Intracellular Ca$^{2+}$ Handling in Vascular Smooth Muscle Cells Is Affected by Proliferation

Olivier Vallot, Laurent Combettes, Philippe Jourdon, Jocelyn Inamo, Isabelle Marty, Michel Claret, Anne-Marie Lompré

Abstract—Despite intensive interest in the dedifferentiation process of vascular smooth muscle cells, very little data are available on intracellular Ca$^{2+}$ signaling. The present study was designed to investigate the evolution of the intracellular Ca$^{2+}$ pools when rat aortic smooth muscle cells (RASMCs) proliferate and to define the mechanisms involved in the functional alterations. RASMCs were cultured in different conditions, and [Ca$^{2+}$]$_i$ was measured by use of fura 2. Expression of the sarco(endo)plasmic reticulum Ca$^{2+}$ pumps (SERCA2a and SERCA2b), Ca$^{2+}$ channels, the ryanodine receptor (RyR), and the inositol trisphosphate receptor (IP3R) was studied by reverse transcription–polymerase chain reaction and immunofluorescence. Antibodies specific for myosin heavy chain isoforms were used as indicators of the differentiation state of the cell, whereas an anti–proliferating cell nuclear antigen antibody was a marker of proliferation. SERCA2a, SERCA2b, RyR3, and IP3R-1 mainly were present in the aorta in situ and in freshly isolated RASMCs. These cells used the 2 types of Ca$^{2+}$ channels to release Ca$^{2+}$ from a common thapsigargin-sensitive store. Proliferation of RASMCs, induced by serum or by platelet-derived growth factor-BB, resulted in the disappearance of RyR and SERCA2a mRNAs and proteins and in the loss of the caffeine- and ryanodine-sensitive pool. The differentiated nonproliferative phenotype was maintained in low serum or in cells cultured at high density. In these conditions, RyR and SERCA2a were also present in RASMCs. Thus, expression of RyR and SERCA2a is repressed by cell proliferation, inducing loss of the corresponding Ca$^{2+}$ pool. In arterial smooth muscle, Ca$^{2+}$ release through RyRs is involved in vasodilation, and suppression of the ryanodine-sensitive pool might thus alter the control of vascular tone. (Arterioscler Thromb Vasc Biol. 2000;20:1225-1235.)

Key Words: vascular smooth muscle • cell proliferation • Ca$^{2+}$ pumps • ryanodine receptors • inositol trisphosphate receptors

Vascular smooth muscle cells, present in normal blood vessels, exhibit a quiescent differentiated phenotype characterized by a unique repertoire of contractile proteins, ion channels, and receptors involved in the contractile function of the cell. They undergo a transition from a contractile phenotype to a proliferative or synthetic phenotype when they proliferate, a situation that is observed in chronic vascular disease and, in particular, in atherosclerosis or restenosis. This is accompanied by changes in the phenotype of many contractile proteins (see Reference 2 for review). Voltage-gated Ca$^{2+}$ currents also vary. Freshly dissociated rat aortic smooth muscle cells (RASMCs) have functional L- and T-type Ca$^{2+}$ channels, but when they proliferate, the proportion of the L-type channels decreases, whereas that of the T-type channels increases. The changes are dependent on the cell cycle. Furthermore, alteration in expression of the α, β, and β subunits of the L-type Ca$^{2+}$ channel was recently reported in culture.

Changes in the response to caffeine, ryanodine, and angiotensin II have been reported during vascular smooth muscle cell culture. This suggests modifications in the intracellular Ca$^{2+}$ stores, but the molecular events underlying these pharmacological properties are unknown. We postulated that alteration in the expression of sarco(endo)plasmic reticulum Ca$^{2+}$ pumps and channels may account, at least in part, for the functional alterations. Ca$^{2+}$ is released from the intracellular stores by 2 types of receptors, the inositol trisphosphate (IP3) receptor and the ryanodine receptor (IP3R and RyR, respectively), the expression of which may vary during proliferation. The type 1 isoform is the major IP3R in adult porcine and rat aorta, whereas type 3 predominates in neonatal aorta. The RyR isoform that is present in vascular smooth muscle cells has not been definitively identified. Refilling of the Ca$^{2+}$ stores depends on the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPases (SERCAs). The 2 spliced variants of the SERCA2 gene, 2a and 2b, are present in the smooth muscle cells, with SERCA2b being the most abundant isoform (see Reference 14 for review). Maturation of the rat aorta is associated with an increase in the level of
SERCA2a mRNA, with the amounts of SERCA2b mRNA being identical in young and adult animals. The goal of the present study was (1) to characterize the intracellular Ca\(^{2+}\) pools in isolated RASMCs and to precisely identify the sarco(endo)plasmic reticulum Ca\(^{2+}\) pumps and Ca\(^{2+}\) channels in this cell type, (2) to study the changes in functional Ca\(^{2+}\) pools and expression of genes encoding the sarco(endo)plasmic reticulum Ca\(^{2+}\) pumps and Ca\(^{2+}\) channels during RASM proliferation in culture, and (3) to gain new insight into the mechanisms involved in the alteration of the phenotype of sarcoplasmic reticulum (SR) proteins during proliferation.

