

Effect of serum withdrawal on the contribution of L-type calcium channels (CaV1.2) to intracellular Ca^{2+} responses and chemotaxis in cultured human vascular smooth muscle cells

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1 Vascular smooth muscle cell (VSMC) chemotaxis is fundamental to atherosclerosis and intimal hyperplasia. An increase in intracellular Ca^{2+} [Ca^{2+}]_i is an important signal in chemotaxis, but the role of L-type calcium channels (CaV1.2) in this response in human vascular smooth muscle cells (hVSMC) has not been examined.

2 hVSMC were grown from explant cultures of saphenous vein. Confluent hVSMC at passage 3 were studied after culture in medium containing 15% foetal calf serum (FCS) (randomly cycling) or following serum deprivation for up to 7 days. Smooth muscle α -actin was measured by immunoblotting and immunofluorescence microscopy. [Ca^{2+}]_i was measured using fura 2 fluorimetry. Chemotaxis was measured using a modified Boyden chamber technique and cell attachment to gelatin-coated plates was also quantified. The number and affinity of dihydropyridine-binding sites was assessed using [5-methyl-³H]PN 200-110 binding.

3 In randomly cycling cells, the calcium channel agonist, Bay K 8644a and 100 mM KCl did not affect [Ca^{2+}]_i. In addition, the rise in [Ca^{2+}]_i induced by platelet-derived growth factor-BB (PDGF) was unaffected by the CaV1.2 antagonists, amlodipine and verapamil. In randomly cycling cells amlodipine did not affect PDGF-induced migration.

4 In serum-deprived cells, smooth muscle α -actin was increased and Bay K 8644a and 100 mM KCl increased [Ca^{2+}]_i. PDGF-induced rises in [Ca^{2+}]_i were also inhibited by amlodipine and verapamil. The ability of Bay K 8644a to increase [Ca^{2+}]_i and verapamil to inhibit PDGF-induced rises in [Ca^{2+}]_i was evident within 3 days after serum withdrawal. In serum-deprived hVSMC Bay K 8644a induced chemotaxis and amlodipine inhibited PDGF-induced migration. Cell attachment in the presence of PDGF was unaffected by amlodipine in either randomly cycling or serum-deprived hVSMC.

5 Serum withdrawal was associated with a decrease in the maximum number of dihydropyridine-binding sites (B_{max}) and a decrease in affinity (K_D).

6 Serum deprivation of hVSMC results in increased expression of smooth muscle α -actin, a marker of more differentiated status, and increased [Ca^{2+}]_i responses and chemotaxis mediated by CaV1.2. These observations may have important implications for understanding the therapeutic benefits of calcium channel antagonists in cardiovascular disease.

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Abbreviations: [Ca^{2+}]_i, intracellular calcium concentration; hVSMC, human vascular smooth muscle cell(s); PBS-A, Dulbecco's phosphate-buffered saline (without calcium and magnesium); PDGF, platelet-derived growth factor-BB isoform

Introduction

Vascular smooth muscle cells (VSMC) are responsible for the regulation of vascular tone and also participate in vascular responses to injury. Studies in animals indicate that following damage VSMC undergo dedifferentiation from a quiescent, 'contractile' state to a 'synthetic' phenotype (Campbell & Chamley-Campbell, 1981) and migrate into the region of injury, where they contribute to intimal hyperplasia (Ross, 1993). Smooth muscle cells in atherosclerotic plaques or intimal hyperplastic lesions more resemble dedifferentiated myofibroblasts, or cultured VSMC (Chamley-Campbell *et al.*,

1979; Thyberg, 1996) than native contractile smooth muscle cells. In cellular terms, the transition from contractile to synthetic phenotype is characterised by reduced expression of a variety of proteins, including contractile proteins, such as smooth muscle α -actin (Owens, 1995). Synthetic cells have limited ability to contract and become more responsive to chemotactic and mitogenic factors (Ross, 1993). In culture, dedifferentiation of smooth muscle cells has been reported to be partially, though not fully, reversible by prolonged withdrawal of growth factors or serum (Chamley-Campbell *et al.*, 1979; Chamley-Campbell & Campbell, 1981). Changes in intracellular Ca^{2+} ([Ca^{2+}]_i) are important in the process of cell migration (Pettit & Fay, 1998). In contractile VSMC

