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Calcium and the replication of human vascular smooth muscle cells: studies on the activation and translocation of extracellular signal regulated kinase (ERK) and cyclin D1 expression

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Abstract

Since the precise role of sarcoplasmic reticular Ca^{2+} in mediating vascular smooth muscle cells (VSMC) proliferation is unknown, the effect of pre-incubation with thapsigargin on extracellular signal regulated kinase (ERK) activation, the translocation of activated of ERK 1/2 to the nucleus, cyclin D1 expression, the onset of S phase and cytosolic Ca^{2+} levels were studied. Human saphenous vein VSMCs (hVSMC) were incubated with 10 nM thapsigargin for 24 h followed by stimulation with fetal calf serum and the activation of ERK1/2 and cyclin D1 assessed by western blotting, the intracellular distribution of ERK1/2 using indirect immunofluorescence, the onset of S-phase with the incorporation of bromodeoxyuridine and sarcoplasmic reticular Ca^{2+} status using FURA-2. Thapsigargin had a marginal effect on ERK1/2 activation only at 5 min and 10 min after stimulation with fetal calf serum. In contrast, the rapid translocation of ERK1/2 to the nucleus was completely blocked by thapsigargin. S phase was delayed by 8 h by thapsigargin which co-incided with the recovery of cytosolic Ca^{2+} levels and cyclin D1 expression. It is concluded that the inhibitory effect of thapsigargin (depletion of Ca^{2+} pools) on hVSMC replication is mediated through the inhibition of translocation of activated ERK1/2 to the nucleus and not to the phosphorylation of ERK, per se, which in turn prevents cyclin D1 expression and thus progression of the cell cycle.

Keywords: Vascular smooth muscle cell, human; ERK (extracellular signal regulated kinase); Cyclin D1 expression

1. Introduction

Neointima formation, which involves the migration and proliferation of vascular smooth muscle cells (VSMCs), is central to the pathophysiology of late saphenous vein graft failure following bypass graft surgery (Motwani and Topol, 1998; Vijayan et al., 2002). However as many as 50% of vein grafts fail within 10 years after graft implantation (Vijayan et al., 2002), for which there is no effective treatment. Obtaining an understanding of the intracellular mechanisms underlying VSMC proliferation is therefore essential for developing novel therapeutic strategies to prevent vein graft failure.

Of the intracellular mechanisms that control VSMC replication, Ca^{2+} release from the sarcoplasmic reticulum may be central (Short et al., 1993; Lapidot and Phair, 1995; George et al., 1997; Shukla et al., 1997; Birkett et al., 1999). Although it is unclear how sarcoplasmic reticulum-derived Ca^{2+} mediates replication, an impact on the early events in the cell cycle are likely since mitogens elicit a rapid increase in cytosolic Ca^{2+} and the inhibition of Ca^{2+} mobilisation inhibits VSMC proliferation (Baran, 1996). Quiescent VSMCs (described as being in Go) enter the G1 phase of the cell cycle when stimulated by mitogen(s) (Sriram and Patterson, 2001). G1 phase, whose duration ranges from 12 to 24 h, is dependent on the continued presence of a mitogen(s) (Sriram and Patterson, 2001). VSMCs then enter the S phase at which point they are committed to replication (Sriram and Patterson, 2001). Sequentially, in the Go/G1

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phase, occupation of mitogenic receptors results in the activation of the ras-raf system, active ras phosphorylating and activating mitogen activated/extracellular signal regulated protein kinase kinase (MEK) (Braun-Dullaeus et al., 1998). MEK then phosphorylates and activates the extracellular regulated protein kinases (ERK1 and ERK2) (Mii et al., 1996). Activated ERK1/2 rapidly translocate to the nucleus (Jaaro et al., 1997; Adachi et al., 2000) where they target transcription factors that regulate progression of the cell cycle, most notably, cyclin D1 (Weber et al., 1997). Cyclin D1 binds to and activates its catalytic partner, cyclin dependent kinase 4 (CDK4) which together drive the cell through the G1 check point into the S phase through phosphorylation of the retinoblastoma gene product (Peeper and Bernards, 1997). How sarcoplasmic reticular-Ca^{2+} is involved in controlling or modulating these mechanisms has not been fully elucidated. However, thapsigargin, an inhibitor of sarcoplasmic reticular Ca^{2+} ATPase pumps, inhibits VSMC replication at concentrations (<10 nM) that also completely deplete sarcoplasmic reticular-Ca^{2+} stores, without eliciting an increase in cytosolic Ca^{2+} levels (Shukla et al., 1997; Birkett et al., 1999). The effect of thapsigargin was also long lasting, in that pre-exposure for 1 h was sufficient to significantly inhibit in vitro VSMC proliferation over 48 h in cultured VSMCs and in cultured saphenous veins for up to 14 days after treatment (George et al., 1997). Crucially, this concentration of thapsigargin elicited no effect on cell viability or apoptosis, whereas other modulators of Ca^{2+}, including cyclopiozonic acid were cytotoxic (Shukla et al., 1997; George et al., 1997). It was therefore suggested that pre-treatment of saphenous veins prior to implantation with thapsigargin may constitute a clinical approach in preventing neointima formation and therefore of reducing late vein graft failure (George et al., 1997). However, before this can be tested clinically, further studies are required to characterise the mechanisms underlying the impact of pre-treatment of vascular tissues with low concentrations of thapsigargin.

