Functional coupling between the caffeine/ryanodine-sensitive Ca^{2+} store and mitochondria in rat aortic smooth muscle cells

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We investigated the role of mitochondria in the agonist-induced and/or caffeine-induced Ca^{2+} transients in rat aortic smooth muscle cells. We explored the possibility that proliferation modulates the coupling between mitochondria and endoplasmic reticulum. Ca^{2+} transients induced by either ATP or caffeine were measured in presence or absence of drugs interfering with mitochondrial activity in freshly dissociated cells (day 1) and in subconfluent primary culture (day 12). We found that the mitochondrial inhibitors, rotenone or carbonyl cyanide *m*-chlorophenylhydrazone, as well as the permeability transition pore inhibitor, cyclosporin A, had no effect on the ATP-induced Ca^{2+} transient at either day 1 or day 12, but prevented caffeine-induced cytosolic Ca^{2+} increase at day 12 but not at day 1. Close connections between ryanodine receptors and mitochondria were observed at both day 1 and 12. Thapsigargin (TG) prevented ATP- and caffeine-induced Ca^{2+} transients at day 1. At day 12, where only 50 % of the cells were sensitive to caffeine, TG did not prevent the caffeine-induced Ca^{2+} transient, and prevented ATP-induced Ca^{2+} transient in only half of the cells. Together, these data demonstrate that rat aortic smooth muscle cells at day 1 have an ATP- and caffeine-sensitive pool, which is functionally independent but physically closely linked to mitochondria and totally inhibited by TG. At day 12, we propose the existence of two cell populations: half contains IP_3 receptors and TG-sensitive Ca^{2+} pumps only; the other half contains, in addition to the IP_3-sensitive pool independent from mitochondria, a caffeine-sensitive pool. This latter pool is linked to mitochondria through the permeability transition pore and is refilled by both TG-sensitive and insensitive mechanisms.

Key words: calcium signalling, sarcoplasmic reticulum, thapsigargin.

INTRODUCTION

Intracellular Ca^{2+} stores actively participate in Ca^{2+} signalling in several cell types, including smooth muscle cells [1]. Endoplasmic reticulum (ER) and its specialized subcompartment, the sarcoplasmic reticulum (SR), are the main dynamic Ca^{2+} stores of these cells. Release of Ca^{2+} from SR/ER depends on two mechanisms: the Ca^{2+}-induced Ca^{2+} release, involving ryanodine receptors (RyRs), and the IP_3-induced Ca^{2+} release, involving IP_3 receptors (IP_3R), which is also modulated by Ca^{2+}. The Ca^{2+} compartments are refilled by SR/ER Ca^{2+}-ATPases (SERCAs), which transport Ca^{2+} by an active process that requires the hydrolysis of ATP. Three genes that encode SERCA have been described in several mammalian species. They give rise to at least six different isoforms by splicing at their 3′-end (reviewed in [2]). All SERCAs isoforms are selectively inhibited by thapsigargin (TG), a tumour-promoting sesquiterpene lactone [3,4]. Other pharmacological substances, such as 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBHQ) and cyclopiazonic acid, can also block SERCAs, but bind with lower affinities [5].

Mitochondria rapidly accumulate and sequester a large amount of Ca^{2+} by the activity of a Ca^{2+} uniporter stimulated by high intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [6]. This Ca^{2+} store not only regulates the activity of mitochondrial enzymes, but also contributes to buffer locally the cytosolic Ca^{2+} concentration. Indeed, recent studies indicate that during Ca^{2+}-signalling events mitochondria sense Ca^{2+} released by the IP_3 receptor [6–9] or the RyR [10,11] from the SR/ER into the cytoplasm. Furthermore, mitochondrial substrates and blockers of mitochondrial respiration alter the propagation of Ca^{2+} waves [12,13]. In addition, close contacts between mitochondria and ER have been demonstrated [14], and functional interactions between mitochondria and ER have been measured in intact cells [9,15]. In smooth muscle, direct evidence has been provided recently that release of Ca^{2+} from SR increases mitochondrial Ca^{2+} concentrations in rat pulmonary artery, guinea-pig colonic smooth muscle cells, aortic smooth muscle cells in culture, and in a smooth muscle cell line [11,16–18].

Ca^{2+} efflux from mitochondria occurs very slowly through the Na^+/Ca^{2+}-exchanger. Ca^{2+} efflux may also occur rapidly by a mitochondrial Ca^{2+}-induced Ca^{2+} release through the permeability transition pore (PTP) [19,20]. The PTP is sensitive to Ca^{2+}, and is selectively inhibited by the immunosuppressor drug, cyclosporin A (CsA), at nanomolar concentrations (reviewed in [21]).

The aim of the present study was to determine: (i) whether mitochondria are involved in the agonist- or caffeine-induced increase in $[Ca^{2+}]_i$; and (ii) whether coupling between mito-

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; SERCA, SR/ER Ca^{2+}-ATPase; RyR, ryanodine receptor; IP_3R, IP_3 receptor; TG, thapsigargin; tBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; PTP, permeability transition pore; CsA, cyclosporin A; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; RASMC, rat aortic smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium.

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chondria and SR/ER is modulated during cell culture. We used rat aortic smooth muscle cells (RASMC) because they express both RyRs and IP_3Rs, the expression of which is regulated by cell proliferation. Indeed, in a previous study [22] we have shown that intracellular Ca^{2+} handling in RASMC is affected by the proliferation status of the cell. When cells proliferate actively, the caffeine/ryanodine-sensitive Ca^{2+} pool is lost and expression of RyR3 and SERCA 2a is down-regulated, whereas IP_3R and SERCA 2b expression is maintained. Re-differentiation in culture is associated with the reappearance of caffeine sensitivity; however, RyR1 is expressed rather than RyR3.

