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Migration of human vascular smooth muscle cells involves serum-dependent repeated cytosolic calcium transients

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SUMMARY

Migration of vascular smooth muscle cells (VSMC) is a key event in the formation of neointima during atherosclerosis. Fura-2 loaded VSMCs were used to investigate calcium homeostasis during cell migration. Multiple spontaneous transient increases in cytosolic free calcium $[Ca^{2+}]_i$ were observed in single human VSMCs migrating on type I collagen. Such $[Ca^{2+}]_i$ transients were dependent on the presence of serum or PDGF-BB. Removal of serum, or loading cells with BAPTA, abolished the transients and decreased cell migration speed. The transients were not affected by disruption of cell polarization by dihydrocytochalasin B. Adhesion was used to investigate the specific role of cell-substrate interactions in the generation of transients. Transients are seen in VSMCs adhering either on collagen or on poly-L-lysine, suggesting that generation of transients is not strictly dependent on integrins. Buffering $[Ca^{2+}]_i$ with BAPTA led to accumulation of β1 integrins at the cellular tail, and to increased release of integrin on the extracellular matrix. These results demonstrate a role for $[Ca^{2+}]_i$ transients in the rapid, serum-dependent migration of VSMCs. These $[Ca^{2+}]_i$ transients are present in migrating VSMCs only when two simultaneous events occur: (1) substrate independent spreading and (2) stimulation of cells by serum components such as PDGF-BB.

Key words: Migration, Smooth muscle, Integrin, Calcium, Collagen

INTRODUCTION

Cell migration involves interactions of the cell with the surrounding extracellular matrix and requires a coordinated series of steps, including attachment to the substrate, generation of force and subsequent detachment from the substrate (Lee et al., 1993). Cell attachment to the substrate is mediated by adhesion receptors such as integrins (for review on adhesion receptors see Aplin et al., 1998). The nature of integrin-induced transmembrane signals controlling cell migration is still unclear. Changes of $[Ca^{2+}]_i$ are of particular interest, since such modifications may well modulate various stages of the migration process. Indeed, many of the key proteins involved in migration can be regulated by $[Ca^{2+}]_i$, including protein kinase C, myosin light chain kinase, actin cytoskeleton regulating proteins such as gelsolin (Cunningham et al., 1991) or fodrin (Harris and Morrow, 1990) and integrins, whose affinity for extracellular matrix proteins can be decreased by increases in $[Ca^{2+}]_i$ (Hendey et al., 1992).

The relationship between changes in $[Ca^{2+}]_i$ and cell migration has been studied only in a few cell types. Multiple transient increases in $[Ca^{2+}]_i$ have been observed in response to the chemoattractant f-Met-Leu-Phe (fMLP) in neutrophils migrating on vitronectin or fibronectin (Jaconi et al., 1991; Marks et al., 1991). Similar calcium transients during migration were observed in the MDCK-F cell line (Wojnowski et al., 1994) or in granule cells migrating from cerebellar microexplant cultures (Komuro and Rakic, 1996).

The migration of vascular smooth muscle cells (VSMC) is one important component in the formation of atherosclerotic plaques (Ross, 1993). The mechanisms that govern this function in this cell type are largely unexplored. Collagens are the dominant surrounding extracellular matrix proteins for VSMC in blood vessels and $\alpha_2\beta_1$ integrin has been shown to be the adhesion receptor implicated in in vitro migration on collagen (Pickering et al., 1997). However, the role of $[Ca^{2+}]_i$ changes in any cell type migrating on collagen has, to our knowledge, never been studied, although it has been reported that $\alpha_2\beta_1$ integrin-mediated adhesion of human platelets to collagen is associated with an increase of $[Ca^{2+}]_i$ (Poole and Watson, 1995). We have thus investigated the importance of changes in $[Ca^{2+}]_i$ in human VSMC migrating on type I collagen.

MATERIALS AND METHODS

Materials

Cell culture media RPMI 1640 and M199, Hepes, L-glutamine, penicillin, streptomycin, amphotericin B and trypsin-EDTA solution (trypsin 0.5 g/l, EDTA 0.2 g/l) were from Gibco (Paisley, UK).

Endotoxin-free human serum was from the Etablissement Régional de Transfusion Sanguine (Strasbourg, France). The serum was prepared from a pool of 13-15 healthy donors negative for hepatitis B virus and HIV, and was complement-inactivated for 30 minutes at 56°C. Monoclonal antibodies (mAbs) to α integrins were all from Immunotech (Marseille, France): HP2B6 (anti-α_1), Gi9 (anti-α_2), C3 (anti-α_3), HP2/1 (anti-α_4), SAM1 (anti-α_5) and GoH3 (anti-α_6). The mAb MOPC21 (Sigma, St Louis, MO, USA) was used as an isotypic control. The mAb P4C10 (anti-β_1 integrin), dihydrocytochalasin B (DHCB) and aphidicolin were from Sigma. Human recombinant platelet-derived growth factor (PDGF-BB) was from R&D systems (Abingdon, UK). FURA-2 acetoxylmethylester (FURA-2/AM) and BAPTA-acetoxylmethylester (BAPTA/AM) were from Molecular Probes. Poly-L-lysine was from Sigma.