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L-type calcium channels (CaV1.2) are a major route of entry for Ca^{2+} ions (Hughes, 1995). Calcium antagonists, such as dihydropyridines and phenylalkylamines, block CaV1.2 and consequently their potential role in inhibiting atherosclerosis has attracted much interest. Calcium channel antagonists have been shown to inhibit smooth muscle cell migration (Nakao *et al.*, 1983) and also inhibit formation of atherosclerosis in some animal models (Nomoto *et al.*, 1987; Jackson *et al.*, 1989). However, evidence in favour of a specific antiatherosclerotic effect in man is less convincing (Borhani *et al.*, 1996; Pitt *et al.*, 2000; Zanchetti, 2001; Hernandez *et al.*, 2003). One possible explanation for this could be that CaV1.2 do not play such an important role in chemotaxis in human vascular smooth muscle cells (hVSMC) as in some other species. This study, therefore, examined the role of CaV1.2 in $[\text{Ca}^{2+}]_i$ responses and chemotaxis in hVSMC.

Methods

Materials

Cell culture plastics, media, foetal calf serum (FCS), supplements and recombinant human PDGF were obtained from LifeTechnologies (Paisley, U.K.). PN200-110, (+)-[methyl- ^3H] (2948.9GBq mmol^{-1}) and polycarbonate filters were purchased from NEN Life Science Products (Boston, MA, U.S.A.), and Poretics (Livermore, CA, U.S.A.) respectively. Bovine serum albumin (BSA) fraction V was obtained from Roche Diagnostics (Lewes, U.K.). Glass coverslips of 13 mm diameter and methanol were purchased from Merck (Poole, U.K.). Monoclonal anti-human smooth muscle α -actin (clone 1A4) (Skalli *et al.*, 1986), secondary antibodies and normal rabbit serum were purchased from Dako Ltd (Ely, U.K.). Normal mouse immunoglobulins were obtained from R & D Systems Europe (Abingdon, U.K.). Nitrocellulose and the enhanced chemiluminescence kit were purchased from Amersham Pharmacia Biotech (High Wycombe, U.K.) and acrylamide and scintillant obtained from National Diagnostics (Hull, U.K.). Amlodipine was a gift from Pfizer U.S. All other reagents were purchased from Sigma (Poole, U.K.).

Cell culture

Explant cultures of human VSMC were grown from saphenous vein as described previously (Chan *et al.*, 1993). Redundant veins were obtained from patients undergoing cardiovascular surgery in accordance with the guidelines of the local Ethics Committee. VSMC cultures were established in Dulbecco's modification of Eagle's medium (DMEM) buffered with 25 mM HEPES and supplemented with 15% (v v^{-1}) FCS 4 mM L-alanyl-L-glutamine (Glutamax-I), penicillin (100 U ml^{-1}), streptomycin (100 $\mu\text{g ml}^{-1}$) and gentamicin (25 $\mu\text{g ml}^{-1}$). hVSMC cultures were maintained in a humidified atmosphere of 5% CO_2 (v v^{-1}) in air at 37°C and characterised by positive immunostaining for smooth muscle α -actin and lack of staining for von Willebrand factor. In this study, confluent VSMC were employed at the third passage for all the experiments, either in a randomly cycling or quiescent state (following up to 7 days of serum deprivation and maintenance in NCTC 109 medium supplemented with 25 mM HEPES, 4 mM, Glutamax-I, 0.25% (w v^{-1}) BSA fraction V

(2.5 mg ml^{-1}) and antibiotics). These conditions were used to induce maximal quiescence as we have previously reported that hVSMC remain viable and do not undergo significant apoptosis following serum withdrawal for up to 7 days (Unlu *et al.*, 2001). Cell cycle analysis of hVSMC by flow cytometry using propidium iodide revealed that the percentages of cells in the S and G0/G1 phases, respectively, were 12.3 ± 0.5 and $74.0 \pm 0.9\%$ in the presence of serum, and 2.6 ± 0.5 and $90.9 \pm 0.6\%$ after 24 h serum deprivation in NCTC 109 medium.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured following loading of hVSMC with 5 μM fura-2AM (90 min, 37°C) as described previously (Clunn *et al.*, 1997a). After loading, cells were washed, briefly trypsinised, centrifuged and resuspended in physiological saline comprising (mM): NaCl 127, KCl 5.9, MgCl_2 1.2, CaCl_2 1, glucose 14 and HEPES 10.6, adjusted to pH 7.2 with NaOH. Fluorescence was measured using excitation wavelengths of 340 and 380 nm with emission at 510 nm using a Deltascan spectrofluorimeter (PTI). Experiments were calibrated and $[\text{Ca}^{2+}]_i$ concentration calculated using 50 μM digitonin to permeabilise the cells. All experiments were performed at 37°C.