**Methods**

**Cell Culture**

RASMCs were isolated from the medial layer of thoracic aorta (aortic cross excluded) of 180- to 200-g male Wistar rats by enzymatic digestion with collagenase (CLS2, 50 U/mL, Worthington) and pancreatic elastase (0.25 mg/mL, Sigma) for 2 hours at 37°C. After periods of 20 minutes, the suspension was centrifuged at 1500 rpm for 5 minutes, and the cells were collected and placed in DMEM+20% FCS. The cells obtained in the first three 20-minute periods were discarded because they had a nonmuscle phenotype. Those obtained in the other cycles were pooled, centrifuged, suspended in DMEM containing 10% FCS, plated onto glass coverslips (aortic cross excluded) of 180- to 200-g male Wistar rats by enzymatic digestion with collagenase (CLS2, 50 U/mL, Worthington) and pancreatic elastase (0.25 mg/mL, Sigma) for 2 hours at 37°C. After periods of 20 minutes, the suspension was centrifuged at 1500 rpm for 5 minutes, and the cells were collected and placed in DMEM+20% FCS. The cells obtained in the first three 20-minute periods were discarded because they had a nonmuscle phenotype. Those obtained in the other cycles were pooled, centrifuged, suspended in DMEM containing 10% FCS, plated onto glass coverslips coated with collagen I (rat tail, Sigma) at a density of 1.3 × 10⁶ cells per square centimeter, and incubated at 37°C in 5% CO\(_2\)/95% air. The coverslips were washed twice with PBS and in PBS containing 50 mmol/L NH₄Cl, and permeabilized with 0.1% Triton X-100 (20°C) at room temperature. For double immunofluorescence studies, cells were fixed in −20°C methanol (8 minutes) and washed in PBS. PCNA was detected with Texas red–conjugated anti-mouse IgG (Amersham), and the antibodies specific for Ca\(^{2+}\) pumps or Ca\(^{2+}\) release channels, with biotinylated anti-rabbit immunoglobulin and fluorescein streptavidin (Amersham). Coverslips were mounted in glycerol/PBS medium (FluoroGuard antifade Reagent, Bio-Rad).

**Loading With Fura 2 and [Ca\(^{2+}\)]\(_i\)**, Measurement

RASMCs were loaded with 3 μmol/L fura 2-AM for 40 minutes at 37°C in 5% CO\(_2\)/95% air. The coverslips were washed twice with saline solution (10 mmol/L HEPES, 116 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl\(_2\), 0.8 mmol/L MgCl\(_2\), 0.96 mmol/L NaH\(_2\)PO\(_4\), 5 mmol/L NaHCO\(_3\), and 2 g/L glucose, pH 7.4). For experiments in extracellular Ca\(^{2+}\)-free medium ([Ca\(^{2+}\)]\(_o\)=0), CaCl\(_2\) was replaced by 0.1 mmol/L EGTA. Observations were made at 34°C with a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy. The ratio was calculated from 340- and 380-nm frame pairs (1 ratio per 3 seconds) to measure a true image subjected to ratio calculation for further analysis. After background correction, Ca\(^{2+}\) values were calculated on whole RASMCs, which are elongated cells (~70 × 10 μm in size) corresponding to an area of ~100 × 15 pixels with the ×40 objective used.

Calibration of fura 2 fluorescence in terms of [Ca\(^{2+}\)]\(_i\) was performed in individual cells and calculated from the ratio of 340-nm/380-nm fluorescence values by using a K\(_j\) (Ca\(^{2+}\) dye) of 250 nmol/L. Maximum and minimum fluorescence ratios were determined as described previously. The resting [Ca\(^{2+}\)]\(_i\), determined by averaging Ca\(^{2+}\) values obtained for 30 seconds on 5 cells from 3 different coverslips and 3 cell preparations (n=45). The cytosolic Ca\(^{2+}\) concentration elevation induced by an agonist (Δ[Ca\(^{2+}\)]\(_i\)) was determined by averaging the values obtained for 36 cells from 3 different cultures.