Cell culture

VSMCs were collected from fragments of human mammary arteries cultured with the explant technique (Ross, 1971). The culture medium was M199/RPMI 1640 (1/1, v/v) containing 10 mM Hepes, 2 mM L-glutamine, antibiotics (100 i.u./ml penicillin and 100 μg/ml streptomycin), amphotericin B (0.25 μg/ml) and 10% human serum. The cells were frozen at the second passage and used in experiments from the third to the tenth passage. Plates were precoated with type I collagen (0.06 mg/ml).

Flow cytometric analysis

Subconfluent VSMCs in culture flasks were rinsed once with PBS containing 1% human albumin and resuspended in FMF medium (RPMI 1640, 5% inactivated goat serum, 0.2% NaN_3), quickly detached by trypsin/EDTA, washed once and resuspended in FMF. The cells were then first incubated with purified mAbs at 20 μg/ml for 30 minutes at 4°C, washed once with PBS, resuspended in FMF containing 10 μg/ml FITC-coupled goat anti-mouse second antibody and incubated for 30 minutes at 4°C in the dark. After dilution in PBS, cells were analysed in a FACsort cytofluorometer (Becton Dickinson).

Migration assay

VSMCs were seeded onto 35 mm diameter Petri dishes coated with type I collagen (0.06 mg/ml), and grown to confluence in a 37°C incubator gassed with 5% CO_2 in air. After 24 hours of serum starvation, a rectangular lesion was created using a cell scraper, the cells were rinsed three times with culture medium containing or not 10% human serum and incubated with the respective experimental medium. After 24 hours of migration, three randomly selected fields at the lesion border were acquired using a 10× phase objective on an inverted microscope (Olympus IMT2; Tokyo, Japan) equipped with a CCD camera (Panasonic). In each field, the distance between the margin of the lesion and the most distant point on migrating cells was analyzed for the 10 most mobile cells. Analysis was made using the UTHSCSA *Image Tool* program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the internet by anonymous FTP from maxrad6.uthscsa.edu). For experiments with BAPTA/AM, cells were loaded for 30 minutes with 30 μM BAPTA/AM prior to the creation of lesions.

Adhesion assay

VSMCs were incubated with culture medium with or without P4C10 mAb at 5 μg/ml. 5×10^4 cells/well were seeded onto 96-well plates coated with type I collagen (0.06 mg/ml). After 5 minutes of adhesion at 37°C, 5% CO_2, the cells were washed twice and culture medium containing Cell Titer 96 AQ (Promega) was added. After 2 hours, absorbance at 492 nm was determined with an ELISA iEMS reader MF (Labsystems, Cergy-Pontoise, France). We verified the linearity between the absorbance and the cell number in the range used (data not shown).

Cytosolic free calcium measurements during migration and adhesion

For $[Ca^{2+}]_i$ measurements during migration, the cells were cultured as above except that cells were grown in 35 mm Petri dishes in which a 2 cm diameter hole had been cut in the base and replaced by a thin (0.07 mm) glass coverslip. These dishes were also coated with type I collagen (0.06 mg/ml). After 24 hours migration, cells were incubated for 30 minutes at 37°C in a Ringer solution (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes and 11 glucose, pH 7.4) containing 5 μM Fura-2/acetoxylmethylester (Fura-2/AM). Cells were then washed for 15 minutes at 37°C with Ringer solution. Digital imaging was performed at room temperature using an IMSTAR (Paris, France) imaging system. Small groups of dispersed cells were viewed using an inverted microscope (Nikon Diaphot, Tokyo, Japan) and an UV-fluor 20× objective (n.a. 0.75, Nikon). Fura-2 fluorescence was excited alternately at 340 and 380 nm, using bandpass filters (±10 nm, Nikon) and a 100 W mercury lamp (HBO, Osram). Emitted fluorescence was bandpass filtered at 510±20 nm (Nikon) and measured using a Darkstar-800 CCD Camera (Photonics Sciences, Milham, UK). Acquired images were analyzed with the *fluo 210* IMSTAR software. Ratiometric Ca^{2+} images were generated at 5 second intervals, using four averaged images at each wavelength. After background compensation, $[Ca^{2+}]_i$ was averaged from pixels within manually outlined regions of interest corresponding to each cell. $[Ca^{2+}]_i$ values were calculated as described elsewhere (Grynkiewicz et al., 1985; Lynch et al., 1994). For calcium measurements during adhesion, confluent VSMCs were loaded with Fura-2/AM as described for migration. Cells were then detached by trypsin/EDTA and resuspended in Ringer solution at 5×10^5 cells/ml under the indicated experimental conditions. This suspension was seeded onto the glass coverslip coated with the indicated protein, and cells were allowed to adhere to the surface for 3-6 minutes. A field in the coverslip was then selected and $[Ca^{2+}]_i$ was determined as described above for each cell in this field. When adhesion was inhibited, observation of the rounded, non-adherent cells sedimented to the bottom of the plate was still possible.