Cell migration

Chemotaxis assays were conducted using blind well chemotaxis chambers (Neuroprobe, Cabin John, MD, U.S.A.) as described previously (Clunn *et al.*, 1997b). Briefly, VSMC were trypsinised and suspended in standard growth medium supplemented with 15% FCS. The resultant cell suspension was centrifuged ($200 \times g$, 10 min) and resuspended in serum-free DMEM supplemented with 0.1% (w v^{-1}) BSA fraction V. Cell viability was assessed using Trypan blue dye exclusion and a cell suspension prepared at a density of 2.25×10^5 viable cells ml^{-1} .

The upper and lower compartments of the blind well chambers were separated by 13 mm gelatin-coated polycarbonate filters with 8 μm pores. The chemoattractant (0.3 ml, PDGF 2 ng ml^{-1}) was added to the lower compartment and cell suspension (0.4 ml, 2.25×10^5 cells ml^{-1}) added to the upper chamber. Cells in suspension were either pretreated with amlodipine or serum-free DMEM (30 min, 37°C) prior to transfer into the upper chamber. The migration assay was allowed to proceed for 5 h in the presence or absence of inhibitor at 37°C in a humidified atmosphere of 5% CO_2 in air. Assays were terminated by washing the cells twice in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS-A) and fixing the cells in absolute ethanol at room temperature (10 min). The polycarbonate filters were stained in a 65 mM toluidine blue solution (15 min) and the cells on the upper side of the filter gently scraped off, leaving the migrated cells on the underside of the filters for counting. The cells present in four fields of view ($\times 200$ magnification) were counted on duplicate filters.

Cell attachment

Cell adhesion was assessed using a modification of the method described by Bilato *et al.* (1997). Briefly, gelatin-coated 96-well plates were prepared by coating overnight with 50 $\mu\text{g ml}^{-1}$

gelatin at 4°C and blocked with 5 mg ml⁻¹ denatured BSA (1 h, 37°C). A cell suspension was prepared at a density of 2×10^5 viable cells ml⁻¹ and 100 μl aliquots of cell suspension added to the wells in triplicate and maintained in a humidified atmosphere of 5% CO_2 in air (5 h, 37°C). The optimal cell density and time course for cell adhesion were determined in preliminary experiments (data not shown). All subsequent steps were conducted at ambient temperature. Nonadherent VSMC were removed by washing twice with PBS-A and adherent cells fixed with 4% formaldehyde in PBS-A. The fixative was removed and the cells stained with 1% (w v⁻¹) toluidine blue in 4% (v v⁻¹) formaldehyde for 15 min. The wells were extensively rinsed with water and the stain eluted from the attached cells using 1% (w v⁻¹) sodium dodecyl sulphate solution. Cell attachment was quantified by the dye adsorption which was determined spectrophotometrically at 630 nm using a microplate reader.

Dihydropyridine binding

The binding of [5-methyl-³H]-PN 200-110 to human VSMC was conducted according to the method of Navarro (1987). Briefly, VSMC were plated into 24-well plates at a density of 10^5 cells ml⁻¹ (1 ml well⁻¹) in standard growth medium supplemented with 15% FCS and allowed to attach overnight. One set of the 24-well plates was used to conduct a binding assay on randomly cycling cells, while cells in the remaining 24-well plates were rendered quiescent (as described earlier) for up to 7 days.

Prior to conducting the binding assays, the hVSMC were washed twice with pre-warmed serum-free DMEM. Cell counts were obtained in triplicate by treatment of the hVSMC with trypsin (0.25% in 1 mM EDTA in PBS-A) in order to normalise the ligand binding with respect to cell number. [5-methyl-³H]-PN 200-110 was prepared in serum-free DMEM to provide a range of final concentrations (10 pM–10 nM). Assays were performed under dim light in triplicate wells (30 min, 37°C) and terminated by removing the ligand and washing the cells three times (5 min each wash) with ice-cold wash buffer comprising 0.9% (w v⁻¹) sodium chloride, 5% (v v⁻¹) dimethyl sulphoxide and 1% (w v⁻¹) BSA (pH 7.0). Nonspecific binding was determined by incubation under the same conditions with excess unlabelled nitrendipine (10 μM). Cells were solubilised with 1 M sodium hydroxide solution and aliquots of the cell lysates transferred to scintillation vials to which Ecocint was added. Radioactivity was measured in a 1900CA TriCarb liquid scintillation counter (Canberra Packard, Pangbourne, U.K.) to determine the amount of [³H]-PN 200-110 bound to the cells.

hVSMC were plated at a density of 2×10^4 cells well⁻¹ onto sterile 13 mm diameter glass coverslips that had been placed in 24-well plates. After overnight attachment, randomly cycling cells were washed twice with PBS-A and fixed with ice-cold methanol (10 min). Parallel cultures were rendered quiescent (as described above) by maintenance in supplemented NCTC 109 growth medium and fixed as described above.