Cells were continuously superfused at a rate of 1.5 to 2 mL/min with control or test solutions by 6 inlet tubes converging on the coverslip chamber. The perfusion chamber volume was ~0.2 mL. Calcium and ATP were dissolved in perfusion medium, and stock solutions of thapsigargin (TG) were prepared in dimethyl sulfoxide.

**Immunofluorescence Study**

The SERCA2a and SERCA2b antibodies (a gift from F. Wuytack, University of Leuven, Leuven, Belgium) have been described elsewhere. The IP3R type I antibody was from Affinity Bioreagents. The polyclonal anti-RyR antibody was against the RyR purified from pig skeletal muscle. It reacted with skeletal muscle and cardiac tissue. Immunolabeling with antibodies specific for the smooth muscle myosin heavy chain (MHC) isoforms, SM1 and SM2, was used as a marker of the contractile phenotype, whereas the nonmuscle MHC-B (NMB-MHC) antibody was a marker of the undifferentiated phenotype. These antibodies were from Yamasa Corp and have been previously described. An anti–proliferating cell nuclear antigen antibody (α-PCNA, clone PC10, Dako) was used as marker of proliferation.

The cells were fixed in paraformaldehyde (4%) for 20 minutes at room temperature, washed in PBS and in PBS containing 50 mmol/L NH₄Cl, and permeabilized with 0.1% Triton X-100 (6 minutes). They were washed 3 times and incubated 2 hours at room temperature with diluted antibodies (PBS containing 3% BSA): anti-(a)-SERCA2a (1/250), -SERCA2b (1/250), -α-IP3R (1/120), -α-RyR (1/150) or -α-SM2-MHC (1/1000), -α-SM1-MHC (1/1000), -α-NMB-MHC (1/2000), and α-PCNA (1/50). They were incubated 1 hour with biotinylated anti-mouse or anti-rabbit IgG (1/200, Amersham) and 45 minutes with streptavidin–Texas red (1/200) at room temperature. For double immunofluorescence studies, cells were fixed in −20°C methanol (8 minutes) and washed in PBS. PCNA was detected with Texas red–conjugated anti-mouse IgG (Amersham), and the antibodies specific for Ca\(^{2+}\) pumps or Ca\(^{2+}\) release channels, with biotinylated anti-rabbit immunoglobulin and fluorescein streptavidin (Amersham). Coverslips were mounted in glycerol/PBS medium (FluoroGuard antifade Reagent, Bio-Rad).

** Polymerase Chain Reaction**

Total RNA from rat brain, aorta, heart, skeletal muscles, cardiomyocytes, hepatocytes, Caco-2 (human colon carcinoma cells), and aortic smooth muscle cells in culture was extracted by the RNA-InstaPure procedure (Eurogentec). Total RNA (1 μg) was used for reverse transcription (RT) by using 2.5 μmol/L random hexamers (Boehringer-Mannheim) or oligo-dT primers (Promega) according to the polymerase chain reaction (PCR) II protocol (Perkin-Elmer). Two PCR reactions were set up from each first-strand reaction. SERCA2b was used as a standard for each reaction. A control without reverse transcriptase was included in each experiment. The reactions were performed in a final volume of 100 μL in a Perkin-Elmer GeneAmp PCR system according to the Perkin-Elmer protocol with 40 cycles of 15 seconds at 95°C, 30 seconds at the specific annealing temperature for the primers, and 30 seconds at 72°C.

Primers for SERCA2a and SERCA2b amplified nucleotides 2615 to 3122 and 3121 to 3630 of published sequences, respectively. RyR1 primers generated a 435-bp fragment from the mouse sequence. RyR2 primers were from the rabbit cardiac RyR2 cDNA and amplified from nucleotides 8603 to 9144. RyR3 primers were as described. Sizes of amplified DNA fragments were 614 bp for RyR3-II and 273 bp for RyR3-I. Primers common to all 3 IP3R subtypes were as in Reference 26. Twenty to 24 nucleotide primers were annealed at 60°C, 50°C, 62°C, 52°C, and 60°C for SERCA2a and SERCA2b, RyR1, RyR2, RyR3, and IP3R, respectively.

The PCR products were subjected to electrophoresis on 8% polyacrylamide gels, stained by using Vistra-green (Amersham), and scanned with a PhosphorfluorImager (Storm, Molecular Dynamics). For RyR3 and IP3R, the DNA was transferred to Hybond N+ membranes and detected by probing with specific oligonucleotides labeled with [γ-32P]ATP (NEN) by using T4 kinase (Eurogentec). The RyR3 oligonucleotide corresponds to a region upstream from the splice site. The IP3R isotype–specific oligonucleotides used in Southern blotting were as described. Hybridization was performed in 6× standard saline citrate (1× SSC contains 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate), 0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.5% SDS, and 100 μg/mL salmon sperm DNA at 60°C. The membranes were washed with 6× SSC and 2× SSC at room temperature and then with 0.5× SSC at 60°C for RyR3, 50°C for IP3R1 and IP3R2, and 40°C for IP3R3. The
membranes were then exposed to the PhosphorImager screen, and signals were recorded by use of Storm.