Immunostaining of β_1 integrin and confocal microscopy

For immunostaining of β_1 integrin on migrating cells, a modified migration model (Paladichuk, 1998) was used. Briefly, 2×10^3 cells were deposited at the top of a 1 mm diameter, 8 mm high sedimentation channel, and allowed to sediment on a collagen-coated glass coverslip for 1 hour at 4°C. After removal of the sedimentation well, coverslips were placed in a 37°C incubator gassed with 5% CO_2 in air, and cells, treated or not with 20 μM BAPTA-AM for 30 minutes, were allowed to migrate for 24 hours before immunostaining. Cells were fixed for 15 minutes with PBS containing 4% paraformaldehyde. Cells were first incubated for 1 hour with the anti-β_1 integrin antibody P4C10 1/400 (v/v) in PBS, and then with an FITC-conjugated goat anti-mouse secondary antibody (Zymed) for 1 hour. Migrating cells were observed with a Bio-Rad 1024 MRC confocal microscope with a 40× epifluorescence objective (Nikon). *Z*-series were collected in 1 μm steps and final images were analyzed after stacking the first six images corresponding to the basal, matrix-associated sections of the cell. The amount of labeling was quantified with NIH Image software and represented as fluorescence intensities using a pseudo-colour scale. For quantification of fluorescence intensities at the front and rear ends of the cells, isolated cells were selected in each condition. The mean of fluorescence intensities was calculated using NIH Image either in the most cortical region of migration front or in the region of the tail where the cytoplasm was negligible. The ratio *r* was then defined as the mean fluorescence intensity of the tail divided by the mean fluorescence intensity of the front of the cell.

RESULTS

To investigate $[Ca^{2+}]_i$ changes during VSMC migration, we used the same cell culture model to assess both migration and $[Ca^{2+}]_i$ under similar experimental conditions. Migration was initiated by making a rectangular lesion in a VSMCs monolayer. After 24 hours, the migration of single VSMCs was measured or $[Ca^{2+}]_i$ quantified from Fura-2 loaded migrating VSMCs using image analysis. Pharmacological modulation of the speed of migration and of $[Ca^{2+}]_i$ in individual migrating VSMCs could thus be followed in parallel.

VSMC migration on type I collagen is accompanied by $[Ca^{2+}]_i$ transients

Spontaneous transient oscillations in $[Ca^{2+}]_i$ were observed in VSMCs migrating on type I collagen-coated coverslips in the presence of 10% human serum, with the frequency of such calcium transients being heterogeneous for different cells (Fig. 1A). Therefore, all observed cells (n=115) were classed into four groups according to the number of $[Ca^{2+}]_i$ transients measured during the 15 minute observation period (group 1, no transient; group 2, 1 or 2 transients; group 3, 3-5 transients; group 4, >5 transients). The observed distribution is summarized in Fig. 1B. In the presence of 10% serum, 44% of the migrating cells displayed at least three transients during 15 minutes. The maximum frequency observed was 17 transients in 15 minutes. The amplitude of the $[Ca^{2+}]_i$ transients was also highly variable between cells, ranging from 20 to 1500 nM (calculated as peak amplitude − basal value), but was constant for any given cell. In contrast, the duration of $[Ca^{2+}]_i$ transients was similar in all cells, with return to baseline values occurring within 30 to 35 seconds. The apparent heterogeneity of $[Ca^{2+}]_i$ transients between different cells was examined during a longer (80 minute) acquisition period. As shown for two representative cells (Fig. 2A), phases of repeated $[Ca^{2+}]_i$ transients alternated with phases without any $[Ca^{2+}]_i$. Over the 80 minute acquisition period, more than 90% of the cells displayed at least 1-2 transients, with 55% of cells exhibiting at least one 15 minute period with six or more transients (Fig. 2B).

$[Ca^{2+}]_i$ transients depend on the presence of serum

We then investigated the possible relationship between the presence of serum and the presence of $[Ca^{2+}]_i$ transients.