Since thapsigargin inhibits VSMC proliferation by depleting sarcoplasmic reticular Ca^{2+} stores, it follows that thapsigargin may exert an impact on the ERK-cyclin D1 system. Studies were therefore undertaken to determine the effect of pre-incubation of human VSMCs to 10 nM thapsigargin for 24 h followed by its removal and serum stimulation on ERK1/2 activation and cyclin D1 expression. Serum was chosen as a promoter of replication since neointima formation in saphenous veins is promoted by a range of disparate mitogens released by leukocytes and platelets, all of which are contained in serum (Jeremy et al., 1997). The use of 10 nM thapsigargin as a test concentration is crucial since it depletes sarcoplasmic reticular Ca^{2+} stores, without eliciting an increase in cytosolic Ca^{2+} levels (Birkett et al., 1999). An integral facet of the ERK1/2 axis is the translocation of the active (phosphorylated) ERK isoforms to the nucleus (Jaaro et al., 1997; Adachi et al., 2000). Fluorescence-linked immunocytochemistry was therefore used to monitor the movement of ERK1/2 in cells after fetal calf serum stimulation. Studies were also carried out using other Ca^{2+} modulators calcium ionophore A23187 and BAPTA-AM.

2. Methods

2.1. Drugs

The following drugs were purchased from the Sigma Chemical (Poole, Dorset, UK): ionomycin, thapsigargin, calcium ionophore A23187, 1,2-bis(2-Aminophenoxy)-ethane *N*,*N*,*N*′ ,*N*′ ,-tetracetic acid (BAPTA-AM).

2.2. Cell replication assay

Saphenous veins were obtained from patients undergoing coronary artery bypass graft surgery CABG, for which ethical approval and patient consent had been obtained. Veins were placed in medium RPMI 1640 (Gibco BRL; Paisley, Scotland) containing 2% amphotericin (Gibco BRL and 0.4% heparin (Sigma Chemical, Poole Dorset, UK). Human VSMCs (hVSMC) were then grown in Dulbecco's Minimum Essential Medium-Glutamax without sodium pyruvate (DMEM; Gibco BRL), containing 100 units penicillamine (Sigma), 100 mg/ml streptomycin (Sigma) and 10% foetal calf serum (Gibco BRL; Shukla et al., 1997). After passage 4, hVSMCs were seeded onto 22 mm diameter coverslips in 12 well plates at a density of 6×10^4 cells per well and cultured for 2 days in DMEM/fetal calf serum. Cells were then rendered quiescent for 4 days in serum-free medium containing with 0.25% lactalbumin hydrolysate (Gibco BRL). Under these conditions, there was no loss of cell numbers over this time course. To elicit proliferation, hVSMCs were incubated in DMEM-10% fetal calf serum containing 10 μM bromodeoxyuridine (BrdU; Sigma) at 37 °C in an O_2/CO_2 (95%/5%) humidified incubator. At different time points, hVSMC were fixed with 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS; Sigma), washed and treated sequentially as follows with: (a) 3% H_2O_2 in methanol for 15 min at 4 °C, (b) 2N HCl for 30 min at 37 °C and (c) 0.1% Triton-X for 10 min at 25 °C, cells being washed with PBS between each step (all chemicals; Sigma). Cells were then incubated with a monoclonal primary antibody against BrdU (ICN, Basingstoke, Hampshire, UK) at 1:10 in 3% (w/v) bovine serum albumin/normal horse serum/PBS for 2 h at 37 °C. Incubation with biotinylated secondary antibody (ICN) diluted 1:200 in 3% bovine serum albumin in PBS; Sigma) was carried out for 45 min at 25 °C. Cells were then incubated with horse radish peroxidase-conjugated ExtraAvidin (Sigma), diluted 1:200 in 3% bovine serum albumin/PBS also for 45 min at 25 °C. Positive nuclei were developed by incubation of cells with 0.05% 3,3-diaminobenzidine (DAB; Sigma)/

0.03% H_2O_2 in PBS at 25 °C for 10 min. Negative nuclei were counterstained with haematoxylin (Sigma). Dehydration of cells through ethanol and histoclear were performed and cells mounted in DPX. Five fields from each sample were counted at ×20 magnification and the % cells positive for BrdU calculated.

In order to assess the effect of 10 nM thapsigargin (Sigma) on the proliferation of human VSMC and S-phase entry, human VSMC were treated with 10 nM thapsigargin for 24 h prior to stimulation with 10% FCS, thapsigargin being removed from the media by washing with DMEM at time of stimulation and proliferation assessed using BrDU incorporation, as above. In other experiments, the effect of addition of calcium ionophore A23187 after the preincubation with thapsigargin on BrdU incorporation over 24 h was studied as well as the effect of the intracellular Ca^{2+} chelator, BAPTA-AM, (Sigma).

2.3. Effect of thapsigargin on ERK1/2 activation and cyclin D1 expression

For Western analysis, hVSMCs were seeded at a density of 8×10^4 cells per well and treated with thapsigaring for 24 h as above. Media was removed and cells washed in ice with PBS. Cell lysis and protein extraction were performed on ice in a final volume of 150 μl of lysis buffer containing 40 mM TrisHCl, 110mM NaCl, 16mM EDTA, 0.1M NaF (all Sigma Chemical), complete inhibitor cocktail (Boehringer Mannheim, Germany) and 10 μg/ml leupeptin (Sigma). Lysates, adjusted to give the same protein concentration for each sample, were diluted 2:1 with reducing loading buffer and boiled for 10 min prior to subjecting sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Concomitantly, pre-stained full range recombinant molecular weight markers (Amersham International, Bucks, UK) were loaded on each gel. An 8% resolving: 5% stacking gel at ratios of 29:1 or 37.5:1 (acrylamide:bisacyrlamide) SDS-containing polyacrylamide (Biorad Laboratories, Hemel Hempstead, Herts, UK) was used. Proteins were separated by passing 180 V constant current through the gel at 4 °C for at least 4 h. Proteins were then electrophoretically transferred to Hybond C nitrocellulose membranes (Amersham) overnight at 4 °C at a constant voltage of 80 mA using a Transblot apparatus (Biorad Laboratories). After protein transfer, membranes were blocked for 4 h with Tris buffered saline (TBS)-Tween buffer (20 mM Tris, 137mM NaCl, 0.1% Tween (TBSTB; Sigma), containing 2.5% skimmed milk powder (w/v). Membranes were then incubated overnight at 4 °C with primary antibodies: anti active (pohosphorylated) ERK1/2 rabbit polyclonal antibody at 1:1000 (New England Biolabs, Hitchin, Herts, UK), anti-inactive total ERK1/2 rabbit polyclonal antibody at 1:1000 (Calbiochem-Novabiochem, Beeston, Nottingham, UK), anti-cyclin D1 mouse monoclonal antibody at 1:500 (Santa Cruz Biotechnology, USA) and as a control for protein loading anti-actin mouse monoclonal antibody at 1:250 (Cedarlanem Ontario, Canada) in TBS-TB containing 3% BSA and 0.01% sodium azide (Sigma). Membranes were then repeatedly washed with 2.5% milk powder in TBSTB (w/v) and then incubated at 25 °C for 45 min with horseradish peroxidase-conjugated secondary antibody of suitable species (DAKO, Ely, Cambridgeshire, UK) diluted to 1:2000 in TBSTB containing 2.5% skimmed milk powder. Unbound secondary antibody was removed by washing the membranes with TBSTB. Bands were visualised using Amersham enhanced chemiluminescence detection system and autoradiography Hyperfilm (Amersham). Bands were quantified with a BioRad GS-690 densitometer and Molecular Analyst Software. Protein concentrations were determined using the Pierce Micro BCA protein assay system (Pierce, Rockford, Illinois, USA). Data are expressed as a % of maximal responses.

