culture was always greater than the percentage which had responded immediately after dissociation of the tissue. The proportion of cells respond-

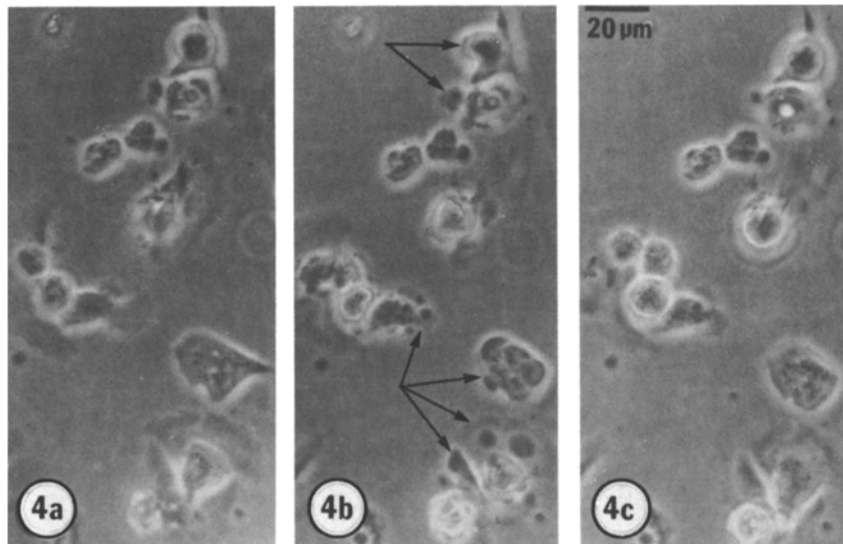


FIG. 4. Phase contrast micrograph of cultured aortic smooth muscle cells contracting in response to AT II. 4a, cells before stimulation; 4b, after exposure to  $10^{-6}$  M AT II for 4 min; 4c, after six exchanges with hormone-free medium. Arrows indicate cells which underwent morphological changes in response to hormone.  $\times 400$ .