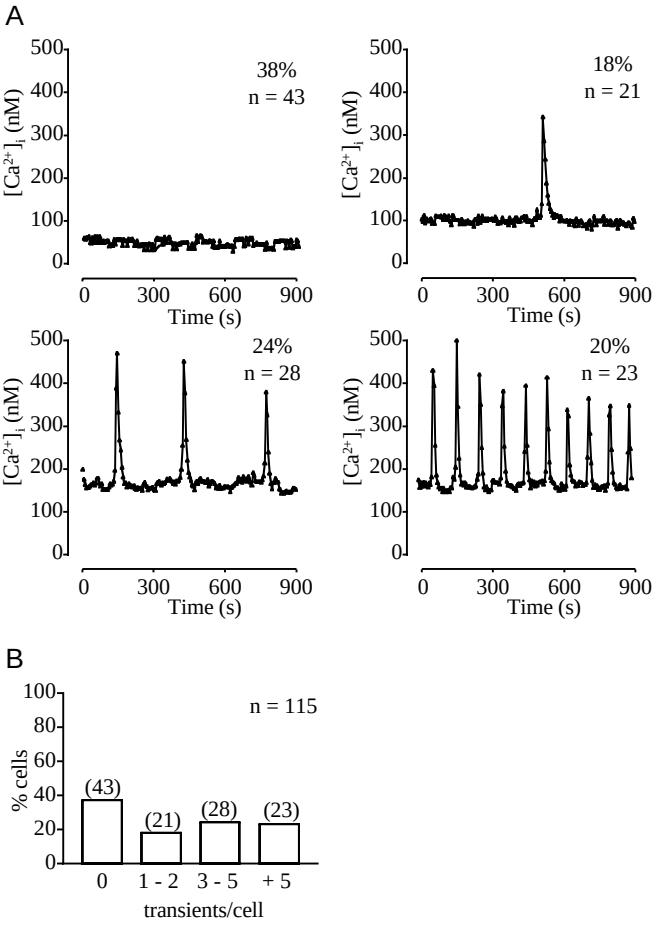

Fig. 1. (A) $[Ca^{2+}]_i$ measurements from individual Fura-2 loaded VSMCs migrating on type I collagen-coated coverslips in the presence of 10% human serum after 24 hours of migration in a cell-culture wound model. The traces are representative of cells from each of four groups (group 1, no changes; group 2, 1 or 2 spontaneous $[Ca^{2+}]_i$ transients; group 3, 3-5 transients; group 4, >5 transients during the 15 minute observation period). The numbers of cells and the corresponding % in each group are given. A total of 115 VSMCs were studied from six different preparations.
(B) Distribution of migrating VSMCs according to the number of spontaneous $[Ca^{2+}]_i$ transients observed within the 15 minute measurement period time (the total number of cells and the number of cells in each group are given).

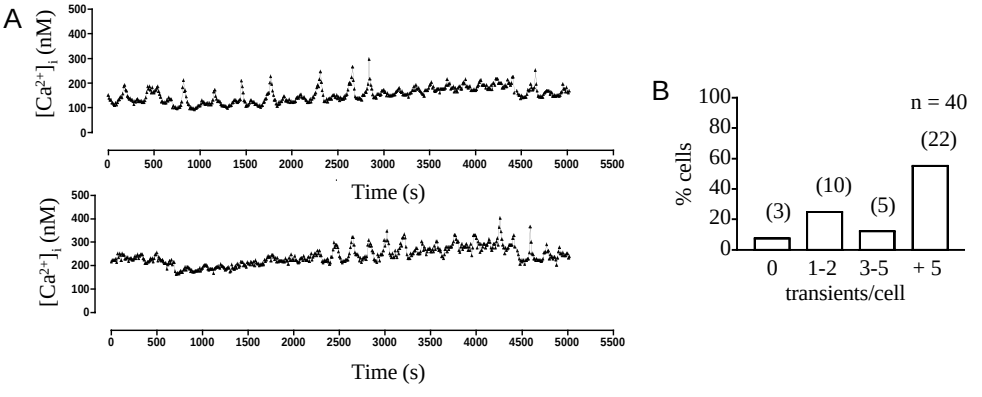

Fig. 2. (A) Examples of $[Ca^{2+}]_i$ measurements during 80 minutes from individual Fura-2 loaded VSMCs migrating on type I collagen-coated coverslips in the presence of 10% human serum after 24 hours of migration in a cell-culture wound model. (B) Distribution of migrating VSMCs according to the maximum number of spontaneous $[Ca^{2+}]_i$ transients observed during a 15 minute segment (measured sequentially for the total 80 minute acquisition period; the total number of cells and the number of cells in each group are given).

