Long-term effects of Ca\(^{2+}\) on structure and contractility of vascular smooth muscle

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Lindqvist, Anders, Ina Nordström, Ulf Malmqvist, Patrik Nordenfelt, and Per Hellstrand. Long-term effects of Ca\(^{2+}\) on structure and contractility of vascular smooth muscle. Am. J. Physiol. 277 (Cell Physiol. 46): C64–C73, 1999.—Culture of dispersed smooth muscle cells is known to cause rapid modulation from the contractile to the synthetic cellular phenotype. However, organ culture of smooth muscle tissue, with maintained extracellular matrix and cell-cell contacts, may facilitate maintenance of the contractile phenotype. To test the influence of culture conditions, structural, functional, and biochemical properties of rat tail arterial rings were investigated after culture. Rings were cultured for 4 days in the absence and presence of 10% FCS and then mounted for physiological experiments. Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)];i) after stimulation with norepinephrine was similar in rings cultured with and without FCS, whereas force development after FCS was decreased by >50%. The difference persisted after permeabilization with β-escin. These effects were associated with the presence of vasoconstrictors in FCS and were dissociated from its growth-stimulatory action. FCS treatment increased lactate production but did not affect ATP, ADP, or AMP contents. The contents of actin and myosin were decreased by culture but similar for all culture conditions. There was no effect of FCS on calponin contents or myosin SM1/SM2 isoform composition, nor was there any appearance of nonmuscle myosin. FCS-stimulated rings showed evidence of cell degeneration not found after culture without FCS or with FCS + verapamil (1 µM) to lower [Ca\(^{2+}\)];i. The decreased force-generating ability after culture with FCS is thus associated with increased [Ca\(^{2+}\)];i during culture and not primarily caused by growth-associated modulation of cells from the contractile to the synthetic phenotype.

CELL CULTURE OF SMOOTH muscle is an indispensable tool in many investigations, but culture of dispersed cells invariably causes rapid modulation from the contractile to the synthetic or proliferative phenotype (4, 19, 25). This involves loss of contractile ability, decreased contractile protein contents, and increased expression of rough endoplasmic reticulum. The synthetic phenotype of the smooth muscle cell is of particular pathophysiological importance in clinical conditions involving growth and proliferation of smooth muscle cells, such as neointima formation as part of the atherosclerotic process (21). However, maintenance of the contractile phenotype in vitro is also of interest, inasmuch as this would allow a stable background against which interventions leading to phenotype modulation could be assessed under controlled experimental conditions. This would open new possibilities for investigating mechanisms behind vascular cell proliferation and damage and also for manipulating cellular constituents to elucidate regulatory pathways.

An important determinant of the cellular phenotype appears to be the presence of extracellular matrix, inasmuch as matrix constituents are known to influence the expression of cellular proteins (23). Therefore, organ culture of smooth muscle cells in their normal environment, embedded in extracellular matrix and with maintained cell-cell contacts, is expected to contribute to maintenance of the contractile phenotype. Indeed, studies have shown that contractility of vascular and intestinal smooth muscle can be well maintained in culture for several days (3, 5, 13, 16, 17, 20, 24). In contrast to dispersed cells, organ cultured smooth muscle survives well in serum-free medium, which keeps cells in a quiescent state. Addition of the growth stimulant FCS to the medium increases DNA synthesis rate and also stimulates contraction, probably because of vasoconstrictors present in serum (5, 17). The contraction suggests increased intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)];i) and can be abolished by dialysis of the serum (5) or addition of 1 µM verapamil to the medium (17), while the increase in DNA synthesis rate is maintained. This is in contrast to the inhibited proliferation in cell culture achieved with a higher concentration (10–100 µM) of verapamil (18). In cultured rat tail arteries, addition of 10% FCS to the culture medium markedly diminished contractility after 4 days of culture, an effect that was prevented by 1 µM verapamil (17). Thus increased DNA synthesis is not invariably linked to modulation from the contractile to the synthetic phenotype, causing decreased contractility. This is also suggested by the maintenance of contractility during a transient increase in DNA synthesis rate over a few days induced by dialyzed FCS in cultured rat renal artery (3).