2.4. Intracellular ERK1/2 localisation using indirect imunofluorescence

hVSMCs were seeded onto 22 mm diameter coverslips in 12-well plates at a density of 6×10^4 cells per well and cultured for 2 days in DMEM-fetal calf serum. Cells were rendered quiescent for 4 days in serum-free medium containing with 0.25% lactalbumin hydrolysate (Gibco BRL). VSMCs were incubated in DMEM-fetal calf serum (±10 nM thapsgargin) at 37 °C in an O_2/CO_2 (95%/5%) humidified incubator. hVSMCs were then fixed with 4% paraformaldehyde (w/v) in PBS, washed with 0.1% Triton-X in TBS. hVSMCs were then incaubated in 20% swine serum/1% Pontamine Sky Blue stain (Sigma) in TBS for 2 h at 25 °C followed by primary antibody directed against dually phosphorylated ERK1/2 or total (inactive non-phosphorylated) ERK1/2 (both rabbit polyclonal; Calbiochem) diluted in 3% BSA/20% swine serum/TBS overnight at 4 °C. Secondary antibodies diluted 1: 200 in 3% BSA/20% swine serum TBS was added to cells for 40 min at 25 °C followed by incubation with FITC conjugated Extra-Avidin (Calbiochem) diluted 1:200 in PBS for 10 min at 25 °C. Coverslips were then removed in vecta shield with nail varnish. Cells were imaged under blue light from a mercury lamp at ×40 magnification with an oil iris lens and photographed immediately from which the percentage of cells demonstrating positive nuclear immunofluorescence was determined by an investigator blinded to the experimental conditions.

2.5. Intracellular calcium imaging with FURA-2

Intracellular Ca^{2+} imaging was carried out using Fura-2 acetoxymethyl ester (FURA-2; Sigma) as previously described (Birkett et al., 1999). hVSMC at passage 4 were grown on 22 mm diameter coverslips in 12-well

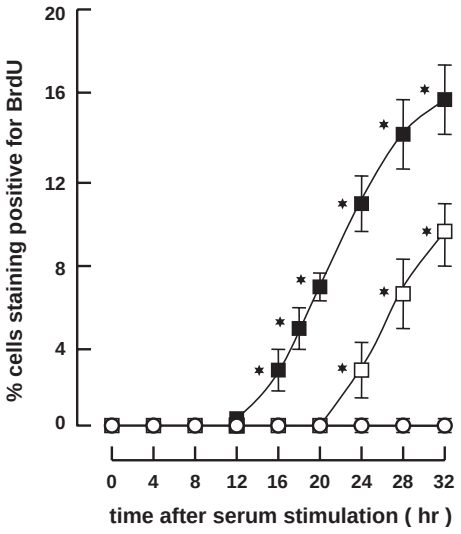


Fig. 1. Effect of 24 h pre-incubation of quiescent human vascular smooth muscle cells with 10 nM thapsigargin (TG), which is removed from the cultures by washing, on bromodeoxyuridine (BrdU) incorporation (% cells positive for BrdU staining) into human VSMC following stimulation with 10% fetal calf serum. Control (untreated) [■]; TG treated [□]. [○] represents BrdU incorporation in cells continually treated with 10 nM thapsigargin after stimulation with serum. Each point=mean±SEM, n=6. ✱ p<0.05 compared to time zero (one-way ANOVA).

plates in DMEM at a cell density of 6–7×10^4 cells/well. Following 24 h incubation with 10 nM thapsigargin, cells were washed 3 times with PBS and incubated for different times over 24 h in DMEM. In two other groups, hVSMC were not pre-incubated with thapsigargin (controls) or cells were continued to be incubated with thapsigargin. Thus, at different times after thapsigargin removal, cells were incubated for 30 min with 2 mM FURA-2 in PBS at 37 °C in a 5%/95% CO_2/O_2 incubator. Extracellular FURA-2 was removed by washing 3 times with PBS. The coverslips were transferred to a stainless steel holder fitted into a thermostatically controlled chamber in which the incubation buffer volume was maintained with a suction device. Mobilisation of sarcoplasmic reticular Ca^{2+} was stimulated with 1 μM ionomycin in Ca^{2+} free medium and the increase in intracellular Ca^{2+} detected using fluorescence with Magical Hardware and Tardis Software (Newcastle upon Tyne, UK). In each experiment fields of view containing 6–8 cells which were evenly loaded with FURA-2 were selected and the mean intracellular Ca^{2+} was calculated for all of the cells in the field of view. Data are expressed as % differences between thapsigargin-treated and maximal responses in untreated cells at equivalent time points.