EXPERIMENTAL

Primary culture of RASMC

RASMC were isolated from the medial layer of thoracic aorta (aortic cross excluded) of 180–200 g male Wistar rats by enzymic digestion with collagenase (CLS2; Worthington Biochemical Corp., Freehold, NJ, U.S.A.; 50 units/ml) and pancreatic elastase (ICN; 0.25 mg/ml) for 2 h at 37 °C, with a continuous slow agitation. After treatment for 20 min, the suspension was centrifuged for 5 min at 430 ***g***, and cells were collected and placed in Dulbecco's modified Eagle's medium (DMEM; ICN) containing 1 % glutamine and 1 % antibiotics (penicillin and streptomycin) and supplemented with 20 % fetal-calf serum (Myoclone plus; Gibco BRL). Several rounds of enzyme treatment were performed. Normally, the cells obtained during the first three 20 min periods were discarded because they displayed a non-muscle phenotype [23]. Cells obtained in the subsequent cycles were pooled, centrifuged, suspended in DMEM containing 10 % fetal-calf serum, and plated on to glass coverslips coated with collagen I (rat tail; Sigma) at a density of 1.3×10^4 cells/cm^2. These cells were then cultured at 37 °C in humidified air/CO_2 (19:1) and maintained by changing the medium every second day with DMEM containing 10 % fetal-calf serum.

Ca^{2+} imaging

RASMC plated on to coverslips were loaded with 3 μM fura 2 acetoxymethyl ester for 40 min at 37 °C in humidified air/CO_2 (19:1). The coverslips were then washed twice with a normal saline solution (10 mM Hepes, pH 7.4, 140 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 4.2 mM $NaHCO_3$ and 11 mM glucose). For experiments in Ca^{2+}-free medium, $CaCl_2$ was replaced by 10 μM EGTA. Cells were continuouly superfused (over-flow method; [24]) at a rate of 1.5–2 ml/min, with control or test solution by six inlet tubes converging on the coverslip chamber. The chamber volume was approx. 0.2 ml and temperature was maintained at 34 °C. Fluorescence images were acquired from an inverted microscope (Zeiss Axiovert 35) set up for epifluorescence microscopy equipped with a xenon UV lamp. Analysis were performed on entire RASMC (approx. 70×10 μm size), corresponding to an area of approx. 100×15 pixels with the $40\times$ objective used. Fluorescence intensity after background correction was averaged (F) and plotted as normalized fluorescence ($\Delta F/F_0$) against time. ΔF was calculated as the difference between the mean value of the first 20 data points prior to stimulation of the cell (F_0) and F. The ratio of fura 2 fluorescence intensities at 340 nm and 380 nm ($\Delta R/R_0$) was calculated to measure a true image every 3 s, for up to 30 min. Transformation of ratios into $[Ca^{2+}]_i$ values was performed for individual cells, using a $K_{D(Ca2+\cdot dye)}$ of 250 nM. R_{max} and R_{min} were determined as described previously [22]. The resting $[Ca^{2+}]_i$ was determined by averaging Ca^{2+} values obtained during 30 s from five cells on three different coverslips and from three different cell preparations.

Caffeine and ATP were used, in absence of external Ca^{2+}, to activate the RyR and IP_3R respectively. TG was used to inhibit SERCA. A mitochondrial uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial complex I inhibitor, rotenone, and a mitochondrial PTP inhibitor, CsA, were used to assess the role of mitochondria. Oligomycin was used to inhibit the ATP synthase. Caffeine, ATP, CCCP, rotenone, tBHQ, oligomycin and cyclosporin A (Sigma) were dissolved in the Ca^{2+}-free perfusion medium. Stock solution of TG was prepared in DMSO, whereas CCCP, cyclosporin A and oligomycin stock solutions were prepared in ethanol.

Confocal analysis of mitochondria and RyR distribution

RASMC at day 1 and 12 were loaded with 0.2 μM MitoTracker Red CMXros (M-7512; Molecular Probes) for 1 h at 37 °C in DMEM containing 10 % fetal-calf serum, either in the presence of 20 μM CCCP added 10 min before the MitoTracker, or in its absence. After washing in PBS, the cells were fixed in 4 % paraformaldehyde for 20 min, permeabilized with Triton X-100 0.1 % for 10 min, and then incubated with a polyclonal anti-RyR antibody [25] diluted 1/200 in PBS containing 3 % BSA. The antibody binding was revealed by incubation with a biotinylated anti-rabbit immunoglobulin (1/200; Amersham) and streptavidin-fluorescein (1/200; Amersham). The entire protocol was performed in the dark.

Samples were examined using a Zeiss LSM 510 equipped with a $\times 63$ objective. To avoid any bleed-through, we used two fluorophors with extremely well separated emission spectra: 520 nm and 579 nm for fluorescein and MitoTracker respectively. Dual excitation, using the multitrack mode (images were taken sequentially), was achieved using the 488 nm argon and 543 nm He/Ne lasers respectively. The gains were such that the red and green signals were approximately the same intensity, but below saturation, and the black level was adjusted to remove the sub-signal noise. Cells displayed for each experiment were representative of many cells imaged over at least two independent experiments.

Data analysis

Sensitivity to caffeine or ATP under control conditions, and after treatment with TG, tBHQ, CCCP, rotenone and cyclosporin A, was estimated by counting the cells which generated a Ca^{2+} transient after addition of ATP or caffeine. Results were obtained from several coverslips from at least two different cultures and were expressed as percentage of responsive cells. The values of Ca^{2+} peaks and resting $[Ca^{2+}]$ were expressed as means $\pm$ S.E.M. and compared by a t test.