The questions arise whether the loss of contractility during culture is an effect primarily of increased [Ca\(^{2+}\)];i and whether it is associated with modulation to synthetic phenotype or with other structural or biochemical alterations. This study was designed to address these issues by analysis of mechanical, biochemical, and structural effects of different culture conditions on the rat tail artery.

MATERIALS AND METHODS

Preparation and organ culture. Female Sprague-Dawley rats, weighing ~200 g, were killed by cervical dislocation. A 5-cm segment of the tail artery, beginning ~1 cm distal to the...
radix, was dissected free under sterile conditions and transferred to a petri dish containing culture medium (1:1 DME/ Ham’s F-12) with the addition of antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The diameter of the vessel segment was ~0.5 mm along its entire length, and the segment was cut into rings of a uniform width under a dissecting microscope, as described elsewhere (17). The rings were transferred to culture dishes containing culture medium as described above or with the addition of 10% FCS (Biochrom, Berlin, Germany). Verapamil (1 µM) was added to some dishes. The dishes were placed in a water-jacketed cell incubator at 37°C under 5% CO₂ in air. In some experiments the arterial rings were suspended on a glass capillary or stainless steel cannula (0.4 mm diameter) during culture.

For force registration, after 4 days in culture the rings were incubated for 20 min in a nominally Ca²⁺-free buffer solution (see Solutions). A thin needle was passed through the lumen of each ring to remove the endothelium. The rings were mounted on parallel stainless steel wires (0.25 mm diameter) and immersed in normal physiological saline solution (PSS). One of the wires was connected to a force transducer (model AE 801, SensoNor, Horten, Norway) and the other to an adjustable support. Solution (0.4 ml) was contained in exchangeable Plexiglas cups fitted into a thermostated metal block (37°C). The rings were contracted with high-K⁺ solution (see Solutions) and allowed to equilibrate for 25 min.

For determination of circumferential-tension relationships, vascular rings were activated by high-K⁺ solution with 10 µM norepinephrine (NE) added. This mode of activation gave a stable and reproducible force response that was greater than the responses to high-K⁺ solution or NE alone. Passive and active tension were determined at increasing circumference with use of the following protocol: 1) accommodation for 5 min in PSS; 2) contraction for 5 min, 3) relaxation for 10 min in nominally Ca²⁺-free solution, and 4) change to new circumference.

Cultured rings mounted for force registration were permeabilized to allow determination of force responses independent of membrane control of intracellular Ca²⁺. Before permeabilization the arterial rings were stretched to optimal length, contracted with high-K⁺ solution, and relaxed in Ca²⁺-free solution containing 2 mM EGTA. Rings were permeabilized using 0.1% (wt/vol) β-escin in an intracellular solution (see Solutions) of pCa 6.0 for 8–10 min and then relaxed in pCa 9.0 solution. The Ca²⁺-force relationship was determined by increasing the Ca²⁺ concentration in steps at 5-min intervals. Force was determined as the steady-state value at each Ca²⁺ concentration. Permeabilization and subsequent procedures were carried out at ambient temperature (21–23°C).

Measurement of [Ca²⁺]. [Ca²⁺] was measured using the fluorescent indicator fura 2. The arterial rings were mounted as described above, equilibrated for 60 min in PSS, stretched to their optimal length, and stimulated with high-K⁺ solution for 5 min once during this time. The rings were then incubated with 10 µM fura 2-AM (Molecular Probes, Eugene, OR) dissolved in a nominally Ca²⁺-free solution for 90 min at room temperature. After washout of fura 2-AM, the rings were incubated for 60 min at 35°C to allow for hydrolysis of the ester bond. [Ca²⁺] was estimated from the ratio of fluorescence at 510 nm for excitation at 340 and 380 nm (9). The fluorescence measurements were carried out using an imaging system (IonOptix) mounted on a Nikon TMD inverted microscope with a Nikon fluor ×20 objective. The fluorescence data were calibrated at the end of each experiment, as described previously (17).