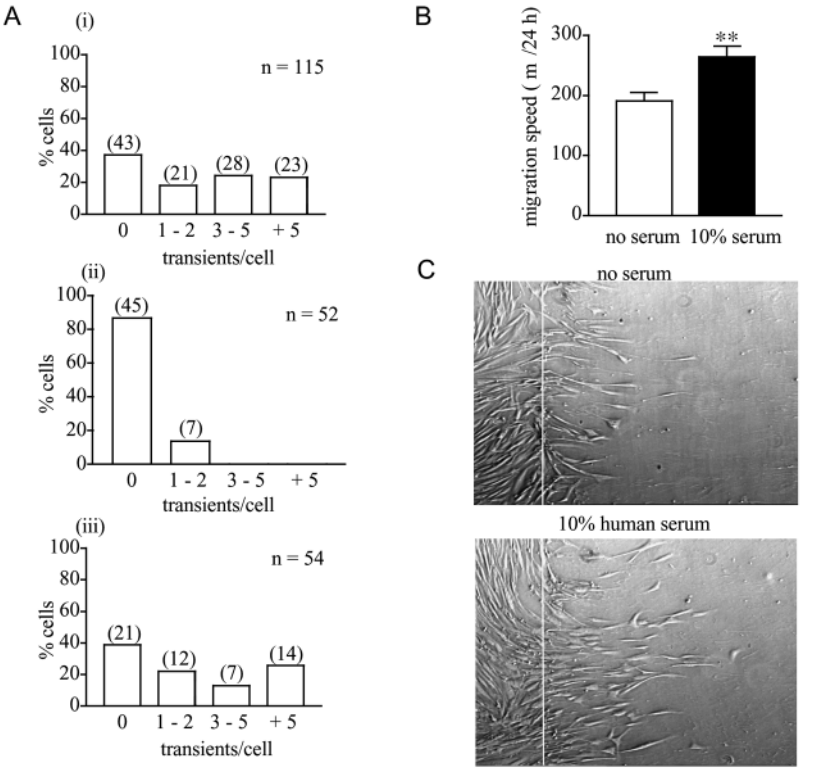

Fig. 3. Effect of serum on $[Ca^{2+}]_i$ transients and migration of VSMCs. (A) Distribution of migrating VSMCs according to the number of spontaneous $[Ca^{2+}]_i$ transients observed within the 15 minute measurement period: (i) after 24 hours migration with 10% human serum; (ii) after 24 hours migration without serum; (iii) after 24 hours migration without serum but following addition of 10% human serum upon acquisition (the total number of cells and the number of cells in each group are given). VSMCs were from at least three different preparations. Using the χ^2-test, distribution (i) was found to be significantly different ($P<0.05$) from distribution (ii), while distribution (iii) was not significantly different from distribution (i). (B) The absence of serum during the 24 hour migration period results in a decrease in migration speed compared to the migration speed in the presence of 10% human serum (**$P<0.01$, Student's unpaired *t*-test). Similar results were obtained in a total of five experiments. (C) Digitized images of representative fields showing VSMC migration after 24 hours in a cell-culture wound model in the presence or the absence of 10% human serum (the white line indicates the border of the lesion).

Removal of serum during the 24 hour period following lesion resulted in a significant drop in the number of cells exhibiting $[Ca^{2+}]_i$ transients, as seen in Fig. 3Aii. Indeed, during the 15 minute observation period, 86% of the VSMCs tested (n=52) in these conditions showed no change in $[Ca^{2+}]_i$, with the remaining 14% of cells never having more than two $[Ca^{2+}]_i$ transients. Remarkably, these serum-starved cells almost instantly regained the capacity to generate $[Ca^{2+}]_i$ transients, following the addition of 10% serum, as shown in Fig. 3Aiii. Thus, the distribution of responding cells (Fig. 3Aiii) was not significantly different from that observed after constant (24 hours) exposure to serum (Fig. 3Ai). In migration assays serum starvation, which, as shown above, inhibited almost completely $[Ca^{2+}]_i$ transients, only lowered the speed of migration by 22% (Fig. 3B). This reveals two types of VSMC motion: a slower motion in the absence of serum, and a serum-induced rapid motion. We verified that the increase in migration speed obtained in the presence of 10% serum was not due to proliferation by performing migration assays in the presence or the absence of 3 or 15 μM aphidicolin, a specific DNA polymerase inhibitor. Migration was identical in aphidicolin-treated and control (untreated) cells, either in the absence or in the presence of 10% serum (data not shown).

An anti-β_1 integrin monoclonal antibody inhibits the generation of $[Ca^{2+}]_i$ transients

The migration of VSMCs on type I collagen is mediated by $\alpha_2\beta_1$ integrins (Skinner et al., 1994). We thus measured the surface expression of the different α integrin subunits in VSMCs by flow cytometry using selective monoclonal antibodies (mAbs; Fig. 4). The α_2 subunit was most abundant in our VSMCs from human mammary arteries, consistent with our previous co-immunoprecipitation study showing that $\alpha_2\beta_1$ is the most abundant β_1 integrin in VSMCs from human kidney artery grown on collagen (Scherberich et al., 1998). To investigate the role of β_1 integrins in the generation of $[Ca^{2+}]_i$ transients, VSMCs were treated with P4C10 (1.5 μg/ml), a function-blocking anti-β_1 integrin mAb, during the 24 hour migration period in the cell-culture wound model described above. The number of cells displaying $[Ca^{2+}]_i$ transients was significantly decreased (Fig. 5A) compared to control,

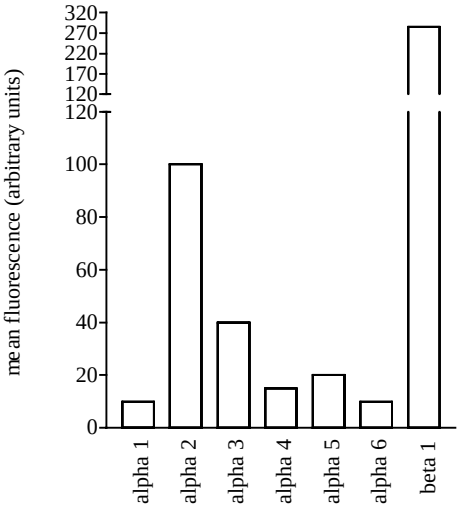

Fig. 4. Surface expression of α_1, α_2, α_3, α_4, α_5 and α_6 integrins and β_1 integrins in VSMCs assessed using specific monoclonal antibodies (see Materials and Methods). Expression was measured by flow cytometry from 10,000 cells. Results are given as the mean of fluorescence intensity in arbitrary units.