RESULTS

Inhibitors of mitochondrial activity do not prevent ATP-induced ER Ca^{2+} release

To investigate the functional relationships between mitochondria and SR/ER Ca^{2+} pools, cells were treated with CCCP, a drug that collapses the mitochondrial proton gradient and thus prevents Ca^{2+} uptake from the cytosol to the mitochondrial matrix. The IP_3-sensitive pathway was stimulated by ATP (20 μM) and the experiments were performed in a Ca^{2+}-free

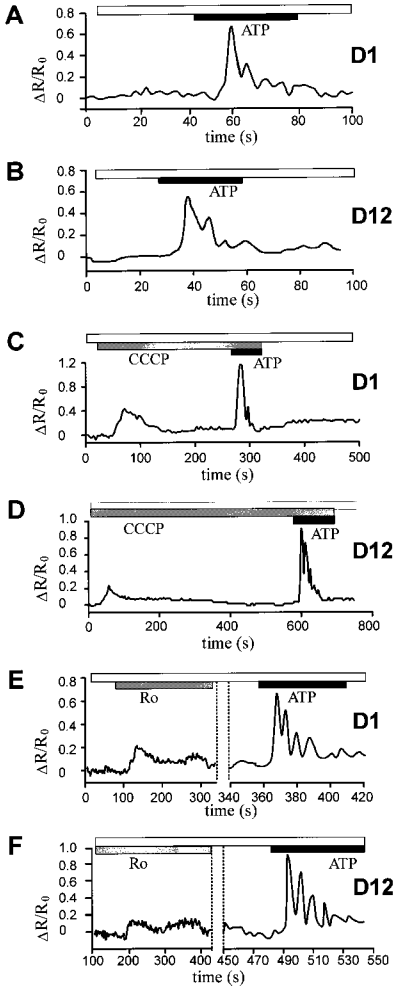

Figure 1 Inhibitors of mitochondrial activity do not prevent ATP-induced ER Ca^{2+} release in individual cells

Representative tracing of an experiment in the absence of external Ca^{2+} (white bar) on RASMC in culture for 1 day (D1; **A**, **C**, **E**) and 12 days (D12; **B**, **D**, **F**). The normalized fluorescence ratio ($\Delta R/R_0$) was plotted against time. The increase in $[Ca^{2+}]_i$ after application of 20 μM ATP was measured under control conditions (**A**, **B**) and after treatment with 20 μM CCCP (**C**, **D**) or 1 μM rotenone (Ro; **E**, **F**). Caf, caffeine.

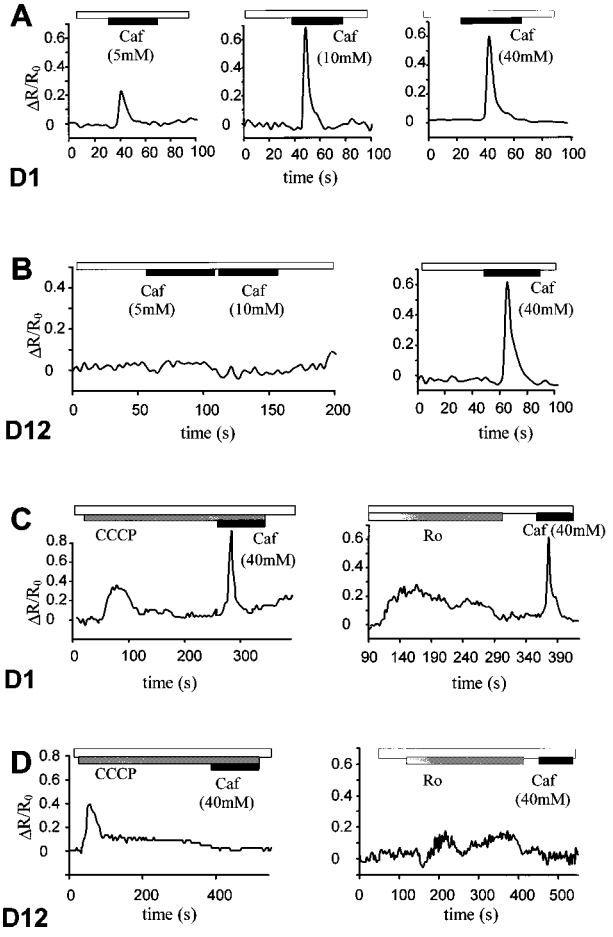

Figure 2 Inhibitors of mitochondrial activity suppress the caffeine-induced cytosolic Ca^{2+} increase in confluent cells

Representative tracing of experiments in the absence of external Ca^{2+} (white bars) on RASMC in culture for 1 day (D1; **A**, **C**) and 12 days (D12; **B**, **D**). The normalized fluorescence ratio ($\Delta R/R_0$) was plotted against time. The increase in $[Ca^{2+}]_i$ after application of caffeine was measured under control conditions (**A**, **B**) and after treatment with 20 μM CCCP (**C** and **D**, left panels) or 1 μM rotenone (Ro; **C** and **D**, right panels).

medium to avoid any possible Ca^{2+} influx. In the absence of CCCP, ATP induced a transient increase in $[Ca^{2+}]_i$ which, after oscillation in most of the cells, returned to the basal level within approx. 1 min on days 1 and 12 cells (Figures 1A and 1B). The resting Ca^{2+} concentration was similar in cells taken on days 1 and 12 (169 ± 6 nM and 159 ± 6 nM respectively, $P = 0.24$). The amplitudes of the first Ca^{2+} peaks were 817 ± 36 nM and 975 ± 60 nM on days 1 and 12 respectively ($P = 0.06$). CCCP (20 μM) induced a small transient increase in $[Ca^{2+}]_i$ and, after CCCP treatment, ATP was still able to induce an increase in $[Ca^{2+}]_i$ in both day 1 and day 12 cells (Figures 1C and 1D).

The effect of rotenone, a mitochondrial respiratory chain inhibitor, was also tested. At 1 μM final concentration, rotenone induced a long-lasting increase in $[Ca^{2+}]_i$ (Figures 1E and 1F). Nevertheless, after rotenone application, ATP could still trigger Ca^{2+} release in both day 1 (Figure 1E) and day 12 cells (Figure 1F). Thus inhibitors of mitochondrial activity do not prevent the ATP-induced ER Ca^{2+} release.