Determination of DNA synthesis rate. DNA synthesis rate in the cultured rings was determined by (methyl-3H)thymidine (Amersham) incorporation during 1 h, as previously described (17).

Analysis of high-energy phosphate compounds and lactate production. Arterial segments were cut to lengths of 4–6 mm. They were weighed in a closed Eppendorf vial after gentle blotting on filter paper, incubated in PSS for 1 h or cultured for 4 days, then weighed again and incubated for another 1 h. The segments were then frozen between metal blocks precooled in liquid N₂ and stored at ~80°C until analysis. The frozen tissues were pulverized and homogenized by brief sonication in methanol-EDTA (1.5 mM), then centrifuged. All extraction procedures were carried out at ~5°C or below. The supernatants were analyzed for adenylylphosphate and lactate contents by isotachophoresis (11). Results were expressed relative to wet weight before extraction. Lactate production was determined by analysis of aliquots from the incubation medium, with blank correction for medium incubated without tissue. There was no change in lactate contents in the blank medium during the 4-day incubation.

Protein separation. The arterial rings were washed once in nominally Ca²⁺-free solution, as described above, and then homogenized by sonication in SDS sample buffer (62.5 mM Tris·HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue). The homogenate was then boiled for 3 min and centrifuged. The proteins were separated using SDS-PAGE on a Bio-Rad Mini gel system. Gradient gels (4–20%) stained with Coomassie brilliant blue were used for determination of total myosin and actin. Fresh and cultured preparations were run in parallel on the gels, with identical number of rings, all prepared to uniform size, in all samples. This basis of normalization allows detection of any changes in protein contents during culture. Actin as well as myosin heavy chain (MHC) bands of cultured samples were expressed relative to the corresponding bands in the fresh sample on the same gel.

For MHC isoform separation, 6% gels with low cross-link were run at 4°C to increase resolution. For Western blot analysis, the proteins were blotted onto a nitrocellulose membrane and then treated as follows: For analysis of nonmuscle myosin isoforms, the blots were blocked with 5% gelatin and incubated with a monoclonal calponin-specific antibody (catalog no. C6687, Sigma Chemical, St. Louis, MO) and an alkaline phosphatase-labeled secondary antibody. For determination of nonmuscle myosin, the blots were blocked with 5% nonfat dry milk and incubated with antibodies against NM-MHC-A (196 kDa; BTI, Stoughton, MA) or NM-MHC-B (200 kDa; BABC0, Richmond, CA) and detected with enhanced chemiluminescence (Amersham).

Histology. The preparations were mounted in an open organ bath at 37°C, as described above for registration of isometric force. They were stretched to the optimal length for force development and, after a 1-h equilibration period, fixed for 2 h in a 0.075 M cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde. The preparations were postfixed in 2% OsO₄, stained en bloc with uranyl acetate, dehydrated, and embedded in Araldite. After polymerization, longitudinal and transverse sections were cut for light and electron microscopy.

Solutions. PSS contained (mM) 135.5 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 11.6 HEPES, and 11.5 glucose, pH 7.35 at 37°C. Nominally Ca²⁺-free solution was prepared by omitting CaCl₂. High-K⁺ solution was obtained by isomolar substitution of NaCl for KCl. The intracellular substitution solution used with permeabilized preparations was of the following composition (mM): 3.2 MgATP, 2 free-Mg²⁺, 12 phosphocreatine, 0.5 sodium azide, and 30 N-tris(hydroxymethyl)-
methyl-2-aminoethanesulfonic acid, pH 6.9 at 21°C. Creatine phosphokinase (15 U/ml) and calmodulin (0.5 µM) were added to the solutions. Desired Ca$_{2+}$ levels were obtained by mixing stock solutions containing EGTA K salt and K$_2$CaEGTA. The total EGTA concentration was 4 mM, and the ionic strength was adjusted with potassium propionate to 150 mM. The cacodylate buffer used for histology contained (mM) 75 sodium cacodylate, 130 sucrose, and 1.2 CaCl$_2$. Chemicals were from Sigma Chemical, unless otherwise indicated.