**Statistical Analysis**

Sensitivity to caffeine, ATP, or caffeine+ATP was estimated by counting the responsive cells on each coverslip. Results were expressed as a percentage of reactive cells. The resting Ca$^{2+}$ level was determined for each cell population by averaging Ca$^{2+}$ values during 30 seconds for 45 cells (from 3 cultures at each time point and 3 coverslips each). The values of Ca$^{2+}$ peaks, $\Delta$[Ca$^{2+}$], after caffeine or ATP stimulation were estimated on 36 cells (6 coverslips in 3 cultures at each time point). The results were expressed as mean±SEM. For each culture, normality was assessed by using the Kolmogorov-Smirnov test. One-way ANOVA or Kruskal-Wallis ANOVA on ranks, followed by the Student-Newman-Keuls test, was used for the statistical comparison of multiple groups. An unpaired $t$ test was used for comparison of the $\Delta$[Ca$^{2+}$] values obtained with caffeine and ATP. Data were analyzed by using the SigmaStat Statistical Software Package, Version 1.0 (Jandel Scientific).

**Results**

**RASMCs Contain Connected RyR-Sensitive and IP3-Sensitive Ca$^{2+}$ Pools**

Experiments were performed in the absence of external Ca$^{2+}$ and in the presence of EGTA to eliminate any possible effect of Ca$^{2+}$ influx. Caffeine induced transient increases in [Ca$^{2+}$], but little or no increase was observed after subsequent ATP application (Figure 1A). After incubation in normal Ca$^{2+}$ medium, an increase in [Ca$^{2+}$], was obtained when ATP was added, but subsequent application of caffeine had no effect (Figure 1A). Thus, both agonists released Ca$^{2+}$ from the same store. Incubation of the same cell with TG (500 mmol/L), a specific inhibitor of the SERCAs, in Ca$^{2+}$-free medium resulted in a slow increase in the intracellular Ca$^{2+}$ level, causing depletion of the Ca$^{2+}$ pools (Figure 1B). Neither caffeine nor ATP induced an increase in [Ca$^{2+}$], in the presence of TG. Thus, the caffeine-sensitive and IP3-sensitive pools are both present in freshly dissociated RASMCs, and they are both TG sensitive.

**RASMCs Lose the Caffeine-Sensitive Ca$^{2+}$ Pool in Culture**

To determine whether the Ca$^{2+}$ pools were stable or were altered during culture, the percentage of cells responding to caffeine, ATP, and both agonists and the percentage of nonresponsive cells were determined on days 1, 3, 5, and 12 of culture (Figure 2). The values of resting Ca$^{2+}$ concentration and of the peaks of Ca$^{2+}$ released by each agonist were also determined (Table). On day 1, a total of 321 cells (7 cultures and 3 to 7 coverslips per culture) were analyzed. No statistical difference in sensitivity to caffeine ($P=0.214$), ATP ($P=0.06$), or caffeine+ATP ($P=0.229$) was observed between the 7 different cultures tested. In our cell population, 77% of cells were sensitive to caffeine and ATP, 3% were sensitive to caffeine only, and 8% were sensitive to ATP only, whereas 12% were insensitive to both drugs (Figure 2). The resting Ca$^{2+}$ concentration was 169±2 mmol/L (n=45), and the increases in cytosolic Ca$^{2+}$ concentration induced by caffeine and ATP were not different (736±25 mmol/L and 817±36 mmol/L, respectively; $P>0.05$; Table).