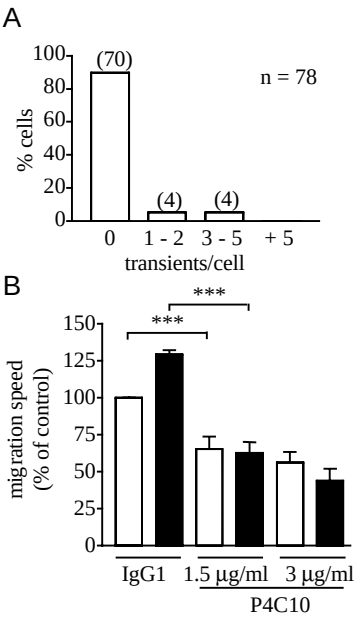

Fig. 5. (A) Effect of the anti-β_1 integrin monoclonal antibody P4C10 (1.5 µg/ml; 24 hours) on the number of VSMCs displaying $[Ca^{2+}]_i$ transients during the 15 minute observation period in a cell-culture wound model. (B) Effect of 1.5 or 3 µg/ml P4C10 on migration speed of VSMCs, in the presence (closed bars) or the absence (open bars) of 10% human serum. After 24 hours of migration following lesion in the presence of P4C10, migration speed was measured as in Fig. 2B. Similar results were obtained in four experiments. (****P*<0.01, one-way ANOVA, Kruskal-Wallis post-testing).

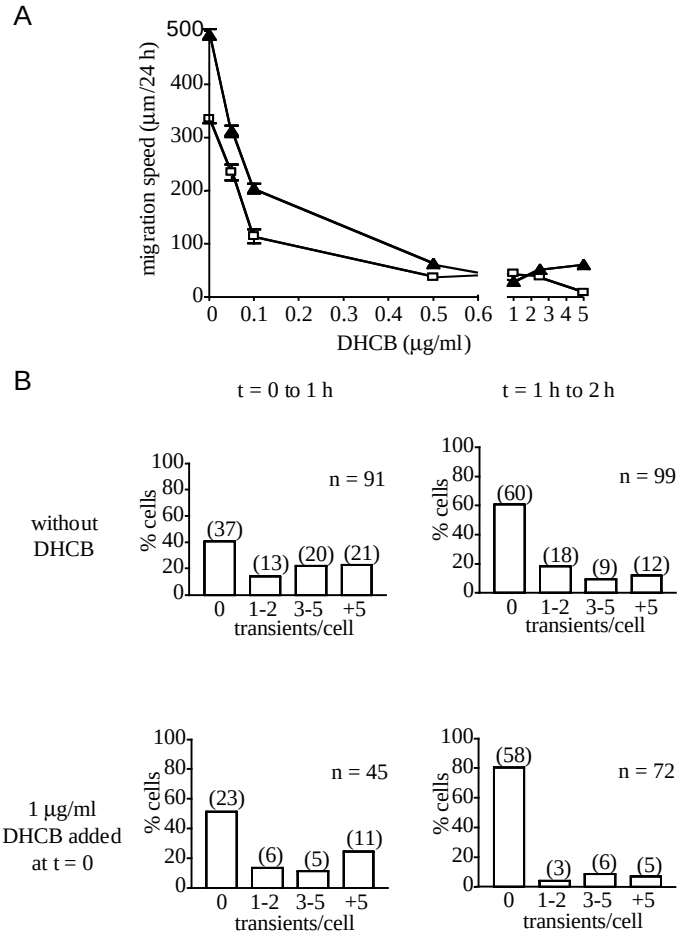

Fig. 6. (A) Effect of dihydrocytochalasin B (DHCB; 24 hours incubation) on migration speed of VSMCs in a cell-culture wound model, in the presence (filled triangles) or the absence (open squares) of 10% human serum. Similar results were obtained in two other experiments performed in duplicate. (B) Effect of DHCB (1 µg/ml) on the distribution of $[Ca^{2+}]_i$ transients observed during 15 minutes in migrating VSMCs, during the first (left panels) and the second hour (right panels) after the addition of DHCB. A significant difference in the distributions between control and treated cells was found only after 1-2 hours treatment (χ^2-test).

untreated cells (see Fig. 3Ai). In the migration assay, a similar 24 hour treatment with 1.5 µg/ml P4C10 caused a total inhibition of serum-dependent motion, and an additional 34±8% inhibition of the serum-independent motion (Fig. 5B). Incubation with 3 µg/ml P4C10 resulted in a stronger inhibition (44% decrease) of the serum-independent motion (Fig. 5B), and 5 µg/ml P4C10 led to the detachment of the cells during the 24 hour migration time (not shown).

Dihydrocytochalasin B inhibits migration dose-dependently but does not modify $[Ca^{2+}]_i$ transients

To investigate the requirement of cell shape changes in the generation of the $[Ca^{2+}]_i$ transients, we used dihydrocytochalasin B (DHCB). Partial inhibition of migration speed was obtained starting at 0.05 µg/ml DHCB, with essentially complete inhibition being observed for DHCB concentrations of 0.5-5.0 µg/ml, both in the presence and absence of 10% serum (Fig. 6A). When Fura-2 loaded, migrating VSMCs were challenged with 1 µg/ml DHCB, a change in cell shape occurred which was complete within 10 minutes, leading to a loss of the polarized aspect of cells (data not shown). Subsequently, no further cell shape changes were observed. In these conditions, the frequency distribution of $[Ca^{2+}]_i$ transients remained unchanged for at least 1 hour compared to controls, while a significant difference in this distribution was only detectable after 2 hours (Fig. 6B). Nevertheless, after 2 hours of treatment with DHCB, about 15% of the cells still displayed more than 3 calcium transients during the 15 minute measurement period.