Statistics. Values are means ± SE. Statistical comparisons were done using Student's t-test for paired or unpaired data as appropriate. For multiple comparisons, ANOVA and Tukey's honest significant difference test were used.

RESULTS

Contractile force was lower in rat tail arterial rings cultured in the presence of 10% FCS than in rings kept in serum-free medium, when mounted for force registration after 4 days of culture (Fig. 1). Addition of FCS to the medium causes increased [Ca$_{2+}$], with associated contraction, and evidence suggests that this increase is the cause of the effects on contractility, inasmuch as these effects were prevented when verapamil was present in the culture medium (17). To further investigate whether the growth-stimulating and [Ca$_{2+}$]-raising effects of FCS can be separated with respect to their effects on vascular contractility, serum was dialyzed with a cutoff molecular weight of 6,000–8,000 Da and subsequently used in culture experiments. Dialyzed FCS had no contractile effect on arterial rings (data not shown). As shown in Fig. 1, culture with dialyzed FCS (10%) did not impair contractility, whereas it stimulated [3H]thymidine incorporation, indicating that the stimulatory effect of FCS on DNA synthesis is separate from the long-term impairment of contractility. In subsequent experiments reported below, undialyzed FCS was used, and the specific effects of [Ca$_{2+}$], during culture were investigated by the inclusion of 1 µM verapamil in the culture medium.

To investigate whether the effect of culture conditions on contractility is related to a possible remodeling of the vessel wall, radius-tension relationships were determined in freshly prepared rings of rat tail artery as well as in rings cultured for 4 days under different conditions (Fig. 2, top). The passive tension at all circumferences tended to be higher in fresh than in cultured rings, but in cultured rings it was identical at all culture conditions (Fig. 2, bottom). To obtain a maximal force response, rings were stimulated by high-K$^+$ solution with the addition of 10 µM NE. The optimal radius for active force development was similar in fresh and cultured rings irrespective of culture condition. Maximal force was the same in rings cultured in the absence of FCS and in fresh rings. Rings...
cultured with FCS alone produced only ~40% of the force of those without FCS. Whereas addition of verapamil to the culture medium with FCS always significantly increases contractility toward that seen after serum-free culture, the restored force level varies between different experimental series but is generally 70–90%.

The present experiments were generally carried out on vascular rings that were kept undistended in the culture medium. This was done to minimize damage to the preparations, and for the same reason the endothelium was not removed before culture. Although this approach leaves out potential modifying factors such as transmural pressure and luminal flow, it has the advantage of being uncomplicated and less susceptible to risks of leakage or infection than a perfusion system.

It is recognized, however, that results may be affected by the mechanical conditions, e.g., the lack of active tension during culture. To evaluate whether distension would alter force production, we suspended rings on glass capillaries (0.4 mm diameter) during culture of intestinal smooth muscle with FCS has been shown to decrease the tension during culture. To evaluate whether distension would alter force production, we suspended rings on glass capillaries (0.4 mm OD) during culture and compared contractile responses to high-K⁺ solution or 10 µM NE with nondistended control rings (Table 1).

There was no significant effect of distension on force production with either mode of stimulation.

Table 1. Effect of distension on contractile properties of cultured arterial rings

<table>
<thead>
<tr>
<th>FCS</th>
<th>Distension</th>
<th>High-K⁺ Solution, mN/mm</th>
<th>Norepinephrine (10 µM), mN/mm</th>
</tr>
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<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>2.67 ± 0.31</td>
<td>5.06 ± 0.58</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>2.46 ± 0.45</td>
<td>4.13 ± 0.83</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.95 ± 0.02</td>
<td>2.18 ± 0.05</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.85 ± 0.32</td>
<td>2.16 ± 0.45</td>
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Values are means ± SE; n = 3–6. Rings of rat tail artery were cultured for 4 days in absence and presence of FCS. For distension, rings were suspended on a glass capillary (0.4 mm diameter) during culture.