When RASMCs were actively proliferating (day 5), caffeine did not induce Ca$^{2+}$ release even at concentrations as high as 40 mmol/L, whereas a transient increase in [Ca$^{2+}$], (882±54 mmol/L) was observed after application of ATP (Table). All cells had lost their functional caffeine-sensitive pool, 80% were sensitive to ATP only, and 20% were insensitive to both agonists (Figure 2). This disappearance was progressive, because on day 3, 17% of the cells (16 of 92 cells from 11 coverslips and 3 cultures) were still sensitive to caffeine, whereas none of the cells tested (0 of 190 cells from 3 cultures and 14 coverslips) were sensitive to caffeine on day 5 (Figure 2). In our conditions, the cells were subconfluent and had stopped proliferating after 12 days. By day 12, 54% of the cells (240 of 442 cells from 4 cultures and 19 coverslips) had reacquired sensitivity to caffeine (Figure 2). The cells did not respond to 5 or 10 mmol/L caffeine, and 40 mmol/L caffeine was required to induce Ca$^{2+}$ release. There was no significant difference between the different cultures on day 12 in ATP sensitivity ($P=0.444$) or ATP+caffeine sensitivity ($P=0.186$). However, when comparing days 1, 3, 5, and 12, there was a significant difference
in ATP\textsuperscript{+}caffeine sensitivity ($P<0.001$) or in ATP sensitivity alone ($P<0.001$).

During the time of culture, the resting Ca\textsuperscript{2+} concentration did not vary significantly ($P=0.092$), and the size of the ATP-sensitive Ca\textsuperscript{2+} pool was also similar ($P=0.063$, Table). Furthermore, the amounts of Ca\textsuperscript{2+} released by caffeine and ATP were not different on day 1 ($P=0.069$) or on day 12 ($P=0.095$). Thus, the difference in SR Ca\textsuperscript{2+} load is not responsible for the absence of sensitivity to caffeine.

Each Freshly Dissociated RASMC Expresses 2 SERCA Isoforms and 2 Ca\textsuperscript{2+} Release Channels

To characterize the phenotype of the isolated RASMCs, the pattern of expression of the genes encoding the SR Ca\textsuperscript{2+} pumps and Ca\textsuperscript{2+} channels was determined, at the protein and RNA levels, on cells exposed to the culture medium for 1 day. Expression of the MHC isoforms and the PCNA antigen were used as markers.

All cells in culture for 1 day in the presence of serum expressed SM1 and SM2 MHCs; NMB-MHC was present only at a low level. This indicates that the cells had a differentiated contractile phenotype. The PCNA antigen was not detected, indicating that the RASMCs were quiescent (Figure 3A).

Two SERCA2 mRNA isoforms are present in rat aorta.\textsuperscript{15,27} We used immunofluorescence and interference contrast images to show that SERCA2a and SERCA2b were both expressed in each individual smooth muscle cell. Similarly, RyR and IP3R were present in each cell (Figure 3B). The immunolabeling with a-IP3R was mainly but not exclusively perinuclear, but confocal images revealed no labeling inside the nucleus (not shown). The a-RyR antibody was not isoform specific and reacted with cardiac as well as skeletal microsomes.\textsuperscript{15,20} No reaction was obtained with a monoclonal antibody specific for RyR2, which also reacts weakly with RyR1 (MA3-916, Affinity Bioreagents; not shown).

We checked for the specificity of RyR in smooth muscle by performing RT-PCR with oligonucleotides specific for RyR1, RyR2, and RyR3 (Figure 4). RyR1 mRNA was present in skeletal muscle and brain but not in cardiac muscle. It was also present in aortic preparations containing endothelium but not in those free of endothelium. RyR2 mRNA was present in cardiac muscle, in brain, and in aortic preparations containing endothelium but only at low levels in skeletal muscle or aortic preparations free of endothelium. Two RyR3 mRNA isoforms were detected: the upper band is compatible with RyR3-II (614 bp), and the lower band is compatible with RyR3-I (273 bp).\textsuperscript{25} RyR3-I was constitutively expressed, whereas RyR3-II was present in brain, total aorta, and aorta without endothelium but not in skeletal muscle or hepatocytes.

RASMCs Lose SERCA2a and RyR but Keep SERCA2b and IP3R in Culture

In agreement with previous results,\textsuperscript{26} the RASMCs had lost the SM2-MHC in culture for 5 and 12 days, but the SM1-
MHC was still expressed in each cell, indicating their smooth muscle phenotype. The NMB-MHC was highly expressed, especially at days 5 and 12 (Figure 5A).

To determine whether change in the SR Ca\(^{2+}\)-transporting enzyme phenotype could account for the loss of caffeine sensitivity that we observed in culture, we first analyzed the protein pattern by immunofluorescence with the use of specific antibodies. The cells were simultaneously labeled with a-PCNA to determine whether proliferation was associated with change in phenotype (Figure 5B). On day 1, all cells were labeled with the antibodies for SERCA2a, SERCA2b, RyR, and IP3R (green), but none were labeled with a-PCNA (red). On day 3, the cells were labeled with a-PCNA, indicating that they entered the cell cycle. SERCA2b and IP3R were expressed at high levels, whereas SERCA2a and RyR were present only at low levels. On day 5, all cells were labeled with a-PCNA. They expressed SERCA2b and IP3R but not SERCA2a and RyR. When cells were subconfluent (day 12), they stopped dividing and did not express the PCNA antigen, and SERCA2a and RyR were detectable again. These data indicate that the expression of SERCA2 and RyR is regulated during culture.