Immunocytochemical studies were conducted at ambient temperatures. Fixed cells were hydrated by incubation in PBS-A (10 min) and blocked with 10% (v v⁻¹) normal rabbit serum in PBS-A for 20 min to reduce nonspecific staining. Cells were incubated with either α -actin antibody (Skalli *et al.*, 1986)

(1/25) or normal mouse immunoglobulins at the same IgG1 concentration for 1 h and extensively washed in PBS-A containing 0.1% v v⁻¹ Tween 20. Secondary antibody (1/15) was applied for 1 h in PBS-A containing 10% normal rabbit serum and the cells thoroughly washed in PBS-A containing 0.1% (v v⁻¹) Tween 20. The coverslips were mounted in Citifluor mountant and viewed on a Carl Zeiss Axiophot microscope under epifluorescence. Photographs were taken using Kodak EliteChrome 400ASA film.

Immunoblotting studies

Proteins were separated on 10% SDS-polyacrylamide gels prior to transfer to nitrocellulose using a Bio-Rad wet gel transfer system as described previously (Patel *et al.*, 2002). Following successful transfer, nitrocellulose blots were blocked using 5% BSA (w v⁻¹) in tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h and washed thoroughly in TTBS prior to probing with primary antibody for 1 h. Blots were probed with the appropriate secondary antibody prepared in TTBS containing 3% BSA, washed three times in TTBS and developed using the enhanced chemiluminescence kit.

Statistics and data analysis

All data are presented as means \pm s.e.m. of *n* observations. Radioligand-binding data were fitted to a logistic function by nonlinear regression using Prism 3.03 (GraphPad Software, San Diego, CA, U.S.A.) to calculate the maximum binding site number (B_{max}) and binding affinity (K_{D}). Statistical comparisons were made with InStat 3.05 (GraphPad Software, San Diego, CA, U.S.A.) using a repeated-measures ANOVA followed by Dunnett's multiple comparison test, or by a Student's paired *t*-test as appropriate. $P < 0.05$ was considered significant.

Results

Serum withdrawal for 7 days resulted in increased levels of smooth muscle α -actin measured by immunoblotting and immunofluorescence microscopy (Figure 1). The effect of 7 days serum deprivation was also examined on changes in $[\text{Ca}^{2+}]_{\text{i}}$ due to CaV1.2 in hVSMC. The calcium channel agonist Bay K 8644a (100 nM) or 100 mM KCl had no effect on $[\text{Ca}^{2+}]_{\text{i}}$ in randomly cycling human VSMC ($n = 3$; data not shown). However, following serum deprivation for 7 days 100 mM KCl (data not shown) or Bay K 8644a increased $[\text{Ca}^{2+}]_{\text{i}}$ (Figure 2a) and this effect was inhibited by amlodipine (3 μM), an inhibitor of CaV1.2 (Figure 2a). In serum-deprived VSMC, pre-incubation with amlodipine (3 μM) also inhibited the rise in $[\text{Ca}^{2+}]_{\text{i}}$ following application of PDGF (10 ng ml⁻¹) (Figure 2b). The effect of amlodipine was more marked on the later sustained rise in $[\text{Ca}^{2+}]_{\text{i}}$ measured 2 min after the initial response to PDGF than the early rise in $[\text{Ca}^{2+}]_{\text{i}}$ measured at 30 s (Figure 3a). In contrast, in randomly cycling cells amlodipine (3 μM) had no effect on the rise in $[\text{Ca}^{2+}]_{\text{i}}$, although NiCl₂ (2 mM), a nonselective blocker of Ca²⁺ entry was able to partially inhibit the response to PDGF (Figure 3b).