2.6. Apoptosis and cell viability

Terminal deoxynucleotidyltransferase dUTP nick end-labeling analysis (TUNEL) was performed on thapsigargin (10 nM) treated cells using the Dead End Colorimetric Apoptosis Detection System (Promega). VSMCS were seeded onto 96-well plates and incubated with 0 or 10 nM thapsigargin as described above. After a further 24 h incubation, cells were washed in sterile PBS and fixed in 4% paraformaldehyde in PBS for 20 min at 4 °C. Cells were washed twice in PBS and then incubated with terminal deoxynucleotidyltransferase and reaction mix including fluorescein-12-dUTP for 1 h at 37 °C. Cells were washed for 30 min in saline-sodium citrate buffer and then incubated with PBS+4′,6-diamidino-2-phenylindole for 15 min at room temperature. 4′,6-diamidino-2-phenylindole is a blue fluorescent nuclear stain and this step ensured that approximately equal cells were imaged in each slide. The cells were visualized by indirect immunofluorescence with excitation at 485nm and emission at 530nm. The percentage of normal or apoptotic cells adherent to culture dish were estimated by an investigator blinded to the experimental conditions.

2.7. Data analysis and statistics

Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Bonferroni's t-test. Differences among means were considered significant at p<0.05. Data were analysed using Graphpad (San Diego, CA, USA).

3. Results

3.1. Effect of thapsigargin on the onset of S-phase in hVSMCs

Following a 4-day period of quiescence (i.e. cells are induced to enter Go) followed by stimulation with 10% fetal calf serum, a statistically significant increase in the percentage of hVSMCs that stained positively for BrdU was observed at 16 h (Fig. 1). Thus, S-phase in human VSMC commences 16 h after stimulation with 10% fetal calf serum. Pre-treatment with 10 nM thapsigargin for 24 h followed by its removal by washing delayed a statistically significant incorporation of BrdU by 8 h (Fig. 1). The continual presence of thapsigargin, however, prevented completely entry into S-phase (Fig. 1). Concomitant addition of calcium ionophore A23187 with fetal calf serum following 24 h pre-incubation with thapsigargin had no

Table 1
Effect of calcium ionophore A23187 (A23) on the inhibition of bromodeoxyuridine incorporation (% cells staining positively for BrdU) elicited by 24 h preincubation with 10 nM thapsigargin (TG) in human VSMC following stimulation with 10% fetal calf serum assessed at 24 h

Untreated	TG treated: zero	TG: 100 nM A23	TG: 1 μM A23	TG: 10 μM A23
19.2±1.2	0.3±0.01	0.28±0.04	0.22±0.02	0.1±0.009

Data=mean±SEM, n=5.

Table 2
Effect of BAPTA-AM on bromodeoxyuridine incorporation (% cells positively stained; index of proliferation) into human VSMC following stimulation with 10% fetal calf serum assessed at 24 h (cells not pretreated with thapsigargin)

Untreated	1 μM BAPTA	10 μM BAPTA	50 μM BAPTA	100 μM BAPTA
20.4±1.9	23.6±3	22.9±4	12.6±1.2[a]	2.2±0.2[a]

Each point=mean±SEM, n=5.
[a] p<0.05 (one-way ANOVA).

effect on thapsigargin-inhibited BrdU incorporation into hVSMCs over a 24-h time course (Table 1). The intracellular Ca^{2+} chelator, BAPTA-AM, at concentrations reported to completely abolish cytosolic Ca^{2+} also had no effect on BrdU incorporation (Table 2), but at higher concentrations did inhibit proliferation (Table 2).

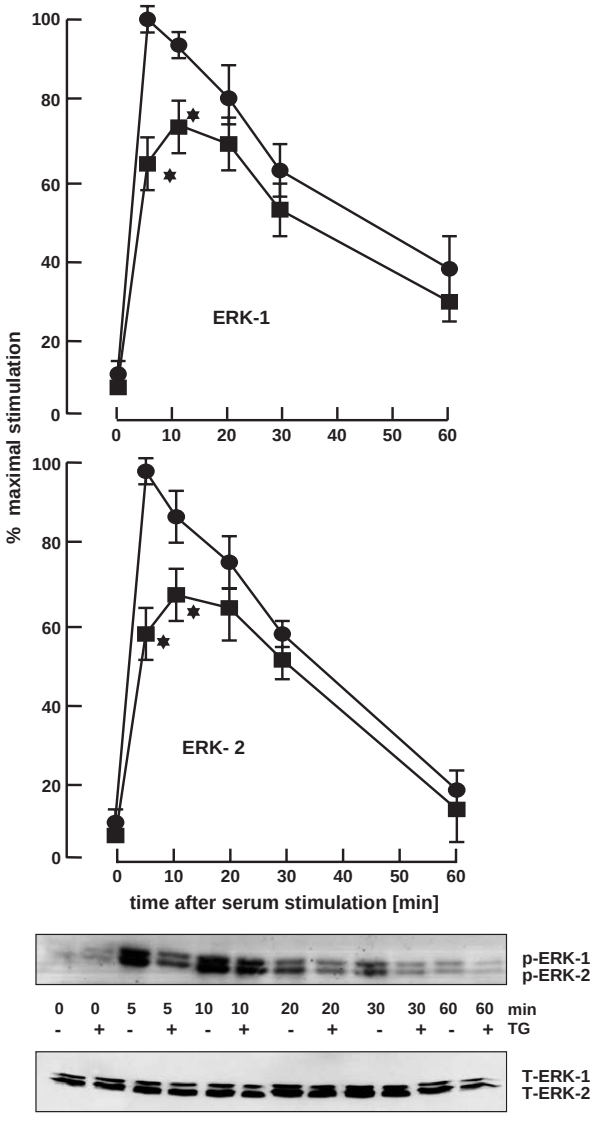


Fig. 2. Effect of 24 h pre-incubation of quiescent human vascular smooth muscle cells with 10 nM thapsigargin (TG), followed by washing, on the early activation (0–60 min) of ERK1/2 (p-ERK1 and p-ERK2) assessed by western blotting and densitometric analysis. Representative western blots of activated phosphorylated ERK1/2 and total ERK1/2±t-ERK1 and t-ERK2) are shown in the panel below. Data are expressed as mean±SEM, n=6. ✸ p<0.05 compared to levels at zero time point (one-way ANOVA).