Fig. 3. Original recording of intracellular Ca²⁺ concentration ([Ca²⁺]i) and force in an arterial ring cultured in presence of FCS (top) and [Ca²⁺]-tension relationships of intact arterial smooth muscle (bottom). Rings were cultured for 4 days in absence and presence of 10% FCS and 1 µM verapamil and then mounted for isometric force registration and loaded with fura 2 for determination of [Ca²⁺]. Values are shown for relaxed rings and for stimulation with 100 nM and 10 µM norepinephrine (NE), giving intermediate and maximal tension, respectively. Values are means ± SE (n = 4–5). *P < 0.05.

To investigate the [Ca²⁺]i-force relationship at the level of the contractile system, arterial rings were permeabilized using β-escin and activated at different Ca²⁺ concentrations in the intracellular range (1 nM–3.5 µM) in solutions containing MgATP and phosphocreatine as energy substrates (see MATERIALS AND METHODS). Maximal force of permeabilized preparations was about one-third that of the corresponding intact preparations, but this factor was similar in the different groups, indicating that the treatment during culture did not influence force loss with permeabilization. Rings cultured without FCS or with FCS + 1 µM verapamil were capable of generating more force at pCa 4.5 than FCS-treated rings (Fig. 4). The Ca²⁺ sensitivity did not differ between the different experimental groups.

To evaluate the effect of culture on tissue viability as evidenced by energy metabolism, preparations were analyzed for adenylate phosphate compounds. These experiments were carried out using 4- to 6-mm-long vascular segments to increase the tissue mass. Culture of segments of this length did not influence function, inasmuch as 0.5-mm-wide rings cut from the segments...
after culture showed normal contractility. The wet weight of the segments was initially 5 mg and decreased by 35 ± 6% and 36 ± 3% during culture in the absence and presence of FCS (n = 12). The contents of ATP, ADP, and AMP were similar in rings cultured with and without FCS as well as in freshly dissected rings (Table 2). Although it was possible to detect phosphocreatine by the presently used isotachophoretic method, detection required larger concentrations of phosphocreatine than of ultraviolet light-absorbing compounds (11), and thus phosphocreatine could not be quantitated in these samples. FCS treatment increased the rate of glycolysis, as evident from a threefold increase in the release of lactate into the medium during culture.

SDS-PAGE gels were used to separate proteins of arterial ring homogenates. Protein contents decreased with culture, but in general the appearance of the protein bands was similar under all culture conditions (Fig. 5). Calponin was detected by Western blot (Fig. 5). Actin content in cultured rings, expressed as the amount of protein per ring, was ~85% of that in fresh rings irrespective of culture conditions. Calponin, which was quantitated from Western blots, was 85–95% in cultured relative to fresh rings, again with no significant effect of culture condition. In contrast, myosin decreased with culture to ~50% compared with fresh tissue. This decrease was similar for all culture conditions (Fig. 6). Thus the loss of proteins with culture is on the average compatible with the loss of weight. Although these two measures cannot be directly compared, neither value was influenced by variations in culture conditions that produce clear differences in contractility.

The major (80%) myosin isoform in the freshly isolated rat tail artery is SM1 (204 kDa), and only minor amounts (20%) of SM2 (200 kDa) can be found. The isoform distribution varies considerably between different vessels in the rat, with about an equal distribution in aorta and dominance of SM1 in renal artery and...

### Table 2. Energetics of cultured tail arteries

<table>
<thead>
<tr>
<th></th>
<th>ATP, µmol/g</th>
<th>ADP, µmol/g</th>
<th>AMP, µmol/g</th>
<th>J_{lac}, µmol·g⁻¹·min⁻¹</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0.37 ± 0.03</td>
<td>0.21 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Cultured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− FCS</td>
<td>0.37 ± 0.05</td>
<td>0.19 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>+ FCS</td>
<td>0.33 ± 0.04</td>
<td>0.19 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.52 ± 0.12*</td>
<td>4</td>
</tr>
</tbody>
</table>