The time course of appearance of CaV1.2 was examined in more detail in subsequent studies using Bay K 8644a (100 nM) and another CaV1.2 inhibitor, verapamil (10 μM). Responses

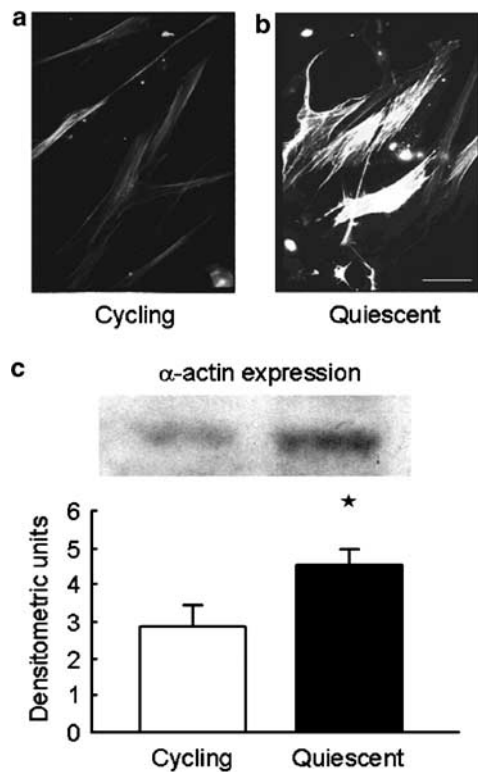


Figure 1 Effect of serum deprivation on smooth muscle α -actin in human saphenous vein-derived VSMC. (a) randomly cycling VSMC and (b) quiescent cells (following serum deprivation for 3 days) were stained with antibody for smooth muscle α -actin and examined using immunofluorescence microscopy (scale bar 50 μm , original magnification $\times 750$). (c) Representative Western blot and average data derived from densitometry of three separate Western blots show α -actin content of cell lysates from randomly cycling and quiescent cells following 7 days serum deprivation. * $P < 0.05$.

to Bay K 8644a (100 nM) increased progressively over a 4-day period (Figure 4) and by day 3 following serum withdrawal verapamil significantly inhibited the rise in $[\text{Ca}^{2+}]_i$ induced by PDGF (10 ng ml $^{-1}$) (Figure 4).

In order to assess the functional significance of these observations, we examined the effect of Bay K 8644a alone, and amlodipine on PDGF-induced chemotaxis of both randomly cycling hVSMC and hVSMC rendered quiescent by serum withdrawal for 7 days. In randomly cycling hVSMC, Bay K 8644a (100 nM) had no effect on chemotaxis and amlodipine (10 μM) did not affect migration in response to PDGF (2 ng ml $^{-1}$), but the nonselective voltage-operated and receptor-operated channel blocker NiCl $_2$ (2 mM) reduced PDGF-induced chemotaxis to nonstimulated levels (Figure 5a).

In contrast, following serum deprivation Bay K 8644a (100 nM) increased chemotaxis to $156 \pm 30\%$ of control ($n = 3$; $P = 0.05$), and amlodipine (10 μM) also significantly inhibited PDGF-induced chemotaxis (Figure 5a). Since the migration of cells through the pores in the filters in the modified Boyden chamber involves the process of attachment as well as directed movement of cells, we examined the effect of amlodipine on attachment to the gelatin-coated filters to establish which process involved CaV1.2. PDGF-induced attachment was unaffected by amlodipine (10 μM) in either randomly cycling or quiescent human VSMC (Figure 5b), suggesting that

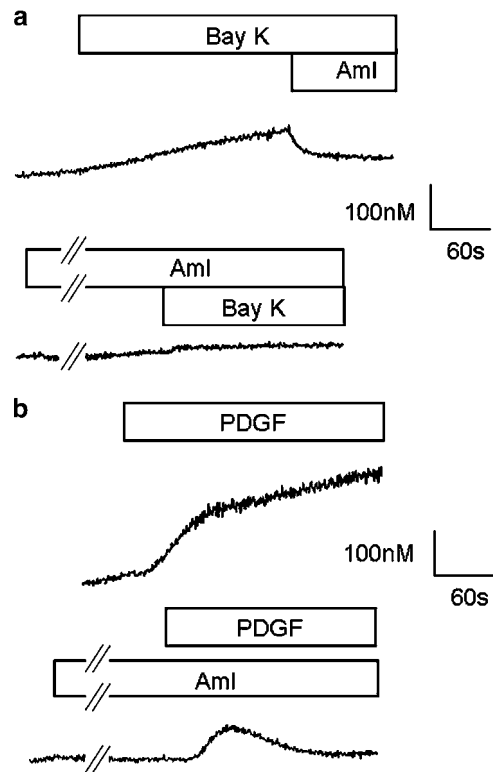


Figure 2 Effect of (a) Bay K 8644a (Bay K; 100 nM) and amlodipine (AML; 3 μM) on $[\text{Ca}^{2+}]_i$ in human VSMC rendered quiescent by 7 days of serum deprivation. (b) Effect of amlodipine (AML; 3 μM) on the rise in $[\text{Ca}^{2+}]_i$ induced by PDGF (10 ng ml $^{-1}$) in human VSMC rendered quiescent by 7 days serum deprivation. Traces are representative of 3–4 similar experiments.

inhibition of CaV1.2 reduced cell migration rather than attachment.