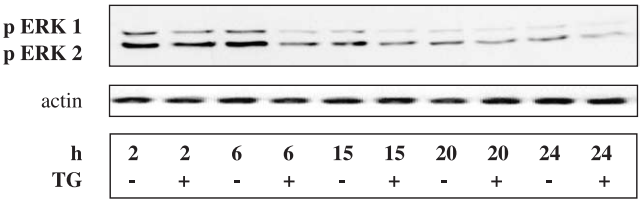
3.2. Effect of thapsigargin on ERK1/2 activation and translocation to the nucleus

Following stimulation with 10% fetal calf serum, there was a marked and rapid increase in the activated (i.e phosphorylated) forms of ERK1 and ERK2 in untreated hVSMCs (Fig. 2). The intracellular concentrations of activated ERK1/2 declined gradually thereafter but still remained elevated for up to 24 h after stimulation (Table 3). Pretreatment for 24 h with 10 nM thapsigargin followed by washing and activation with 10% fetal calf serum caused a significant reduction in ERK1/2 activation only at 5 min and 10 min after stimulation (Fig. 2). Pretreatment for 24 h with 10 nM thapsigargin followed by washing and activation with 10% fetal calf serum had no effect on the levels of total inactive ERK1/2 over 60 min after stimulation (Fig. 2.). Over the longer term (2–24 h), thapsigargin had no statistically significant effect on levels of activated ERK1/2 following stimulation with 10% FCS (Table 3).

Within 5 min after stimulation with fetal calf serum, activated ERK1/2 was located in the nucleus, where it persisted for 8 h and beyond (Fig. 3 and Table 4). In hVSMC pre-incubated with 10 nM thapsigargin for 24 h prior to fetal calf serum stimulation, there was a marked inhibition of acute activated ERK1/2 translocation which had reversed significantly by 8 h after the onset of stimulation (Fig. 3 and Table 4). By contrast, thapsigargin had no effect on the intracellular localisation of inactive ERK1/2 (Fig. 4).

Table 3
Effect of preincubation of human vascular smooth muscle cells with 10 nM thapsigargin (TG) for 24 h on the phosphorylation of (a) ERK1 (p-ERK-1) and (b) ERK2 (p-ERK2) from 2 to 24 h after stimulation with 10% fetal calf serum

	2 h	6 h	15 h	20 h	24 h
(a)					
Control	90 ± 9	76 ± 7	73 ± 7	52 ± 6	37 ± 4
TG	89 ± 10	74 ± 7	67 ± 5	49 ± 6	35 ± 3
(b)					
Control	97 ± 9	84 ± 4	77 ± 5	44 ± 6	38 ± 5
TG	92 ± 10	76 ± 6	72 ± 5	40 ± 7	33 ± 8



Representative western blots of activated phosphorylated p-ERK1 and EER-2 and actin loading control) are shown in the panel below Data=% of maximal activation at 60 min (Fig. 2). Each point=mean±SEM, n=5.