Inhibitors of mitochondrial activity suppress the caffeine-induced cytosolic Ca^{2+} increase in subconfluent cells

We then used caffeine to activate SR/ER Ca^{2+} release through RyRs. As shown in Figure 2(A), addition of caffeine to day 1 cells in control conditions induced a transient increase in $[Ca^{2+}]_i$. The amplitude of the transient was maximal at 10 mM and 40 mM caffeine (Figure 2A) and the average variation in $[Ca^{2+}]_i$ at 10 mM was 736 ± 25 nM. On day 12, the addition of caffeine at concentrations of 5 mM and 10 mM was unable to induce an increase in $[Ca^{2+}]_i$, although $[Ca^{2+}]_i$ transients were induced by 40 mM caffeine (Figure 2B). After CCCP treatment, 40 mM caffeine was active in increasing $[Ca^{2+}]_i$ in day 1 (Figure 2C, left panel), but not in day 12 (Figure 2D, left panel) cells. The same was true after rotenone treatment (Figures 2C and 2D, right panels). Thus cell sensitivity to caffeine was reduced at day 12, and Ca^{2+} pools still responsive to caffeine appeared to be coupled to mitochondria.

Since mitochondrial depolarization by CCCP could result in reduction in cellular ATP and modification of the ATP/ADP

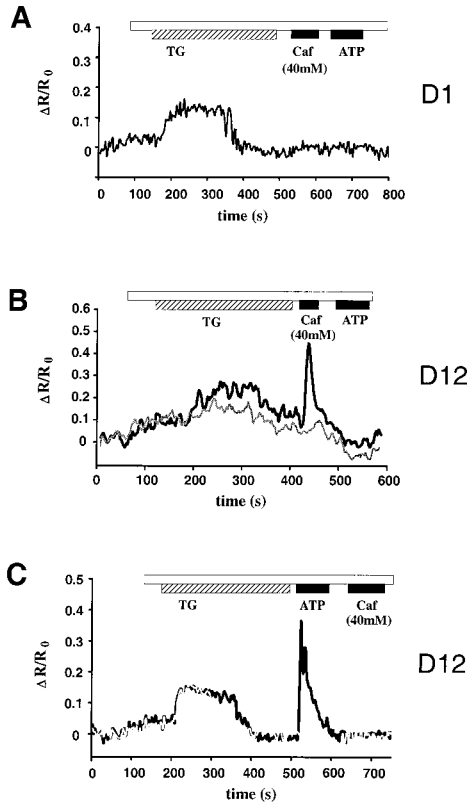

Figure 3 Inhibition of SERCA activity does not completely inhibit the caffeine (Caf)- and ATP-induced Ca^{2+} transients

Day 1 (D1; **A**) and day 12 (D12; **B**, **C**) RASMC were incubated in a Ca^{2+}-free medium (white bar) and treated with 500 nM TG. No Ca^{2+} transient was elicited after sequential application of 10 mM caffeine and 20 μM ATP in day 1 cells (**A**). In day 12 cells, 40 mM caffeine induced an increase in $[Ca^{2+}]_i$ in 52% of the cells (black trace), but not in the others (gray trace) (**B**). Similarly, 45% of the day 12 cells were sensitive to ATP after TG treatment (black trace) (**C**). It is noteworthy that after application of a first agonist (caffeine or ATP), the addition of subsequent agonists was without effect, suggesting that a common ATP- and caffeine-sensitive Ca^{2+} pool was present.

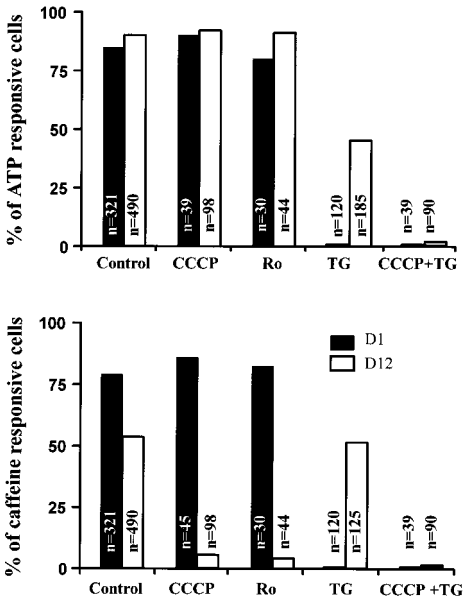

Figure 4 Percentage of ATP or caffeine-responsive cells after inhibition of mitochondria and SERCA activities

The percentage of ATP-responsive (top panel) and caffeine-responsive cells (bottom panel) in day 1 (D1; filled bars) and day 12 (D12; open bars) under control conditions (Ca^{2+}-free medium) or in presence of CCCP, rotenone (Ro), TG and both CCCP and TG. *n*, number of cells.

ratio [26], we performed experiments in the presence of CCCP and oligomycin (1 μM), an inhibitor of ATP synthase. Similar results were obtained in presence of CCCP alone or CCCP + oligomycin: caffeine was unable to release Ca^{2+} at day 12 (results not shown), indicating that the absence of caffeine-induced Ca^{2+} response was not due to alteration in the ATP/ADP ratio. Furthermore, in the presence of the mitochondrial complex I inhibitor, rotenone, which does not alter the ATP/ADP ratio, the caffeine-evoked transient was also abolished.

Inhibition of SERCA activity does not completely inhibit the caffeine- and ATP-induced Ca^{2+} transients

TG, a specific inhibitor of SERCAs, was used to block the rapid Ca^{2+} transport from the cytosol to the lumen of the SR and, consequently, to deplete the SR/ER stores by unopposed leakage across the membrane.

In the absence of external Ca^{2+}, application of 500 nM TG to day 1 or day 12 RASMC produced a slow increase in $[Ca^{2+}]_i$, which soon returned to basal level (Figure 3). After TG application to day 1 cells, the $[Ca^{2+}]_i$ responses to caffeine (10 mM or 40 mM) and ATP (20 μM) were completely abolished (Figure 3A). In contrast, in day 12 cells, although application of 500 nM TG also induced a slow transient increase in $[Ca^{2+}]_i$, subsequent addition of 40 mM caffeine still triggered a $[Ca^{2+}]_i$ transient in 52% of the cells tested (Figure 3B, black trace). Futhermore, ATP was able to induce a Ca^{2+} transient in 45% of the cells tested (Figure 3C, black trace). In all cases, after a first Ca^{2+} transient increase, a secondary application of ATP or caffeine had no effect, suggesting a unique Ca^{2+} pool.