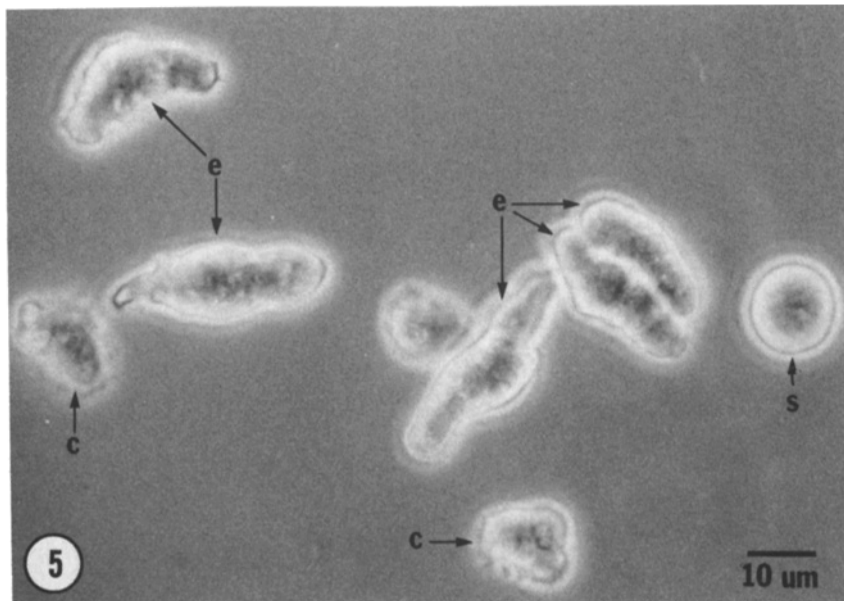
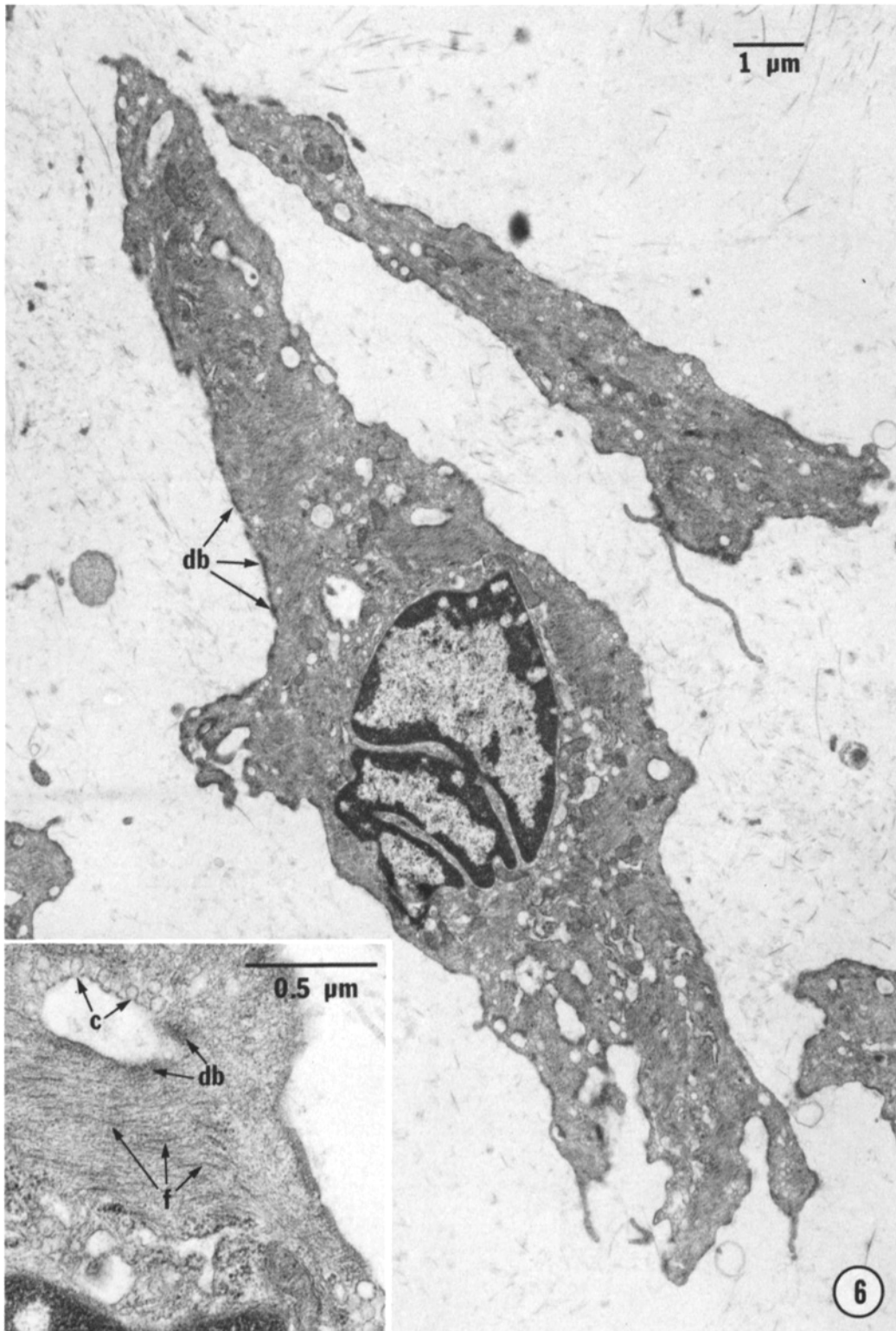


FIG. 5. Phase contrast micrograph of several aortic smooth muscle cells after tissue dissociation. Types of cells in the preparation have been labeled: e, elongated cell; c, crenated cell; s, spherical cell.  $\times 900$ .