To determine whether regulation of gene expression is translational or pretranslational, we analyzed the Ca\(^{2+}\) pumps and Ca\(^{2+}\) channel mRNA levels by RT-PCR (Figure 6). SERCA2 (Figure 6A), IP3R (Figure 6B), and RyR (Figure 6C) mRNA isotypes were amplified from total RNA from the aorta, from RASMCs cultured for 1, 3, 5, and 12 days, and from control RNAs. SERCA2b was used as a control in the PCR reaction. SERCA2a was present in RASMCs on day 1 but was absent on days 3 and 5 and was detected again on day 12 (Figure 6A). SERCA2a mRNA was not detected again on day 3, whereas the corresponding protein was still present (Figure 5B). This can be explained by differences in the half-lives of mRNA and protein. SERCA2a mRNA was detected again on day 12, together with the corresponding protein.

IP3R mRNA was detected in each mRNA preparation tested, whereas SERCA2b was below the detection level in isolated cardiomyocytes (Figure 6B). IP3R-1 was the major isoform in the aorta and RASMCs, and its level was rather constant during culture, whereas IP3R-2 was present at low but constant levels in all RASMC preparations. In cardiomyocytes, type 2 was present at high levels as demonstrated,\(^{26}\) but some IP3R-1 was also detected in our experiments. IP3R-3 was not detected in RASMCs at any stage of culture but was present at very high levels in Caco cells, as previously demonstrated (J. Mesonero, personal communication, 1999). Thus, 2 IP3R isotypes were detected in RASMCs, but their mRNA levels did not change during culture.

RyR3-I and RyR3-II mRNAs were present in total aorta and in cultured cells (Figure 6C). RyR3-I but not RyR3-II was also present in skeletal and cardiac muscle. RyR3-I mRNA was produced at all stages of proliferation, whereas RyR3-II disappeared progressively on days 3 and 5 and was not present on day 12. This suggests that splicing of the RyR3 gene is regulated during proliferation as for the SERCA2 gene. Surprisingly, large amounts of RyR3-I mRNA were detected on day 5, whereas there was no RyR protein (Figures 5B and 7B) and no caffeine sensitivity (Figure 2).
Loss of SERCA2a and RyR Is Associated With the Proliferative State of RASMCs

To gain better insight into the mechanisms of alteration of SR Ca\(^{2+}\) pump and channel phenotypes, RASMCs were cultured in different conditions of medium and density, and the phenotype was analyzed by immunofluorescence and RT-PCR.

Expression of SM2- and NMB-MHC was used as a marker of the differentiated and undifferentiated phenotype, respectively (Figure 7A). In 10% FCS, SM2-MHC was absent, and NMB-MHC was present at high levels. In low serum (0.1%), the differentiated phenotype and expression of SM2-MHC were maintained. Addition of PDGF-BB to low serum reversed the phenotype of MHC toward the undifferentiated NMB-MHC. When grown at high density in presence of 10% FCS, the cells had a differentiated phenotype, as shown by the presence of the SM2-MHC.

Double immunolabeling with a-PCNA (in red) and antibodies specific for the SR Ca\(^{2+}\) pumps and channels (in green) were performed on the same cell cultures (Figure 7B). In 10% FCS, RASMCs were labeled with a-PCNA and had lost SERCA2a and RyR. In low serum, the cells did not proliferate and were not labeled with a-PCNA. In these conditions, they still expressed SERCA2a and RyR. In the presence of PDGF-BB, the PCNA antigen was detected, indicating that the cells had entered the cell cycle, and SERCA2a and RyR were absent. Plating at high density in the presence of 10% FCS prevented proliferation (no PCNA antigen detected) and loss of SERCA2a and RyR.

To confirm the immunofluorescence data and to determine whether regulation of SERCA2a and RyR gene expression was translational or pretranslational, SERCA2a, SERCA2b, and RyR mRNA levels were measured on the same cultures by RT-PCR (Figure 8). SERCA2a mRNA was present in RASMCs cultured in low serum or at high density, whereas it was absent from cells cultured in 10% FCS or PDGF-BB. RyR3-I was ubiquitously expressed. RyR3-II was absent from cells cultured in high serum or PDGF-BB but was present in cells cultured in low serum or at high density.

These 2 sets of data demonstrate that expression of SERCA2a and RyR3 is regulated at a pretranslational level when RASMCs proliferate.