Sensitivity to tBHQ, another SERCA-blocking drug, was also tested by the same protocols (results not shown). Treatment of day 1 RASMC with tBHQ for 5 min completely prevented the ATP-induced Ca^{2+} increase. In contrast, on day 12, after tBHQ application ATP-induced Ca^{2+} transient was observed in 52% of the RASMC tested and caffeine-triggered Ca^{2+} release in 48% of the cells tested.

Finally, inhibition of both SR/ER and mitochondrial stores by co-application of TG and CCCP completely inhibited ATP- and caffeine-triggered Ca^{2+} increase in $[Ca^{2+}]_i$. Indeed, only 1% of day 12 cells, and none of the day 1 cells, responded to ATP or caffeine under these conditions (Figure 4).

Percentage of ATP or caffeine-responsive cells after inhibition of mitochondria and SERCA activities.

The percentages of day 1 and day 12 RASMCs responsive to ATP in control conditions, and in the presence of CCCP, rotenone and TG, are summarized in Figure 4 (top panel). On day 1, 85% of the cells were responsive to ATP in control conditions, 90% and 80% after CCCP and rotenone treatment respectively. On day 1, all of the RASMCs tested expressed Ca^{2+} pumps sensitive to TG and the Ca^{2+} store was depleted by TG or TG + CCCP.

On day 12, 90% of the cells were sensitive to ATP in control conditions, 92% and 91% in the presence of CCCP and rotenone respectively. In the presence of TG, ATP could still release Ca^{2+}

in 45% of the cells, but TG + CCCP completely inhibited ATP-induced Ca^{2+} transient.

The proportion of cells sensitive to caffeine under various conditions is summarized in Figure 4 (lower panel). On day 1, 80% of cells were sensitive to caffeine under control conditions, 87% and 83% after CCCP and rotenone treatment respectively. TG and TG + CCCP completely prevented caffeine-induced Ca^{2+} response at day 1.

On day 12, 54% of the cells were responsive to caffeine under control conditions. Thus most of the day 1 cells, but only half of the day 12 cells, released Ca^{2+} in the cytosol after caffeine stimulation. The proportion of caffeine-sensitive cells was reduced to 6% after CCCP treatment and to 4% after rotenone treatment. The percentage of day 12 cells sensitive to caffeine under control conditions and in the presence of TG was approx. 50% in both cases, indicating that TG did not prevent caffeine-induced Ca^{2+} release. Caffeine response was totally prevented by co-treatment with CCCP and TG.

In conclusion, in the vast majority of the day 1 cells, the ATP- and caffeine-induced Ca^{2+} transient was prevented by the SERCA inhibitors. In contrast, by day 12, after treatment with TG or tBHQ, Ca^{2+} transients could still be induced by ATP or caffeine in half of the cells. The two mitochondrial inhibitors, CCCP and rotenone, did not alter the number of ATP or caffeine-responsive cells on day 1. On day 12, these inhibitors did not prevent the ATP-evoked Ca^{2+} transient but prevented caffeine-induced Ca^{2+} release in the majority of RASMC. These data suggest that caffeine mobilizes Ca^{2+} from a mitochondria-linked Ca^{2+} store in cells from day 12 but not from day 1, and that Ca^{2+} release is entirely dependent on TG-sensitive SERCA pumps on day 1 but not on day 12.

The PTP is involved in the caffeine-induced Ca^{2+} release in confluent cells

Since PTP is one of the putative mechanisms for Ca^{2+} efflux from the mitochondrial matrix, we investigated the effect of PTP inhibition on the caffeine-induced Ca^{2+} release using CsA. As expected from the experiments using mitochondrial inhibitors (Figures 1C–1F), CsA (500 nM) did not prevent the ATP-induced Ca^{2+} response in day 1 and 12 cells (Figure 5, left panels). However, although the caffeine-induced Ca^{2+} response was not affected by CsA in day 1 cells, this effect was completely suppressed in day 12 cells (Figure 5B, right panel). The percentage of day 1 and 12 cells responsive to ATP and caffeine is summarized in Figure 5(C).

Analysis of the distribution of mitochondria and RyRs

We have previously shown that in RASMC the IP_3R was mainly perinuclear [22]. Here we demonstrate that the RyRs and the mitochondrial network spread from the nucleus throughout the cytosol at both day 1 and day 12 (Figure 6). No striking variation was observed in the distribution of mitochondria (Figure 6A) and RyRs (Figure 6B). At both stages, the MitoTracker Red fluorescence and the fluorescein labelling of RyRs were co-localized (Figure 6C). However, some cells at day 12 did not express the RyR (Figure 6, D12-B), which is in agreement with the fact that not all of the cells were sensitive to caffeine. The absence of fluorescein labelling in one of the cells in Figure 6 (D12-B) indicated that the secondary antibody gives only background signal. In the presence of CCCP no labelling was observed with the MitoTracker (results not shown).