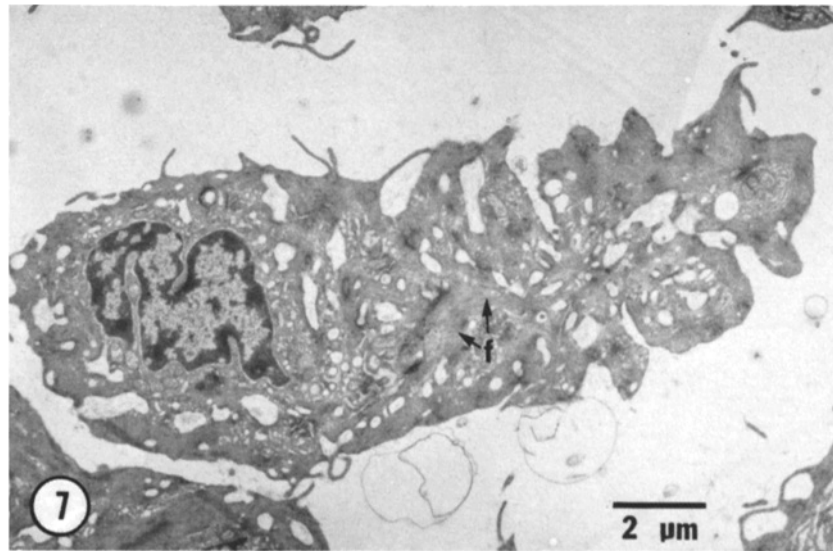


FIG. 7. Electron micrograph of a crenated cell in the preparation. This cell is identifiable as smooth muscle by its content of filaments (f).  $\times 6,000$ .

ing did not increase further after 24 h and for up to 5 days in culture. In a representative experiment, 25% of the cells responded to  $10^{-6}$  M AT II immediately after dissociation; this increased to 90% after 3 days in culture.

*Morphology of Dispersed Aortic Cells.* The morphology of freshly dispersed cells was studied by phase and Nomarski differential interference contrast microscopy. Approximately 50% of the cells were ellipsoidal,  $\approx 10 \mu\text{m} \times 30 \mu\text{m}$  in size, and had smooth or slightly crenated surfaces (Fig. 5) consistent with their appearance in intact aorta (20). Other cells were either very crenated or smooth and spherical. These latter cell types could be identified as originating from smooth muscle by electron microscopy. Although they did not contract in response to hormones, they occasionally regained hormone responsiveness after culture as discussed above.

In the electron microscope, the cytoplasm of the ellipsoidal cells consisted of closely packed arrays of thin (6 nm) and thick (10 nm) filaments (presumably actin and myosin) with occasional dense bodies characteristic of smooth muscle (20, 21) (Fig. 6). The majority of the intracellular organelles (elements of rough endoplasmic reticulum, Golgi complex, and mitochondria) were well preserved and usually located in the perinuclear cytoplasm. The plasmalemma was devoid of basement membrane but appeared to be closely associated with numerous extracellular filaments reminiscent of the microfilaments of elastin (2, 22). Organized collagen fibrils were generally absent from the cell pellets after thorough washing.

The highly crenated, noncontractile cells (Fig. 7) were identified as smooth muscle on the basis of their content of thick and thin filaments. The nucleus was frequently

FIG. 6. Electron micrograph of isolated aortic smooth muscle cell after dissociation but before washing. Note that plasma membrane associated dense bodies (db) have been preserved and that the basement membrane found in situ is absent. Collagen fibrils seen here in abundance can be removed by washing.  $\times 11,000$ . Inset shows 6 and 10-nm filaments (f), dense bodies (db), and caveolae (c) in greater detail.  $\times 40,000$ .