Buffering of $[Ca^{2+}]_i$ transients inhibits the increase in migration speed induced by serum

Migrating VSMCs were loaded with BAPTA-AM (30 µM) in order to strongly buffer any increases in $[Ca^{2+}]_i$. The effect of serum on migration speed was totally abolished in BAPTA-loaded cells, which migrated at the same speed as unloaded cells in the absence of serum (Fig. 7). A non-significant decrease of migration speed in BAPTA-loaded cells in the absence of serum was also detected.

PDGF-BB stimulates VSMC migration and gives rise to $[Ca^{2+}]_i$ transients

We hypothesized that growth factors contained in serum might underly the generation of $[Ca^{2+}]_i$ transients, and we thus tested the effects of platelet-derived growth factor (PDGF-BB) on $[Ca^{2+}]_i$ transients and on migration. After 24 hours of serum starvation, a control observation period of 15 minutes

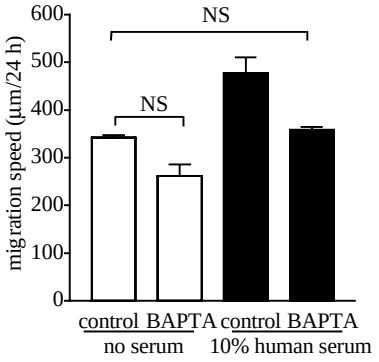

Fig. 7. Effect of $[Ca^{2+}]_i$ buffering with BAPTA/AM on migration speed of VSMCs in a cell-culture wound model, in the presence (closed bars) or the absence (open bars) of 10% human serum. Cells were loaded for 30 minutes with 30 µM BAPTA/AM, and after 24 hours, migration speed was measured as described previously. Values are mean ± s.e.m. of three experiments performed in duplicate. (NS, $P>0.05$; one-way ANOVA, Kruskal-Wallis post-testing).

confirmed that very few cells displayed $[Ca^{2+}]_i$ transients (Fig. 8Ai). Upon addition of 20 ng/ml PDGF-BB, the proportion of cells having $[Ca^{2+}]_i$ transients increased (Fig. 8Aii). This effect was maintained over the following 30 minutes, with a shift towards higher numbers of cells having several $[Ca^{2+}]_i$ transients (Fig. 8Aiii,iv). An original tracing from a representative cell is shown in Fig. 8B. In migration assays, PDGF-BB significantly stimulated the rate of migration (Fig. 8C).

PDGF-BB or serum induce $[Ca^{2+}]_i$ transients during VSMC adhesion and spreading on collagen

The role of β_1 integrins as well as PDGF-BB or serum on the generation of $[Ca^{2+}]_i$ transients was investigated during VSMC adhesion. Adhesion of VSMCs on type I collagen or on fibronectin gave rise to $[Ca^{2+}]_i$ transients comparable to those observed during VSMC migration on type I collagen (Fig. 9B).

The anti-β_1 integrin mAb P4C10 or serum starvation both caused a complete inhibition of adhesion on collagen (Fig. 9A) and calcium transients (Fig. 9B, lower panels). Such $[Ca^{2+}]_i$ transients were also absent for poorly adherent cells on uncoated glass (Fig. 9B, lower panel). VSMCs were also plated on poly-L-lysine in the absence of serum. In these conditions, adhesion as well as spreading of VSMCs was complete, but without any $[Ca^{2+}]_i$ transients (Fig. 10Ai). Adhesion on poly-L-lysine-coated coverslips, in the presence of 20 ng/ml PDGF-BB or 10 % serum, was accompanied by $[Ca^{2+}]_i$ transients. The frequency of the transients was classed as described above during VSMCs migration (Fig. 10Aii,iii). No significant difference in the distribution of cells exposed to PDGF-BB or serum could be detected (χ^2-test). However, when similar experiments were performed for cells plated on type I collagen, the addition of serum induced a significantly higher frequency of $[Ca^{2+}]_i$ transients than the addition of PDGF-BB (Fig. 10Bii versus iii). The distribution of cells according to the frequency of $[Ca^{2+}]_i$ transients was similar in cells exposed to serum, whether plated on poly-L-lysine or collagen (Fig. 10Aiii versus Biii). Note that these distributions were also comparable with that observed during cell migration on collagen in the presence of serum (Fig. 1B). Pretreatment of cells plated on poly-L-lysine in the presence of PDGF-BB with the anti-β_1 integrin blocking antibody P4C10 partially inhibited the occurrence of transients (Fig. 10Cii), whose frequency was comparable with that observed in cells plated on collagen (Fig. 10Bii versus Cii).