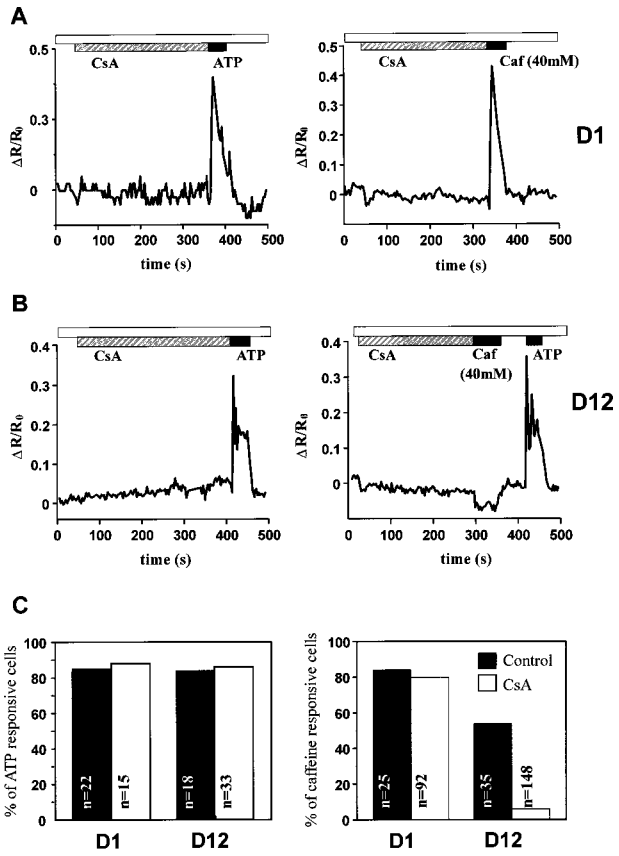

Figure 5 PTP is involved in caffeine-induced Ca^{2+} release in confluent cells

Representative trace of measured $\Delta R/R_0$ fluorescence ratio from day 1 (D1; **A**) and day 12 (D12; **B**) RASMC after 5 min incubation with 500 nM CsA in Ca^{2+}-free medium (white bar). Ca^{2+} transient was induced either by 20 μM ATP (left panel) or 40 mM caffeine (Caf; right panel). CsA did not affect ATP-induced Ca^{2+} transient; it suppressed caffeine-evoked transient increase in $[Ca^{2+}]_i$ in day 12, but not in day 1 cells. (**C**) A summary of the fraction of cells responding to ATP (left panel) or caffeine (right panel) at day 1 (filled bars) and day 12 (open bars) in control conditions or after treatment with 500 nM CsA. *n*, number of cells.

DISCUSSION

Our data demonstrate that functional coupling between the SR/ER and mitochondria is regulated during the de-differentiation/redifferentiation process which occurs in rat aortic smooth muscle cells in culture. Differentiated RASMC at day 1 have a common ATP-sensitive, caffeine-sensitive Ca^{2+} pool which is not linked to a mitochondrial Ca^{2+} pool. This pool is filled by TG-sensitive, ATP-dependent Ca^{2+} pumps of the SERCA type (Figure 7, D1). At day 12, after cells pass through the cell cycle and are subconfluent, all cells are still sensitive to ATP, but two sub-populations can be distinguished: one in which TG prevents ATP-evoked Ca^{2+} signal, and one in which it does not. However, TG evokes a slow increase in $[Ca^{2+}]_i$ in the two sub-populations, indicating that they both expressed SR/ER Ca^{2+} pumps. Furthermore, in half of the cell population, caffeine induces an increase in $[Ca^{2+}]_i$ by a mechanism linked to mitochondrial activity. The caffeine-induced Ca^{2+} transient is completely blocked by CsA, indicating that the PTP is involved in Ca^{2+} release but it is not prevented by TG. Based upon these results, we propose that by day 12 of culture two types of cells are present (Figure 7, D12). In some cells (Figure 7, C1) only the IP_3Rs are functional, whereas other cells (C 2) are sensitive to

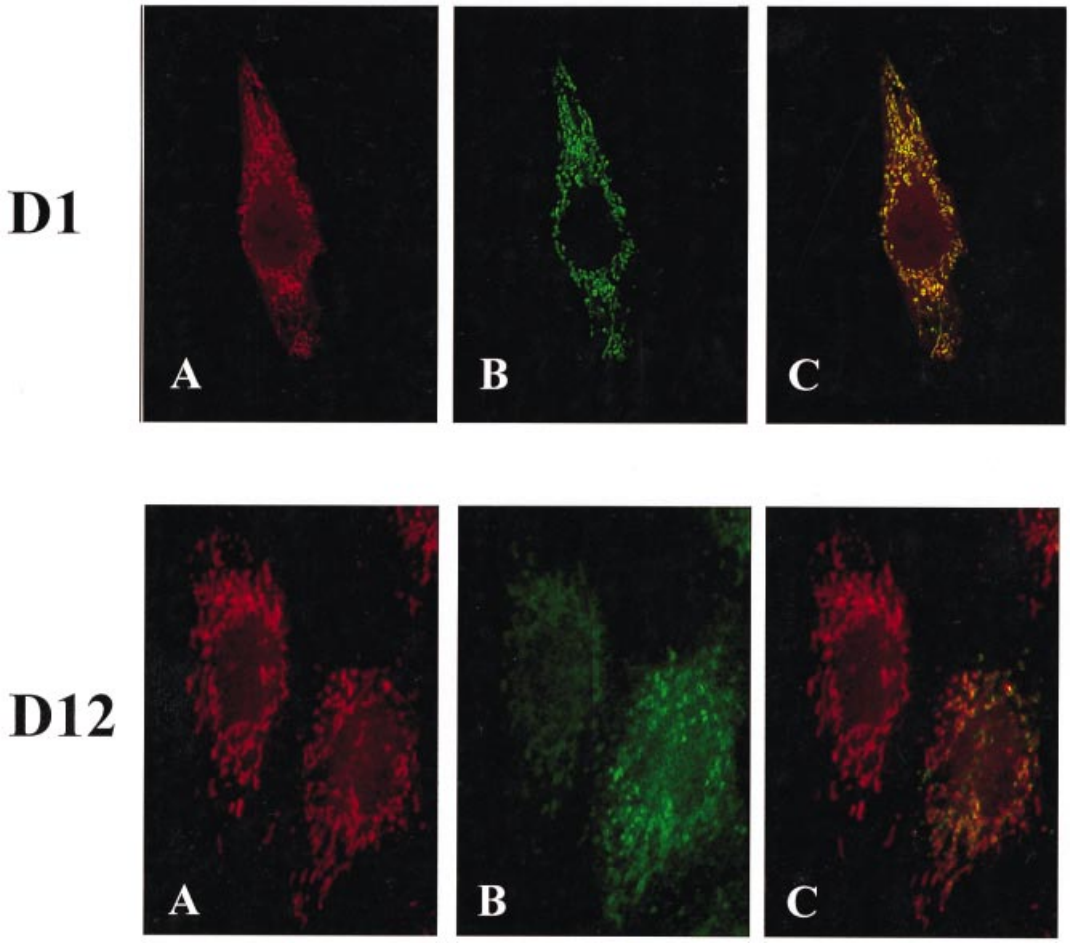

Figure 6 Analysis of the distribution of mitochondria and RyRs

Day 1 (D1) and day 12 (D12) RASMC were labelled with the MitoTracker Red (**A**) and with fluorescein-labelled anti-RyR antibody (**B**). A merge image (**C**) shows co-localization of RyRs and mitochondria. Control experiments were performed either with the secondary antibody alone or in presence of MitoTracker + CCCP. In both cases no fluorescence was observed, indicating the specificity of the labelling (results not shown).