In view of the evidence suggesting an increased functional role of CaV1.2 in $[\text{Ca}^{2+}]_i$ responses and chemotaxis in serum-deprived cells, we examined whether this was due to changes in the number and/or affinity of dihydropyridine-binding sites. Radioligand-binding studies using [^3H]-PN 200-110 showed a significant reduction in the B_{max} and a progressive decrease in K_D in hVSMC after serum deprivation for 3 or 7 days (Figure 6a) compared with randomly cycling cells.

Discussion

In this study, we have examined the role of CaV1.2 channels in $[\text{Ca}^{2+}]_i$ responses and chemotaxis in randomly cycling and serum-deprived hVSMC derived from saphenous vein. In randomly cycling cells cultured in medium containing 15% FCS depolarisation by KCl or the selective CaV1.2 opener Bay K 8644a had no effect on $[\text{Ca}^{2+}]_i$ and inhibition of CaV1.2 with amlodipine did not affect migration in response to PDGF. Serum withdrawal caused growth arrest as evidenced by the reduced number of cells in S phase. In addition, there was an increase in smooth muscle α -actin expression, suggesting that serum withdrawal results in some degree of re-differentiation towards a contractile state as reported previously (Fager *et al.*, 1989). Serum deprivation was also accompanied by evidence of a contribution of CaV1.2 to

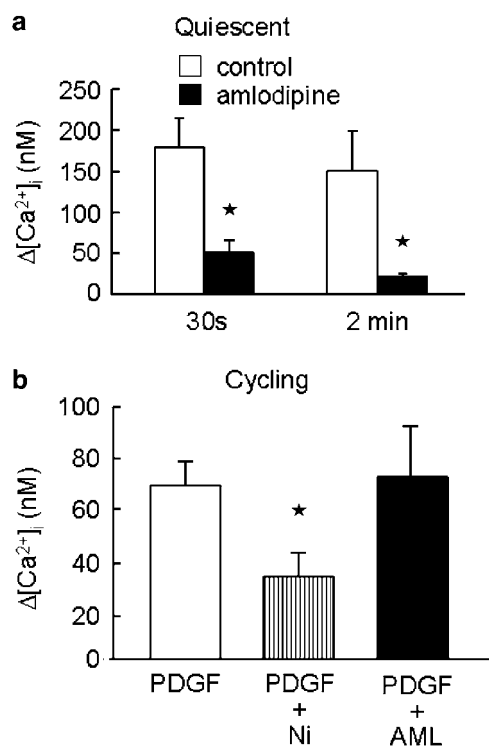


Figure 3 (a) Effect of amlodipine ($3 \mu\text{M}$) or vehicle (control) on the increase in intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) induced by PDGF (10 ng ml^{-1}) in hVSMC deprived of serum for 7 days (quiescent) 30 s and 2 min after the initial response to PDGF. (b) Effect of NiCl_2 (Ni; 2 mM) or amlodipine (AML; $3 \mu\text{M}$) on the change in intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) induced by PDGF (10 ng ml^{-1}) in randomly cycling human VSMC. Data are mean \pm s.e.m. of 3–5 observations. * $P < 0.05$.

$[\text{Ca}^{2+}]_i$ responses and chemotaxis. In the case of $[\text{Ca}^{2+}]_i$ responses, this was evident after 3–4 days of serum withdrawal. An action of amlodipine on cell attachment was excluded as the basis for its inhibitory action on cell migration and this effect is therefore likely to involve interference with Ca^{2+} -dependent processes involved in migration (Nomoto *et al.*, 1988). The concentrations of amlodipine used in these studies were quite high, but we have previously reported that the IC_{50} for amlodipine under nondepolarised conditions is $\sim 0.5 \mu\text{M}$ (Garcha *et al.*, 1993) and consequently high concentrations are required for effective inhibition of $\text{CaV}1.2$. The absence of an effect on amlodipine on randomly cycling cells argues against nonspecific inhibitory effects of these concentrations of amlodipine. These observations contrast with earlier studies in animal-derived VSMC in which dihydropyridines and other calcium channel antagonists have been reported to inhibit chemotaxis in randomly cycling cells (Nomoto *et al.*, 1988; Corsini *et al.*, 1996).