Discussion

Our data demonstrate the following: (1) In freshly isolated RASMCs, Ca\(^{2+}\) is released from internal stores by 2 mechanisms, a caffeine- and ryanodine-sensitive mechanism and an IP3-sensitive mechanism, both of which are sensitive to TG. This is supported by the presence in each individual cell of 2 Ca\(^{2+}\) release channels, RyR3 and IP3R, and 2 Ca\(^{2+}\) pumps, SERCA2a and SERCA2b. (2) Reorganization of the intracellular Ca\(^{2+}\) compartments is part of the dedifferentiation process that occurs during culture: the RASMCs lose their caffeine- and ryanodine-sensitive store because of pretranslational changes in the expression of the RyR3 and SERCA2 genes. (3) Expression of RyR3 and SERCA2a is repressed at a pretranslational level when cells acquire a proliferative phenotype.

We have demonstrated that the caffeine sensitivity is lost in proliferating cells but reappears when cells are subconfluent. This observation is consistent with previous results showing that the ryanodine-sensitive Ca\(^{2+}\) store was absent from proliferating cultures of RASMCs but was present in subconfluent cultures. Furthermore, it has been shown that 80% of quiescent RASMCs in the early phase of culture responded to high K\(^{+}\), angiotensin II, and caffeine by an increase in [Ca\(^{2+}\)], but that most of the cells had lost their [Ca\(^{2+}\)] responsiveness to the 3 agents in the proliferating phase.

Several explanations may be found for changes in reactivity to pharmacological agents affecting Ca\(^{2+}\) release from
Figure 5. A, Immunodetection of SM1, SM2, and NMB-MHCs in RASMCs in culture for 3, 5, or 12 days. B, Double-immunolabeling of RASMCs in culture for 1, 3, 5, and 12 days with a-SERCA2a, a-SERCA2b, a-RyR, and a-IP3R (green) and with a-PCNA (red). Bar=10 μm.
internal stores. Our data indicate that changes in the caffeine sensitivity cannot be attributed to changes in the SR Ca\(^{2+}\) load because the SR Ca\(^{2+}\) content, estimated by the amount of Ca\(^{2+}\) released by high concentrations of caffeine or ATP, was similar throughout the culture period. The basal cytosolic Ca\(^{2+}\) concentration was also unchanged. We show that alteration in caffeine sensitivity is associated with changes in the phenotype of SR Ca\(^{2+}\) pumps and channels. Caffeine-insensitive cells do not express RyR but do express IP3R. Similarly, SERCA2a is absent, but the ubiquitous SERCA2b isoform is still present. Subconfluent cells regain sensitivity to high doses of caffeine, but RyR1 rather than RyR3 is present. The low sensitivity to caffeine cannot be explained by the presence of a different RyR isoform because both are sensitive to caffeine. Other mechanisms that control the activity of the Ca\(^{2+}\) channels, such as the level of intrareticular proteins that regulate Ca\(^{2+}\) release and the Ca\(^{2+}\) sparks, are also regulated during proliferation. Alternatively, the frequency of the Ca\(^{2+}\) sparks, the physiological stimuli of the RyRs, may vary independently of the fact that the RyRs are functional and Ca\(^{2+}\) is released by caffeine. Such a situation has been described recently during ontogeny of the cerebral arteries. All these changes will participate to alter the sensitivity of the vessel to pharmacological vasoconstricting drugs.

Very little is known about the mechanisms of control of the expression of the SR Ca\(^{2+}\) pumps and Ca\(^{2+}\) channels in smooth muscle. The 2a isoform of the SERCA2 gene is the muscle-specific variant, and splicing of the SERCA2 gene is regulated by myogenic factors during differentiation of a muscle cell line. A similar phenomenon may occur in smooth muscle cells, and myogenic factors that are induced when smooth muscle cells differentiate may favor splicing of the SERCA2 gene toward SERCA2a. We have previously shown that the level of SERCA2a mRNA increases in the rat aorta during maturation of the vessel, whereas the level of SERCA2b mRNA remains stable. These 2 sets of results are consistent, and in both cases, the differentiated smooth muscle phenotype was associated with the presence of SERCA2a. We demonstrate that SERCA2a is downregulated by PDGF-BB in RASMCs, whereas upregulation of SERCA2a was observed in porcine aortic cells after 16 to 34 hours in the presence of PDGF-BB. Differences in the initial phenotype of the cells and in the level of action of PDGF-BB may account for some of the discrepancies. Indeed, porcine aortic cells were passaged, and the effect of PDGF-BB was mainly translational, whereas we used primary cultures, and our results indicate that regulation was pretranslational. Differentiation of muscle cell lines was also associated with changes in the caffeine sensitivity and expression of the ryanodine receptor gene, but at that time, the various RyR isoforms were not yet described. Transforming growth factor-β was shown to induce the expression of RyR3 together with the release of Ca\(^{2+}\) in response to ryanodine in mink lung epithelial cells.