Values (means ± SE) are expressed relative to tissue wet weight. Rat tail arteries were frozen after incubation for 1 h in physiological buffer solution (fresh tissue) or for 4 days in culture medium ± 10% FCS. Frozen tissue was extracted in methanol-EDTA and analyzed by isotachophoresis. Lactate production (J_{lac}) was estimated from release of lactate into incubation medium. *P < 0.05, by Student’s t-test (unpaired).
portal vein as well as tail artery (Fig. 7). Culture, irrespective of condition, does not change the myosin isoform distribution, and furthermore, nonmuscle myosin (NM-MHC-A, 196 kDa or NM-MHC-B, 200 kDa) could not be detected by Western blot (see MATERIALS AND METHODS). NM-MHC-A and NM-MHC-B were, however, readily detected in primary culture of rat tail arterial cells (data not shown).

Figure 8 shows electron micrographs of fresh and cultured rings. In cultured rings the extracellular space was expanded compared with that in fresh rings. The cytological appearances of cells in fresh rings and cells cultured in the absence of FCS were identical (Fig. 8, A and B). In rings cultured in the presence of 10% FCS the cells showed a considerable pleomorphism (Fig. 8C). The cells varied with respect to electron density from cells with a normal appearance to almost white cells (Fig. 8C, top and bottom). Degeneration of cells was rarely seen in fresh rings and in rings cultured in the absence of FCS. In rings cultured with FCS and verapamil the cell configuration in the medium of the rings was similar to that of rings cultured in the absence of FCS (Fig. 8D), even though degenerating cells were more often observed. Large intracellular vacuoles were often seen in cultured preparations, particularly those cultured with FCS.

Figure 9 shows higher magnification of a fresh preparation (A) as well as areas from an FCS-cultured ring (same as in Fig. 8C) with normal (B) as well as disrupted (C) cell morphology. These latter cells main-

![Fig. 6. Relative contents of actin, myosin, and calponin in rings of rat tail artery cultured for 4 days in absence and presence of 10% FCS ± 1μM verapamil. Values are means ± SE (n = 4–5). *P < 0.05; ***P < 0.001 for comparison with fresh tissue.](http://ajpcell.physiology.org/)

![Fig. 7. Separation of myosin isoforms (SM1 (204 kDa) and SM2 (200 kDa)) on 6% polyacrylamide gels in fresh and cultured tail artery (top) and in rat aorta, renal artery, and portal vein (bottom).](http://ajpcell.physiology.org/)
tained their general configuration and integrity of the cell interior but with different degree of breakdown of plasma membranes and organelles. In some areas only ghostlike cell remnants were observed. Cells with high electron density showed the normal appearance of myofilaments and organelles of the contractile smooth muscle phenotype.

DISCUSSION

The correlation of structural, biochemical, and mechanical data obtained here shows that vascular preparations can be kept in organ culture for several days with maintained contractility and no evidence of a significant modulation of smooth muscle cells from the contractile to the synthetic phenotype. Loss of contractility under conditions of increased basal \( [Ca^{2+}]_i \), as induced by undialyzed FCS, is associated with morphological alterations, which, however, do not suggest modulation to the synthetic phenotype. Although only a single time point (4 days) was extensively investigated, preliminary observations suggest that longer FCS-free incubations (7 days) are associated with loss of contractility (data not shown). Thus the cultured preparations are not stable over a long time and with the presently used methodology probably exist in a catabolic state, although tissue viability is maintained. Given these limitations, the time frame of 4 days is sufficient for many investigations involving alteration of metabolism or signaling mechanisms.

Overall, there is no clear correlation between contractility and contractile protein contents under different culture conditions, inasmuch as culture with 10% FCS results in a considerable reduction in contractility but does not affect myosin, actin, or calponin contents relative to serum-free culture. On the other hand, culture with FCS causes morphological alterations that seem to account for the change in contractility, inasmuch as these alterations were largely prevented, and contractility was improved, when the \( Ca^{2+} \) channel blocker verapamil was included in the medium along with FCS. This points to \( [Ca^{2+}]_i \) as a determinant of structural and functional integrity in cultured preparations.