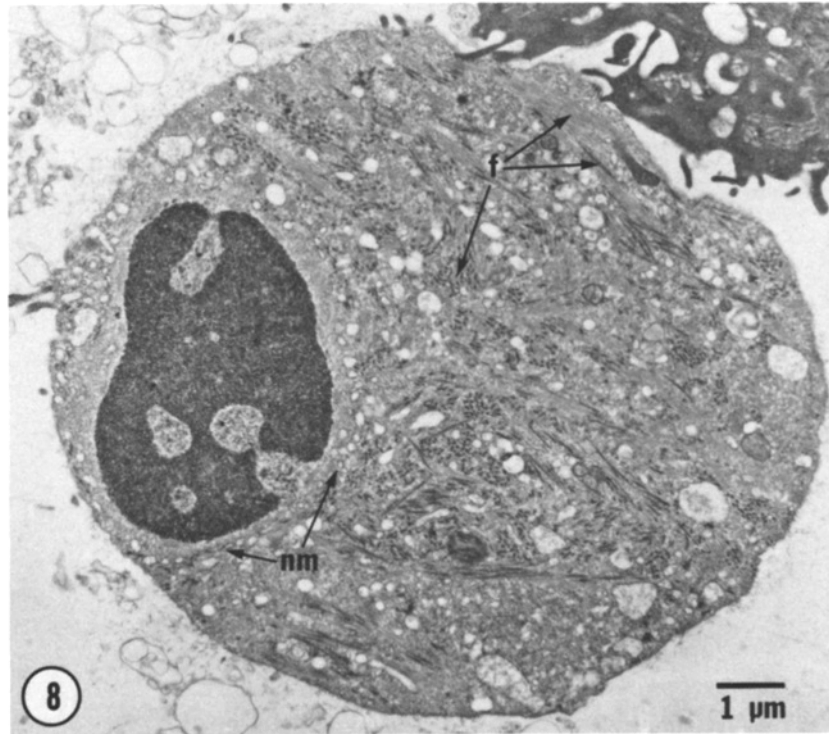


FIG. 8. Electron micrograph of a spherical cell in the preparation. 6 and 10-nm filaments (f), though present, are in disarray. Fragments of the degenerating nuclear envelope (nm) can also be seen.  $\times 9,000$ .

located peripherally and often deeply indented or even pinched into two or more lobes. The nuclear infoldings usually contained arrays of parallel microtubules which were not seen in the elongated cells. The plasma membrane of this population was highly contorted and many were probably nonviable. The spherical cells were also clearly of smooth muscle origin when observed by electron microscopy, although their arrays of actin and myosin filaments were highly disorganized (Fig. 8).

Although a number of cells in all of our freshly dispersed preparations had undergone various degrees of damage, greater than 95% could be identified in the electron microscope as having originated from smooth muscle, on the basis of their abundance of organized filaments resembling actin and myosin.

After 1–5 days of culture in plastic Petri dishes, cells were prepared for light and electron microscopy and sections were cut parallel to the dish face. The cultured smooth muscle cells were similar in morphology to those already described by Ross (2), Gimbrone and Cotran (6), and others (5, 7). During the first 5 days in culture, greater than 90% of the cells could be identified as smooth muscle on the basis of their content of intracellular filaments.

#### Discussion

The mammalian aorta has been used for biochemical and pharmacologic studies of vascular smooth muscle cells because of its large size compared to other vessels, the

ease with which it can be removed and dissected, the homogeneity of the cell population in its media, and its possible role in disease processes (23). Studies of vasoactive drug or hormone action on the aorta in situ or in vitro are subject to a number of limitations which may necessitate the use of isolated cell preparations such as the one described here.

In comparison to isolated vascular smooth muscle cells prepared by others, a significant proportion of the cells prepared by our procedure respond immediately after dissociation to agonists that normally cause contraction of the aorta in situ or in vitro. Both norepinephrine and AT II caused contraction of the cells at concentrations within an order of magnitude of that giving a half maximal response in intact tissue and are blocked by specific inhibitors. The contractile response observed in the single cells is therefore interpreted by us to represent the physiological response which these cells undergo in situ. Also, the morphological changes observed in contracting vascular smooth muscle cells (shortening with formation of membranous evaginations) appear similar to those described by Fay and Delise (10) for smooth muscle cells isolated from frog stomach. This suggests there may be similarities in the contractile mechanism for smooth muscle cells from these two sources.