The process of dedifferentiation of vascular smooth muscle cells has been subject to intensive interest because of its importance in vascular pathology. It is clearly demonstrated that when vascular smooth muscle cells reenter the cell cycle, they undergo a transition from a contractile to a synthetic phenotype, and this is associated with major changes in the

**Figure 6.** Detection of SERCA2 (A), IP3R (B), and RyR (C) mRNA isoforms by RT-PCR in total aorta and RASMCs in culture. Total RNA from skeletal muscle (Sk.muscle), heart, cardiomyocytes, and Caco cells were used as controls. In panel A, PCR products were detected by using Vistra-green. In panel B, IP3R mRNA was amplified by using primers common to all isoforms. Total PCR products were stained by Vistra-green with SERCA2b used as control (2 upper lanes). Each IP3R mRNA isoform was then detected by Southern blotting with specific oligomers (3 lower lanes). Southern blot hybridized with type 1 was exposed for 4 hours, whereas the membranes hybridized with types 2 and 3 were exposed for 16 hours. In panel C, RyR isoforms were detected by using SERCA2b as a control. The cDNAs corresponding to RyR1 and RyR2 were stained by using Vistra green and detected with a FluorImager, whereas the 2 RyR3 isoforms were visualized by Southern blotting. Two alternatively spliced RyR3 mRNAs, RyR 3-I (273 bp) and RyR 3-II (614 bp), were present; RyR3-II disappeared progressively during culture.
Figure 7. A, RASMCs in culture for 6 days in different conditions were immunolabeled with antibodies specific for SM2- or NMB-MHCs. Culture conditions were as follows: 6 days in 10% FCS (a and b), 2 days in 10% FCS and 4 days in 0.1% FCS (c and d), 2 days in 10% FCS, 1 day in 0.1% FCS, and 3 days in 0.1% FCS+50 ng/mL PDGF-BB (e and f), and high-density cell culture (10⁶ cells/cm²) with 10% FCS for 6 days (g and h). Bar=10 μm. B, Double immunolabelings of RASMCs cultured as in panel A, with a-SERCA2a, a-SERCA2b, a-RyR, and a-IP3R (in green) and with a-PCNA (in red). Bar=10 μm.
arteries, which are characterized by the presence of undifferentiated smooth muscle cells. It remains to be demonstrated whether the RyRs are absent from proliferating smooth muscle cells in vivo as they are from cells in culture. If loss of RyR occurs in vivo, this may play an important role in the pathology of arterial and coronary vasospasm and may be an important factor in myocardial ischemia and infarction.

Many factors may turn on and off the smooth muscle program, such as growth factors, cell-cell interaction, extracellular matrix, and mechanical stresses. The extracellular matrix cannot be involved in the modifications described in the present study because the same cell substrate, collagen-type I, was used in all experiments. Several soluble factors present in the serum may also control gene expression. However, in the presence of 10% FCS, the cells keep the differentiated phenotype when grown at high density or at subconfluence, whereas in the proliferative phase, they are dedifferentiated. Thus, the medium by itself is not sufficient to explain the changes in phenotype that we observed. Cell-cell interaction is certainly an important factor because redifferentiation occurs when cells are confluent and are kept in high-density cultures. The absence of pressure and flow and the absence of the endothelial cell lining may also be important factors in the dedifferentiation process. Additional experiments are now needed to define the factors involved in the regulation of SERCA2, RyR, and IP3R gene expression in vascular smooth muscle cells.

Acknowledgments

This study was supported by the Ministère de l’Education nationale, de l’Enseignement Supérieur, de la Recherche, et de l’Insertion professionnelle (ACC-SV No. 9509012), Recherche et Partage, and Fondation pour la Recherche Médicale. The authors are grateful to Dr F. Wuytack (University of Leuven, Belgium) for providing the a-SERCA2. They thank José Mesonero (INSERM U442, Orsay) for sharing unpublished data and providing the Caco cell total RNA.

References


Intracellular Ca^{2+} Handling in Vascular Smooth Muscle Cells Is Affected by Proliferation
Olivier Vallot, Laurent Combettes, Philippe Jourdon, Jocelyn Inamo, Isabelle Marty, Michel Claret and Anne-Marie Lompré

doi: 10.1161/01.ATV.20.5.1225
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/5/1225

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/