FCS stimulates DNA synthesis in the cultured arterial rings, as evidenced by increased \(^{3}H\)thymidine incorporation. However, this effect seems not to be causally related to an increase in \( [Ca^{2+}]_i \), or to a loss of contractility, inasmuch as dialyzed serum stimulated \(^{3}H\)thymidine incorporation but did not cause any contraction and did not decrease contractility. De Mey et al. (5) likewise showed that dialysis of their serum preparation abolished its contractile effect but did not influence the rate of DNA synthesis in cultured rat renal arteries. They tentatively identified at least one

Fig. 8. Low-power electron micrographs of media of fresh tail arterial rings (A) and rings cultured in absence (B) and presence (C) of 10% FCS and in 10% FCS + 1 µM verapamil (D). Scale bar, 2 µm.
The results obtained here suggest that effects of growth stimulation itself on contractile properties of cultured arterial rings are minor, a result that may indicate only limited alteration from the contractile to the synthetic phenotype, even in the presence of strong growth stimulation, as represented by the 10% FCS ordinarily used in cell cultures. This result is unexpected on the basis of the, perhaps reasonable, hypothesis that reduced contractility would reflect altered phenotype. Evidently, conditions in cultured arterial tissue differ from those in dispersed cells, to the effect that the tissue is more sensitive than the cells to Ca$^{2+}$ overload while its phenotypic differentiation is better maintained, probably owing to influence from the extracellular matrix and cell-cell contacts. It is, however, quite likely that cells locally modulate to the synthetic phenotype and proliferate, e.g., near cut ends and at the borders of the muscle layer. This could account for at least a fraction of the increased $[^3]$H$^{3}$H]thymidine incorporation during growth stimulation but no appreciable influence on overall contractility. This type of response would be expected on the basis of the neointima formation observed in cultured arterial tissue (12), a phenomenon of considerable interest for the understanding of the atherosclerotic process.

The biochemical composition of the cultured arteries differed from that of the fresh arteries, in that the amounts of contractile proteins were decreased, irrespective of culture conditions. Calponin, which is considered to be a marker for the contractile smooth muscle phenotype (7), was somewhat lower in cultured than in fresh preparations but did not vary significantly with culture condition. The loss of weight during culture correlates with the decrease in contractile protein contents, although wet weight is expected to be influenced by many other factors, such as alteration of the extracellular space, which was expanded in cultured tissue, as judged by our electron micrographs.

Even though the decrease in total myosin indicates that the organization of the contractile system has been altered, there was no change in the relative abundance of SM1 and SM2 MHC isoforms. Efforts to detect the appearance of nonmuscle myosin isoforms A or B (15, 22) were unsuccessful. In contrast, both nonmuscle myosin isoforms were abundant in tail artery cells kept in primary culture, demonstrating that the typical modulation to a synthetic phenotype occurs under these conditions.

To our knowledge, the only study besides ours that has investigated contractile protein expression relative to force in cultured arterial preparations is that of Ishibashi and Bukoski (14). These authors reported that serum-free culture for 48 h of rat mesenteric resistance arteries decreased the force response to high-K$^+$ + NE and simultaneously decreased total MHC and SM2 isoform contents, whereas SM1 contents were unchanged. There was no correlation between total MHC or SM1 contents and force, whereas SM2 contents correlated ($r^2 = 0.48$) with active force. The steroid hormone 1,25-dihydroxyvitamin D$_3$ reversed the effects of culture on force and myosin
expression, whereas culture with a high concentration of insulin (5 µg/ml) further decreased force and caused the appearance of nonmuscle (196-kDa) myosin. In the tail artery the distribution of SM1/SM2 isoforms is highly uneven, with ~80% SM1. Thus a small shift in isoform composition may have gone undetected in our experiments. In comparison with the profound changes in myosin isoforms occurring in culture of dispersed cells, it is, however, fair to conclude that a major change of cellular phenotype from the contractile to the synthetic form during culture of intact smooth muscle tissue is not supported by the biochemical data.