The reasons why vascular smooth muscle cells prepared by others fail to contract in response to agonists immediately after dissociation are presently unclear. We feel that the lack of control of protease levels present in crude enzyme preparations could cause damage to receptors or to the cytomuscular skeleton and may result in loss of the contractile response. The enzyme preparations used by others may have contained high levels of proteases and phospholipases as we have found in some commercial preparations (14). Crude enzyme preparations in our hands have never yielded cells that contract and in addition the omission of STI in our dissociation procedure with purified enzymes produced cells that appear to be insensitive to agonists. Finally, it is conceivable that phospholipases present in crude enzyme preparations may damage cells, as our studies on the pancreatic acinar cell (14) have shown.

We have demonstrated in these studies that some cells respond to both norepinephrine and AT II, suggesting the presence of at least two different agonist receptors on the same cell. However, some individual cells were responsive to norepinephrine but not to AT II, indicating that there may be some heterogeneity in the cell population with respect to their hormone receptors or differences in lability of these receptors during tissue dissociation. In addition, many cells in our own preparation did not respond at all and there was considerable variability (5–50%) in the number of cells which did respond when various preparations were compared.

While it is likely that many viable cells were damaged during the dissociation, it is conceivable that some do not respond to hormones even in situ. Contraction of such cells in the intact tissue may, in principle, be mediated by gap junctions (24) coupled to one or a few cells which respond to either hormonal agonists or neurotransmitters. Studies are planned using ferritin labeled AT II to determine whether all cells in the preparation have binding sites for this hormone.<sup>2</sup> In addition, fluorescent labeled AT II may be used to ascertain by light microscopy whether all cells which bind hormone are capable of responding by contraction.

Studies of isolated vascular smooth muscle cells maintained in tissue culture for

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<sup>2</sup> R. E. Galardy et al. 1978. *J. Med. Chem.*

relatively short periods indicate that a higher percentage of such cells respond to AT II than when freshly isolated. Quantitative binding studies using  $^{125}\text{I}$ -labeled AT II will help to establish if replacement of damaged receptors is occurring during culturing. Alternatively, the improved responsiveness may be due to repair of another portion of the stimulus-contraction coupling mechanism or simply that detection of the contraction phenomenon is improved in culture because of the extended, flattened morphology of these cells.

### Summary

A procedure for dissociating the rabbit aorta into single, functional smooth muscle cells is described. After removal of adventitia and intima, slices of media were incubated with purified collagenase, elastase, and soybean trypsin inhibitor in a Krebs-Ringer buffer modified with Hepes, amino acids, and a  $[\text{Ca}^{2+}]$  of 0.2 mM. After enzymatic digestion and mechanical shear, the yield of dispersed cells was approximately 25% based on DNA recovered. Greater than 95% of the cells excluded trypan blue and approximately 80–90% adhered to tissue culture dishes. By phase contrast microscopy, most of the cells were elongate and approximately  $10\ \mu\text{m} \times 30\ \mu\text{m}$  in size. The remainder were either spherical or highly crenated and contracted. Electron microscopy of the cells showed that immediately after dissociation greater than 95% could be identified as smooth muscle, though most had undergone some degree of structural change compared to cells in situ. Depending on the preparation, from 5 to 50% of these cells contracted in response to agonists. Cells shortened by 10–15% and developed numerous evaginations when stimulated by angiotensin II, norepinephrine, or carbamylcholine. Cells relaxed after washout of agonists and could subsequently be restimulated. Specific inhibitors of each of the agonists blocked the contractile response. Dispersed cells cultured for 1–5 days contracted in even higher numbers than the freshly prepared cells, suggesting restoration of hormone binding and/or contractile function in culture. This preparation provides a system in which the physiology of individual vascular smooth muscle cells may be studied.

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