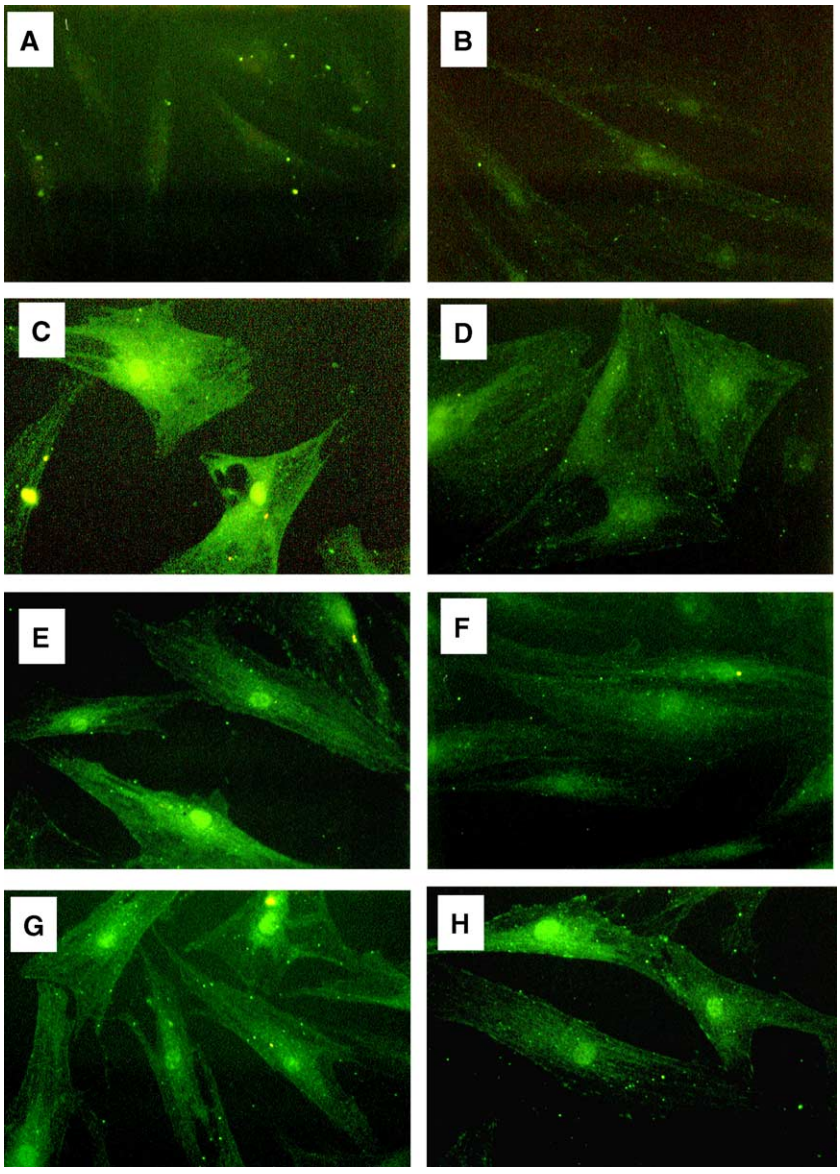


Fig. 3. Representative photomicrographs demonstrating the effect of a 24-h pre-incubation of human vascular smooth muscle cells with 10 nM thapsigargin followed by washing and activation with fetal calf serum on the translocation of phosphorylated ERK1/2 to the nucleus, assessed by indirect immunofluorescence: (A) control, time 0; (B) thapsigargin, time 0; (C) control, 5 min; (D) thapsigargin, 5 min; (E) control, 30 min; (F) thapsigargin, 30 min; (G) control, 8 h; (H) thapsigargin, 8 h.

3.3. Effect of thapsigargin on cyclin D1 levels, cytosolic calcium and cell viability

Following stimulation with 10% fetal calf serum, cyclin D1 protein levels in hVSMCs increased in a time-dependent manner compared to time zero, reaching statistical significance at 4 h after stimulation and thereafter reaching a maximal (a 12-fold increase compared to that at time zero) at 24 h (Fig. 5). In hVSMC pre-incubated with 10 nM thapsigargin for 24 h prior to fetal calf serum stimulation, cyclin D1 levels only began to rise significantly above basal at 8 h after stimulation with fetal calf serum (Fig. 5).

Table 4
Effect of preincubation of human vascular smooth muscle cells with 10 nM thapsigargin (TG) for 24 h, compared to untreated control cells (CON) on the translocation of activated ERK1/ERK2 to the nucleus after stimulation with 10% fetal calf serum

	0 min	5 min	10 min	20 min	30 min	60 min	240 min	480 min
CON	2±0.2	96±12	99±9	98±8	95±11	96±9	88±11	79±8[a]
TG	3±0.2	5±0.6	4±0.3	3±0.05	6±0.7	6±0.5	8±0.8	24±2[a]

Data=% of cells with positive nuclear immunofluorescence. Each point=mean±SEM, n=6.
[a] $p<0.05$ compared to 0 min (one-way ANOVA).

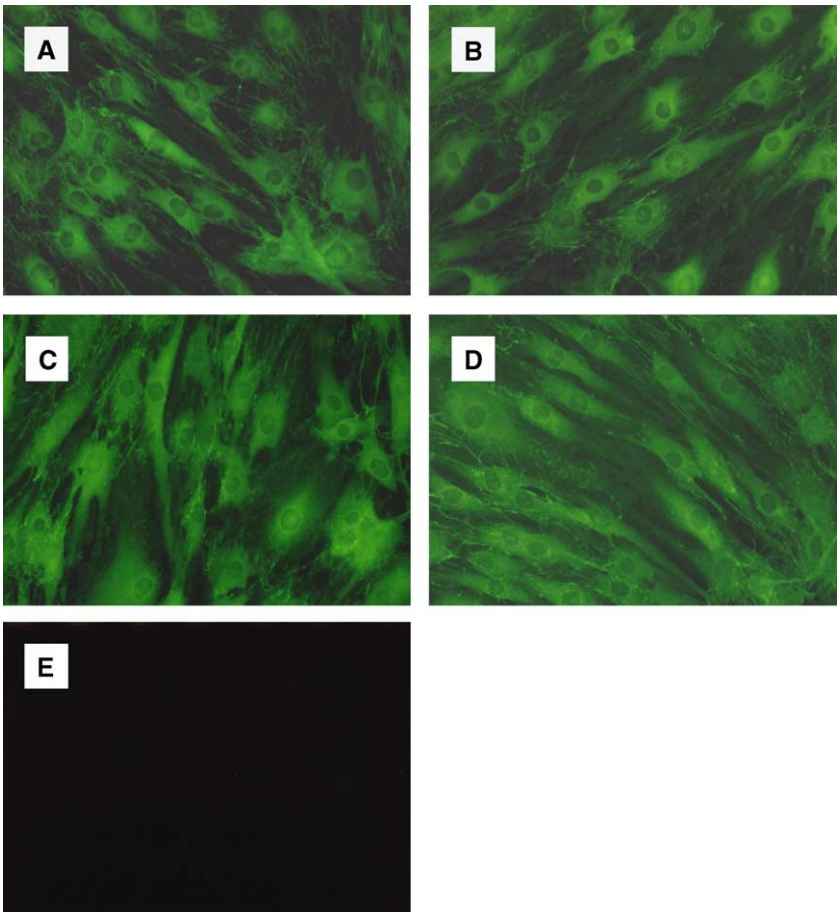


Fig. 4. Representative photomicrographs demonstrating effect of a 24 h pre-incubation of human vascular smooth muscle cells with 10 nM thapsigargin followed by washing and activation with fetal calf serum on the intracellular distribution of total inactive ERK1/2: (A) control, time 0; (B) thapsigargin, time 0; (C) control, 30 min; (D) thapsigargin, 30 min; (E) non-immune control. Note lack of nuclear fluorescence.

In hVSMC pre-incubated with 10 nM thapsigargin for 24 h prior to fetal calf serum stimulation, a statistically significant increase in ionomycin-stimulated cytosolic Ca^{2+} compared to cells in which thapsigargin was present was apparent 8 h after thapsigargin removal (Fig. 6).