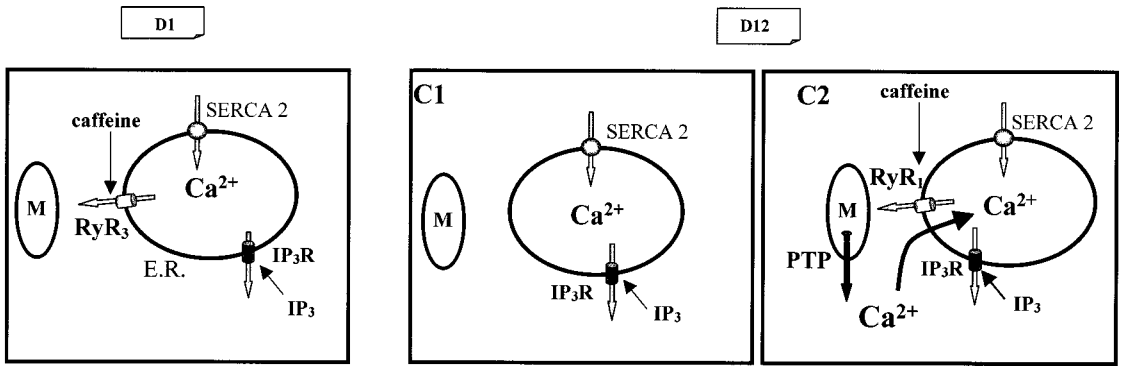

Figure 7 Ca^{2+} pools in day 1 and day 12 RASMC in culture

The definition of these pools is based on data from the present study and a previous paper [22]. On day 1 (D1), the SR/ER is independent from mitochondia (M). Ca^{2+} accumulates into the SR/ER by TG-sensitive pumps, SERCA 2a and SERCA 2b. Each cell has two types of SR/ER Ca^{2+} channels, RyR3 and IP_3R1. On day 12 (D12), TG induced a transient increase in $[Ca^{2+}]_i$ in all the cells tested, indicating that they express SERCA-type pumps. In half of the cells (**C1**) Ca^{2+} is released by IP_3R, and RyR is not expressed in these cells. This pool is independent from mitochondria. In the remaining cells (**C2**), Ca^{2+} is released by both IP_3-sensitive and caffeine-sensitive mechanisms, and the two pools are interconnected. The IP_3-sensitive pool is independent from mitochondria, whereas Ca^{2+} released by RyR1 activates Ca^{2+} release by mitochondria through the PTP. After depletion by TG, Ca^{2+} accumulates in this pool by a mechanism resistant to TG.

both caffeine and IP_3. In Figure 7 (C2) only the caffeine-sensitive pool is coupled to mitochondria, and the PTP amplifies the caffeine-induced Ca^{2+} release. Both cell types express SERCAs, but in C2 when the Ca^{2+} store has been depleted by TG it can be refilled by another mechanism and Ca^{2+} transients could be generated by caffeine or ATP.

In freshly isolated RASMC, the Ca^{2+} stores of more than 80% of the cells are sensitive to both ATP and caffeine. The ATP- and caffeine-sensitive Ca^{2+} pool is independent of the mitochondrial store since it is not inhibited by CCCP or rotenone. We have previously shown [22] that these cells have a differentiated phenotype and that they express two SR/ER Ca^{2+} channels, RyR3 and IP_3R1, and Ca^{2+} pumps, SERCA 2a and SERCA 2b. Monteith and Blaustein [18] have shown that, in RASMC cultured in serum-free medium for less than 3 days (they also display a differentiated contractile phenotype), a high dose of ATP (100 μM) increased both cytosolic and mitochondrial [Ca^{2+}], but with low doses of ATP (1 μM) they observed an increase in cytosolic [Ca^{2+}] without any change in mitochondrial [Ca^{2+}]. This latter result is in agreement with the effects we observed by the addition of 20 μM ATP, and it suggests that, in differentiated RASMC at day 1, the SR/ER and the mitochondrial Ca^{2+} pools are independent. However, in other smooth muscle cells, such as rat pulmonary artery [16], guinea-pig colonic cells [17] or the smooth muscle cell line A10 [10], release of Ca^{2+} from the SR increases mitochondrial [Ca^{2+}]. Elevation in mitochondrial Ca^{2+} occurs either through the IP_3 pathway [16,17] or through the ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release mechanism [10,16]. In the present study, we find that in subconfluent cells caffeine evoked a Ca^{2+} transient in 52% of the cells, and that the Ca^{2+} response was inhibited by CCCP or rotenone. Because these mitochondrial inhibitors did not affect IP_3-induced Ca^{2+} transient generated by ATP, we propose that the IP_3-sensitive pool is not connected to mitochondria. When the same type of cells were treated with serotonin or low doses of ATP, agonists which act through the IP_3 pathway, the mitochondrial [Ca^{2+}] did not change [18]. Thus, in RASMC, activation of the IP_3 pathway alone does not seem to generate an increase in mitochondrial [Ca^{2+}].