Previous studies have reported that cell culture conditions affect expression of calcium channels. In rat aortic myocytes, Richard *et al.* (1992) reported that low-voltage-activated calcium channel currents were expressed transiently, while cells were proliferating, whereas high-voltage-activated currents were seen in the first 5 days in primary culture and after the cells had reached confluence. Ihara *et al.* (2002) reported L-type calcium channels were lost during proliferation and reappeared following serum deprivation, inhibition of MEK,

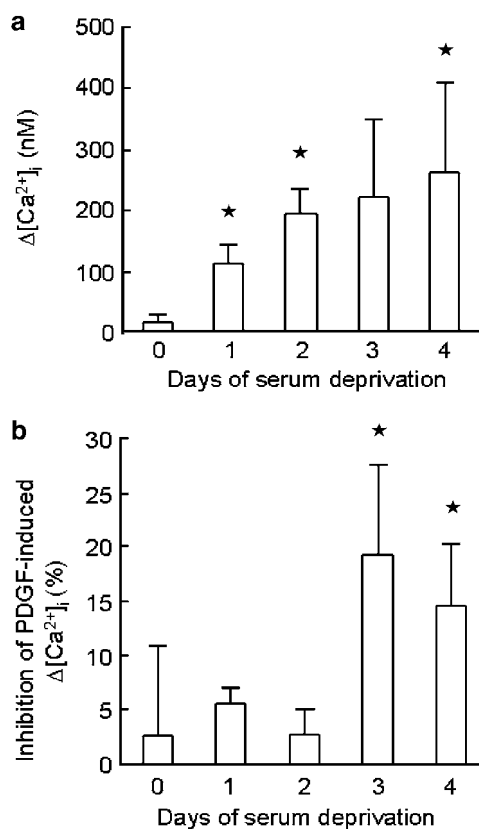


Figure 4 (a) Effect of Bay K 8644a (100 nM) on the peak increase in intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) after serum deprivation up to four days. (b) Effect of verapamil ($10 \mu\text{M}$) on the peak increase in intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) induced by PDGF (10 ng ml^{-1}) after serum deprivation up to 4 days. Data represent mean \pm s.e.m. of 3–5 observations. * $P < 0.05$.

or once cells had achieved confluence. Similarly, Kuga *et al.* (1996) reported that expression of both L- and T-type currents in rat aortic myocytes varied between different phases of the cell cycle with greatest expression of L-type currents in G1 phase and T-type currents in S phase. In human coronary myocytes, only dihydropyridine-sensitive L-type currents were seen in freshly isolated myocytes, whereas both L- and T-type currents were recorded from cells in primary culture (Quignard *et al.*, 1997); the effect of more prolonged culture was not examined.

Surprisingly, in our studies of hVSMC, radioligand-binding assays showed a decrease in the number and affinity of dihydropyridine-binding sites following serum withdrawal for up to 7 days. These data suggest that reduced expression of $\alpha 1$ -subunits of $\text{CaV}1.2$ (which correspond to dihydropyridine binding sites) does not account for the reduced functional role of $\text{CaV}1.2$ in randomly cycling hVSMC. This finding contrasts with a previous study, examining the A7r5 cell line, derived from rat embryonic thoracic aorta, which reported that retinoic acid-induced re-differentiation resulted in increased calcium channel currents, higher levels of dihydropyridine binding and increased expression of mRNA for $\alpha 1$ calcium channel subunits (Gollasch *et al.*, 1998). While the decrease in dihydropyridine-binding sites with serum deprivation in hVSMC was unexpected and remains to be explained, it should be noted that the number of dihydropyridine-binding