4. Discussion

The present study demonstrates that pre-treatment with 10 nM thapsigargin exerts a marked inhibitory effect on hVSMC replication, delaying S phase entry by 8 h. This delay is temporally related to cyclin D1 expression and sarcoplasmic reticular Ca^{2+} pool status as well as to acute ERK1/2 activation (phosphorylation) and translocation to the nucleus. The effect could not be ascribed to apoptosis and/or cell death or a reduction in cell numbers.

Previous studies have indicated that the anti-proliferative effect of 10 nM thapsigargin on VSMC replication is mediated through depletion of sarcoplasmic reticular Ca^{2+} pools since 10 nM thapsigargin completely inhibited Ca^{2+} release from the sarcoplasmic reticulum in hVSMCs (Birkett et al., 1999). This indicated (but did not prove) that an increase in cytosolic Ca^{2+} is obligatory for VSMC replication to occur. However, if cytosolic Ca^{2+} levels, per se, are responsible for the progression of the cell cycle, then it would be expected that an agent that promotes an increase in Ca^{2+} may negate or reverse the inhibitory action of thapsigargin. However, the prolonged presence of calcium ionophore A23187 had no effect on VSMC proliferation following thapsigargin pre-treatment and serum stimulation in this study. Furthermore, the cell-permeable Ca^{2+} chelator, BAPTA-AM, at concentrations known to chelate cytosolic Ca^{2+} (Waser et al., 1997), had no effect on VSMC replication. However, at higher concentrations (50–100 μM) BAPTA-AM inhibited proliferation, which is also consistent with previous reports that these concentrations of BAPTA-AM deplete sarcoplasmic reticular-Ca^{2+} pools (Gissel et al., 1997). Taken together, these data indicate that an elevation of cytosolic Ca^{2+} derived from the sarcoplasmic reticulum, per se, may not mediate proliferation and that sarcoplasmic reticular-Ca^{2+} status alters cell cycle progression though mechanisms other than an elevation of cytosolic Ca^{2+}.

In keeping with previous reports on VSMCs from other species, serum promoted two phases of ERK1/2 activation

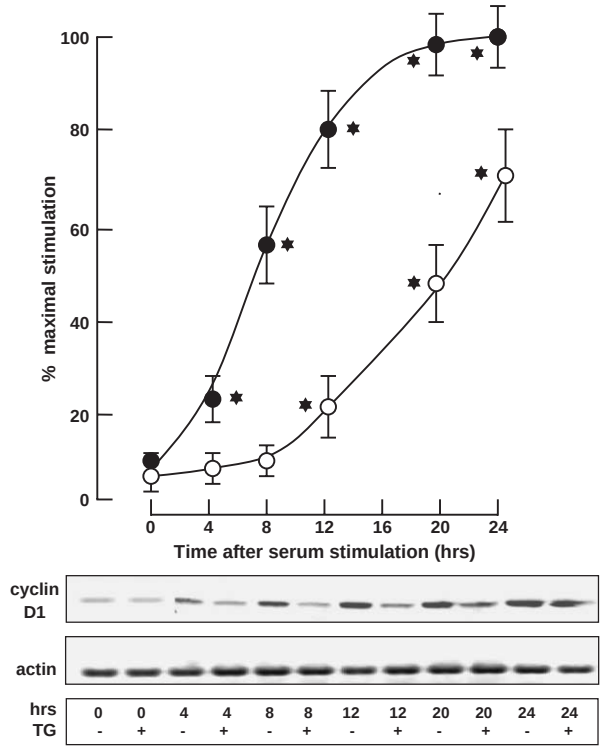


Fig. 5. Effect of 24 h pre-treatment of human vascular smooth muscle cells with 10 nM thapsigargin on the accumulation of cyclin D1 protein (% maximal at 24 h) [▲] compared to controls (untreated) [●] following stimulation with 10 % fetal calf serum. Representative western blots of cyclin D1 and actin are shown in panels beneath the graph. Data are expressed as mean±SEM, n=6. ✸ p<0.05 compared to levels at zero time point (one-way ANOVA).

in human VSMCs: a rapid response that occurs over 30 min and a prolonged elevation that persists over 24 h (Weber et al., 1997). Phosphorylation of ERK1/2 is essential for VSMCs to progress into the S-phase (Mii et al., 1996) and probably reflects the presence of the many mitogen(s) present in serum (Jeremy et al., 1997). In the present study, thapsigargin pre-treatment had a marginal, but still statistically significant, effect only at 5 and 10 min for ERK-2 and 5 min for ERK-1 after serum stimulation. It is possible that this effect of thapsigargin on the phosphorylation of ERK2 and ERK1 is due to do a Ca^{2+} responsive element(s) upstream of ERK activation, for example, Ca^{2+} tyrosine kinase (Yamamoto et al., 1998) or protein kinase C (Brinson et al., 1998). However, it is reasonable to suggest that sarcoplasmic reticular-Ca^{2+} pools play a minimal role in the activation of ERK1/2 in response to serum, since maximal ERK1/2 stimulation is only marginally reduced. Besides, the prolonged activation of ERK1/2 is deemed more important than the initial burst of ERK activation (Weber et al., 1997). It is also entirely possible that individual components of serum (e.g. PDGF and thromboxane A_2) are more sensitive to this depletion. However, the pathobiology of neointima formation involves a plethora of mitogens and growth factors and not one single mitogen or growth promoting factor (Jeremy et al., 1997).