Despite the structural and functional effects of culture with FCS, there was no extensive cell death after 4 days of culture, insomuch as measurements of high-energy phosphates showed no decrease in ATP, ADP, or AMP after culture with FCS. However, there was an increased glycolytic metabolism after culture with FCS, as indicated by increased lactate production. A possible reason for this is increased Ca$^{2+}$ transport, inasmuch as there is evidence for association of Ca$^{2+}$-Mg$^{2+}$-ATPase with glycolytic activity in the plasma membrane (10).

Besides alterations in structure of the contractile apparatus, possible effects of culture conditions on mechanisms involved in activation of contraction need to be considered. Culture of guinea pig ileum muscle in the presence of FCS causes a decrease in the density of inward (Ca$^{2+}$-) current over the cell membrane. This is prevented when verapamil is added to the culture medium (8). Because FCS itself causes an increase in [Ca$^{2+}$], the effect may represent a Ca$^{2+}$-dependent downregulation of channel availability. This effect does not seem to account for the present results from the tail artery, insomuch as stimulation by NE caused the same increase in [Ca$^{2+}$], in arterial rings that had been cultured with or without FCS or with FCS + verapamil, whereas force development at any given [Ca$^{2+}$], was lower in FCS-treated rings. Furthermore, effects of FCS and of verapamil persisted when the arterial rings were permeabilized and then activated under conditions of damped Ca$^{2+}$ and calmodulin concentrations. Thus altered excitation-contraction coupling, involving membrane properties or cytoplasmic composition, does not seem to be a major factor in the effect of culture on contractility.

The optimal circumference (or radius) for force development was not changed in cultured arteries, suggesting that the compliance of the tension-generating units has not been significantly affected. These results also demonstrate that the decrease in force development observed after culture with FCS is not due to a shift along the circumference-force relationship. Because the vessel rings used here were not distended during culture, the contraction induced by FCS may possibly influence subsequent mechanical responses. However, this does not seem to be the case, insomuch as mechanical properties were unaffected when vessel rings were cultured suspended on glass capillaries to prevent constriction. The possibility remains, however, that the absence of active force development (tone) against a mechanical resistance representing the intraluminal pressure has contributed to atrophy and loss of myosin during culture. Culture of perfused rabbit aorta has indicated that flow and transmural pressure have differential effects on DNA and total protein expression (1).

The effect of the endothelium was not systematically investigated here, insomuch as most preparations were cultured with intact endothelium. However, the functional integrity of the endothelium after 4 days of culture is not known, insomuch as our mechanical recording system did not allow mounting without destruction of the endothelium. Suspending vessel rings on glass capillaries would be expected to destroy the endothelial layer, which suggests that an intact endothelium during culture is not a prerequisite for the effects reported here. However, Wang et al. (27) reported an endothelium-dependent effect of retinol in preserving contractility of rat aortic segments during 24 h of culture. Thus endothelium-derived factors may act in concert with plasma factors to influence structure and contractility of the vessel wall (2).

The ultrastructural features seen with culture in the presence of FCS compare reasonably with those described by Todd and Friedman (26). They observed well-preserved structural integrity of tail arteries for >2 wk of culture. In the presence of FCS there was outgrowth of cells from the adventitia and into the lumen and increased collagen contents in the intercellular space. Large droplets that were considered to represent lipid accumulation were observed in cultured vessels. The effects were more pronounced with 10% than with 2% FCS. Pale cells were observed in cultured preparations, as in our study. The pronounced effects seen with 10% FCS in our experiments seem more extensive than those reported by Todd and Friedman, although they were principally similar. Possibly this can be explained by differences in contractile, and thus [Ca$^{2+}$]-raising, actions of the FCS preparations used.

In summary, this study has shown that vascular smooth muscle kept in organ culture maintains functional, biochemical, and morphological characteristics in serum-free medium and also during growth stimulation with FCS, provided Ca$^{2+}$-inflow is inhibited. Ca$^{2+}$ load associated with the contractile action of FCS causes morphological alterations leading to decreased force development. The mechanisms behind these effects of Ca$^{2+}$ remain to be established. These results should provide a basis for examining long-term effects of trophic or contractile stimuli on vascular smooth muscle in vitro.

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