To clarify the role of Ca^{2+} pumps in the process, we have blocked the SR/ER activity using the specific inhibitors, TG and tBHQ. TG (or tBHQ) induced a slow, long-lasting Ca^{2+} transient in all of the cells at both day 1 and day 12, indicating that the cells expressed SERCA-type Ca^{2+} pumps. TG and tBHQ prevented ATP and caffeine-dependent Ca^{2+} transients in all day 1 cells, and the ATP-evoked transient in half of the cells at day 12 (Figure 7, D12-C1). By day 12, in the remaining ATP-sensitive cells and in all of the caffeine-sensitive cells, TG did not prevent cytosolic Ca^{2+} increase (Figure 7, D12-C2). Either this pool was not completely depleted of Ca^{2+} by TG or it was filled by another mechanism. The first hypothesis is very unlikely because we used high doses of TG (up to 2 μM). Several studies have described the release of Ca^{2+} by caffeine and/or IP_3-generating systems that is not inhibited by TG in different cell types, including the vascular cell line A7R5 and rat aortic smooth muscle cells in primary culture [27,28]. Recently [29], a bradykinin-sensitive, TG-insensitive Ca^{2+} pool distinct from a TG-sensitive pool was also described in vascular smooth muscle cells from spontaneously hypertensive rats, but not in cells from normotensive Wistar–Kyoto rats, where bradykinin- and TG-sensitive pools were common. This latter result indicates that a functionally important reorganization of the intracellular Ca^{2+} compartments occurs in pathological situations.

How is the SR/ER filled in the TG-resistant cells? Many studies have discussed the possibility of the existence of TG-insensitive Ca^{2+} pumps [30], but the molecular identity of this pump remains unknown. At least six different SERCA isoforms have been identified (reviewed in [2]). However, SERCA 1, SERCA 2a and 2b, as well as SERCA 3a, isoforms have been shown to be inhibited irreversibly by TG [4], and therefore are unlike to be candidate pumps. More recently other SERCA3 isoforms have been described. These isoforms differ only in their C-terminal region, but have a conserved N-terminal region to which TG is believed to bind, suggesting that they are also likely inhibited by TG. In cells adapted to become resistant to TG, mutation of the SERCA pump or overexpression of the multidrug transporter, p-glycoprotein, have been described [31,32]. We cannot exclude the possibility that such a phenomenon also occurs in culture. Furthermore, other non-identified TG-resistant pumps, such as the Pmr1 identified in yeast and *Caenorhabditis elegans* [33], may still exist.

We have shown that on day 12, the caffeine-induced Ca^{2+} transient is TG-insensitive and dependent on mitochondrial activity. When treated with both CCCP and TG, the percentage of caffeine-responsive cells is as low as that after CCCP treatment alone. Surprisingly, the percentage of ATP-responsive cells was also reduced to nearly 0 by co-treatment with CCCP and TG, although it was not affected by CCCP alone and was reduced only by half after TG treatment. Thus the ATP-induced Ca^{2+} response is not dependent on mitochondria in the absence of TG. TG alone does not affect ATP-response in half of the cells (Figure 7, D12-C2), but inhibition of SERCA by TG, associated with inhibition of mitochondrial function by CCCP, prevents the ATP-evoked Ca^{2+} release, suggesting that in some cells (Figure 7, D12-C2), refilling of the Ca^{2+} store may be dependent on mitochondria.

To understand how the caffeine-induced Ca^{2+}-release mechanism is dependent of mitochondria, we investigated the effects of CsA, a specific inhibitor of mitochondrial PTP (reviewed in [21]. CsA treatment in day 12 cells almost suppressed the caffeine-induced Ca^{2+}-release mechanism, indicating that the mechanism of Ca^{2+} release by caffeine requires PTP activation. The percentage of inhibition of the caffeine response by CsA was the same as that inhibited by CCCP, indicating that CsA targeted mitochondria. Although CsA also inhibits calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase [34], it is unlikely that the results we describe in the present study are due to regulation of RyRs by CsA, since CsA does not affect FKBP12 or RyR channel gating [35]. Rather, we favour the possibility that CsA inhibits cyclosporine D, which regulates the activity of the PTP [36].

Close associations between ER and mitochondria have been reported in a variety of non-muscle cells (reviewed in [37]). The present data also show that RyRs and mitochondria are physically in close apposition. However, we could not detect any difference in the distribution of RyRs and mitochondria, which could explain why the ryanodine-sensitive store is coupled to mitochondria at day 12 and not at day 1. We have previously shown that when RASMC enter the cell cycle they lose the caffeine/ryanodine-sensitive Ca^{2+} pool [22]. This change in Ca^{2+} signalling is associated with the down-regulation of RyR3 and SERCA 2a. When the cells stop proliferating as they reach confluency they become sensitive to high doses of caffeine and express RyR1 rather than RyR3 [22]. The presence of RyR1 rather than RyR3 may contribute to the functional difference. In rat portal vein, in contrast with the aorta, the three RyR subtypes are expressed, but only RyR1 and RyR2 are involved in the Ca^{2+}-induced Ca^{2+}-release mechanism in normal conditions [38]. RyR3 is activated only in conditions of increased SR loading [39]. Furthermore, a reorganization of the channels may

also occur, as demonstrated during development of cerebral arteries [40]. Based on our previous results and on those presented here, we propose that on day 1 caffeine activates RyR3 and generates a Ca^{2+} transient. On day 12, caffeine might still trigger isolated Ca^{2+} sparks that would not be sufficient to induce a large Ca^{2+} transient increase, but that would induce a small and highly localized $[Ca^{2+}]_i$ increase near SR microdomains in close contact with mitochondria. This small $[Ca^{2+}]_i$ increase might be sufficient to trigger the PTP opening and activate the mitochondrial Ca^{2+}-induced Ca^{2+} release. Previous work has shown that Ca^{2+} wave propagation was supported by locally specialized ER-release sites which, by a mechanism of regenerative Ca^{2+} release, support wave propagation over long distances [41]. Mitochondria were found in intimate association with sites possessing high density ER markers, such as IP_3R, SERCA and calreticulin [13,38,42].

In conclusion, we have shown that differentiated RASMC have a unique SR/ER caffeine-sensitive and agonist-sensitive Ca^{2+} pool containing TG-sensitive pumps that are independent of mitochondrial calcium stores. After cells have gone through the cell cycle and start to redifferentiate, a reorganization of the Ca^{2+} pools occurs and the caffeine-sensitive pool is connected to mitochondria. The presence of functional coupling between ER and mitochondria in redifferentiating cells that have RyRs which cannot elicit Ca^{2+} transients may help to propagate the Ca^{2+} signal throughout the cell.

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