In contrast to activation of ERK1/2, thapsigargin had an emphatic effect on the translocation of ERK1/2 to the nucleus. Within 5 min after stimulation with serum in control cells (i.e. without thapsigargin), ERK1/2 was detected using immunofluorescence within the nucleus and remained there for 30 min and beyond. Pre-treatment of cells with 10 nM thapsigargin completely inhibited the acute translocation of ERK1/2 to the nucleus. Since translocation of activated ERK1/2 to the nucleus is obligatory for progression of the cell cycle (Brunet et al., 1999), it is reasonable to suggest that this effect is central to the anti-proliferative effect of thapsigargin. Of the mechanisms known to mediate translocation and sequestration of phosphorylated ERK1/2 to the nucleus, both passive diffusion and active transport are possible, since ERK1 and ERK2 with molecular sizes of 44kDa and 42kDa, respectively, are close to the theoretical maximal size for unaided protein transport (Rothman, 1994; Adachi et al., 1999; Fukuda et al., 1995; Khokhlatchev et al., 1998; Whitehurst et al., 2002). Unphosphorylated (inactive) ERK1/2 also remains in the cytosol due to a combination of binding to the cytoskeletal proteins, MEK1 or MAPK phosphates 3 (MKP-3) (Reszka et al., 1995; Lenormand et al., 1998). Phosphorylation of ERK1/2 by MEK results in dissociation of ERK1/2 from its anchorage sites and its translocation to the nucleus (Adachi et al., 1999). Another possibility is that depleted sarcoplasmic reticular-Ca^{2+} stores disrupts the synthesis of ERK1/2 anchorage proteins, protein synthesis inhibitors having been shown to reduce ERK1/2 retention (Brunet et al., 1999). Thapsigargin may also influence the function of nuclear pores, through which all macromolecules must pass in order to enter the nucleus (Rothman, 1994). Indeed, depletion of sarcoplasmic reticular-Ca^{2+} stores with thapsigargin and BAPTA in kidney

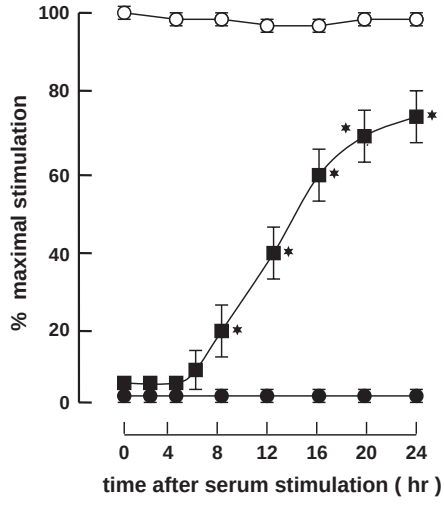


Fig. 6. Effect of 24 hr pre-treatment of human vascular smooth muscle cells with 10 nM thapsigargin on ionomcyin-stimulated cytosolic Ca^{2+} levels (% untreated control cells). Data are expressed as mean±SEM, n=6. ✸ p<0.05 compared to levels at zero time point (one-way ANOVA).

cells inhibits signal-mediated (active) transport of proteins into the nucleus (Greber and Gerace, 1995). Further studies are required to investigate this complex area.

As mentioned, a principal target for ERK1/2 is cyclin D1 expression (Lavoie et al., 1996). Cyclin D1 is essential for cell cycle progression through the G1/S checkpoint in response to all growth factors (Peeper and Bernards, 1997), transcriptional control of the cyclin D1 promoter being activated by phosphorylated ERK1/2 (Lavoie et al., 1996). In the present study, cyclin D1 levels increased significantly above basal at 4 h after serum stimulation, reaching a maximal at 24 h. In cells pre-treated with thapsigargin, however, significant expression of cyclin D1 was delayed by 8 h, similar to the delay in the onset of S-phase as described above. This effect is consistent with an inhibition of ERK1/2 translocation by thapsigargin, which then delays cyclin D1 expression.

Finally, thapsigargin pre-treatment also inhibited the release of Ca^{2+} in response to ionomycin, delaying it for 8 h, after which time the Ca^{2+} release response recovered to 50% of maximal at 24 h. Temporally, the recovery of Ca^{2+} pool refilling correlated with the onset of significant cyclin D1 expression (8 h), as well as the delay in the onset of the S-phase (8 h). These data therefore provide strong presumptive evidence for a functional link between sarcoplasmic reticular-Ca^{2+} pool status, cyclin D1 expression and the onset of S-phase. The recovery of the Ca^{2+} release responses is likely to be due to the neosynthesis of sarcoplasmic reticular Ca^{2+} ATPase pumps, since thapsigargin irreversibly binds to these pumps (Waldron et al., 1994). Indeed, in DDTMF-2 kidney cells pretreated with thapsigargin followed by its removal, the recovery of the proliferative capacity was restored within 6 h, a response ascribed to the expression and de novo synthesis of sarcoplasmic reticular Ca^{2+} ATPase pumps (Waldron et al., 1994).

It is concluded that pre-incubation of hVSMC with thapsigargin, at a concentration that completely depletes sarcoplasmic reticular Ca^{2+} stores without promoting an increase in cytosolic Ca^{2+} (i.e. 10 nM), followed by its removal, delays the progression of the cell cycle in hVSMCs by 8 h. This effect is not mediated by the inhibition of ERK1/ERK2 phosphorylation but the inhibition of ERK1/2 translocation to the nucleus, which in turn prevents the expression of cyclin D1. These effects are not mediated by an increase in cytosolic Ca^{2+}, per se. The overall effect of thapsigargin is reversible, in that the sarcoplasmic reticular Ca^{2+} pump function is restored within 8h which would explain the 8 h delay in the advent of the S-phase and cyclin D1 expression. It has been previously proposed that pre-treatment of saphenous veins with thapsigargin may constitute a therapeutic approach to preventing neointima formation. However the present data suggest that despite a marked early influence, the effect of thapsigargin may not be long-lasting enough to be of therapeutic value. However, the present studies have shed light on the role of Ca^{2+} in mediating the cell cycle, as well as the potential importance of translocation of ERK1/2 as a therapeutic target for inhibiting VSMC proliferative disorders such as vein graft failure and restenosis following balloon angioplasty.

Acknowledgments

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