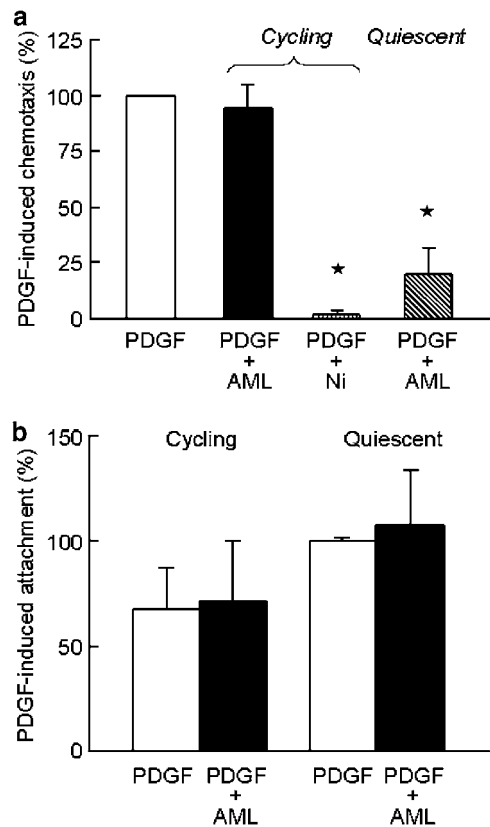


Figure 5 (a) Effect of calcium channel blockers on chemotaxis of hVSMC in response to PDGF (2ng ml^{-1}). Studies were performed in cycling and quiescent cells (after 7 days of serum deprivation). PDGF was placed in the lower chambers of the modified Boyden chambers and amlodipine (AML; $10\ \mu\text{M}$) or NiCl_2 (Ni; $2\ \text{mM}$) was present in both chambers. (b) Effect of amlodipine (AML; $10\ \mu\text{M}$) on attachment of human VSMC in the presence of PDGF (2ng ml^{-1}). Both randomly cycling and quiescent cells (after 7 days of serum deprivation) were preincubated with either serum-free medium or amlodipine ($10\ \mu\text{M}$) for 30 min prior to conducting the attachment assays in gelatin-coated 96-well plates. Data are mean \pm s.e.m. of 4–7 separate experiments. * $P < 0.05$.

sites does not correspond to functional $\text{CaV}1.2$ in the membrane. Indeed, the number of dihydropyridine-binding sites generally exceeds the estimated number of functional $\text{CaV}1.2$ channels by 50–100-fold (Schwartz *et al.*, 1985; Aiba & Creazzo, 1993). It has been reported that the level of expression of β subunits is important in regulating membrane localisation of α_1 subunits (Perez-Garcia *et al.*, 1995; Gao *et al.*, 1999) and further studies exploring the effect of serum deprivation on calcium channel β (and other accessory) subunit expression would be of interest in understanding this issue.

The majority of evidence supporting the use of calcium antagonists as antiatherosclerotic agents has been based on animal studies in which these agents reduce the severity of atherosclerosis, independent of actions on plasma lipids and blood pressure (Paoletti *et al.*, 1995). In humans, however, studies which have examined the action of calcium channel antagonists on atherosclerosis and intimal hyper-

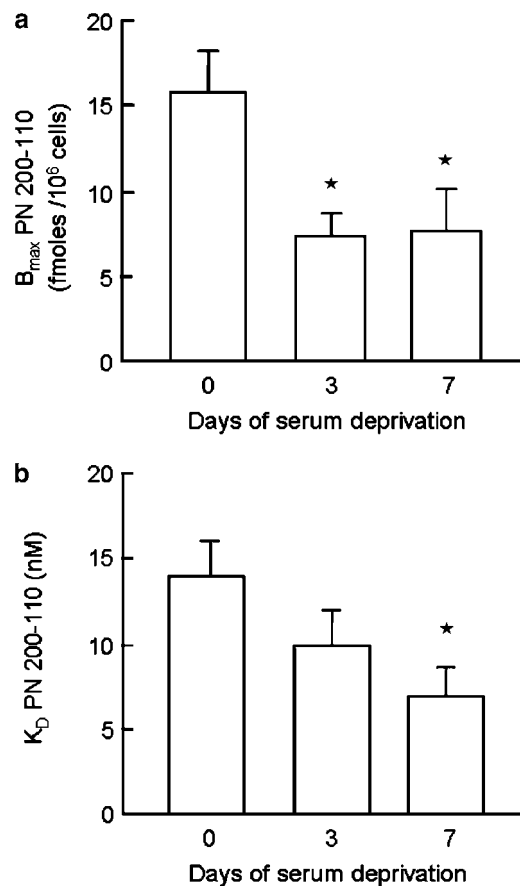


Figure 6 Changes in (a) B_{max} and (b) K_{D} for the binding of [5-methyl- ^3H]-PN 200-110 to human VSMC. Radioligand-binding assays were conducted as described in Methods. Both B_{max} and K_{D} were determined from experiments performed on randomly cycling VSMC (day 0) and following serum deprivation for 3 and 7 days. Data represent mean \pm s.e.m. from five separate experiments. * $P < 0.05$.

plasia have been less clear cut (Borhani *et al.*, 1996; Pitt *et al.*, 2000; Zanchetti, 2001; Hernandez *et al.*, 2003). It is possible that this is related to the influence of phenotypic state on $\text{CaV}1.2$ and its function in the migration of hVSMC.

In summary, this study shows that serum deprivation increases functional $\text{CaV}1.2$ in hVSMC and that this is not attributable to a reduction in the number of dihydropyridine-binding sites (i.e. the α_1 subunit of $\text{CaV}1.2$). These findings may have implications for better understanding the effects of calcium channel antagonists in human cardiovascular disease.

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