Buffering of intracellular calcium modifies β_1 integrin distribution on migrating VSMCs

To investigate a potential effect of $[Ca^{2+}]_i$ transients on integrin distribution, β_1 integrins were observed by immunostaining and confocal microscopy on the surface of VSMCs migrating on collagen in the presence of 10% serum. The cells show a randomly distributed labeling for β_1 integrins (Fig. 11), except for an intense labeling at the migration front (yellow arrow). For BAPTA-loaded cells migrating in the same conditions, labeling of β_1 integrins at the migration front of the cell was also intense

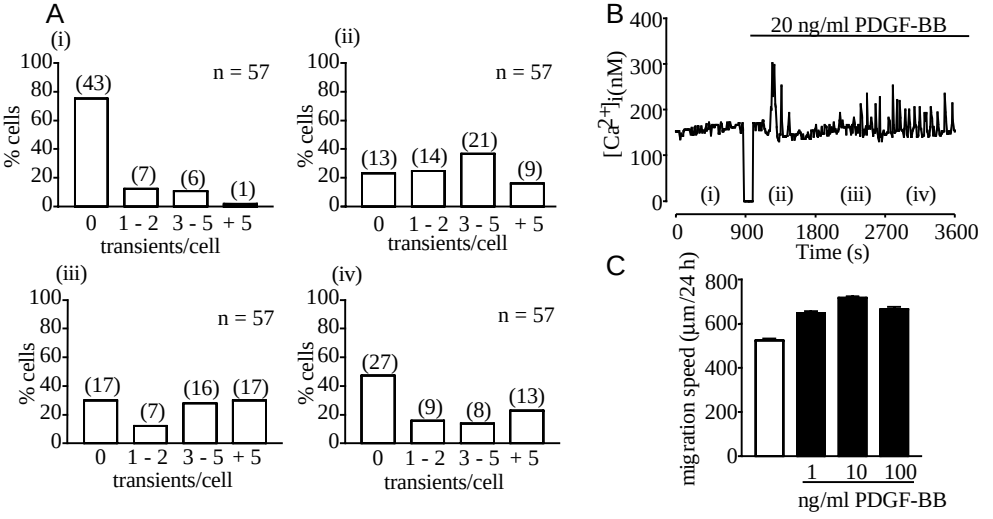

Fig. 8. (A) Effect of PDGF-BB on the number of $[Ca^{2+}]_i$ transients in serum-starved VSMCs. After 24 hours migration without serum, cells were loaded with Fura-2 and the distribution of cells having $[Ca^{2+}]_i$ transients was established in the absence of serum during an initial 15 minute period (i). Then, PDGF-BB (20 ng/ml) was added and the distribution of cells having $[Ca^{2+}]_i$ transients was determined for three consecutive 15 minute periods (ii, iii and iv). The data are from 57 cells from four separate experiments. (B) A tracing from a single cell showing the effect of PDGF-BB on $[Ca^{2+}]_i$. (C) Effect of PDGF-BB on migration speed of VSMCs in a cell-culture wound model. After 24 hours of migration in the absence of serum but with the indicated concentration of PDGF-BB, migration speed was determined as described previously. Values are means ± s.e.m. of two experiments performed in duplicate.

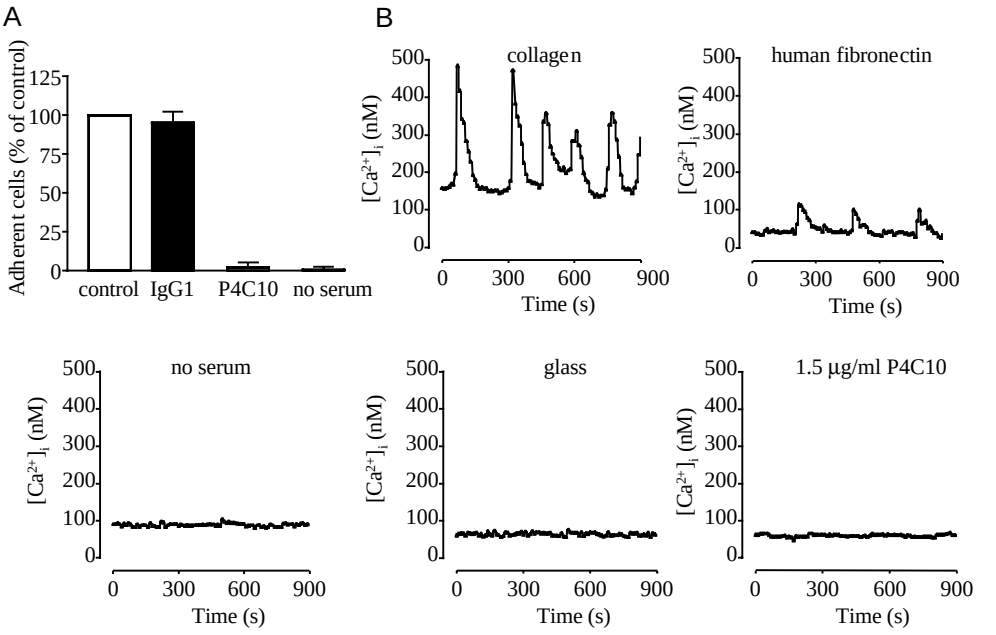

Fig. 9. (A) Effect of 5 µg/ml P4C10 or serum starvation on adhesion of VSMCs on a collagen-coated surface. Incubation time for adhesion was 5 minutes in the presence of 10% human serum. Results are expressed as % of adherent cells compared with control. (B) Examples of $[Ca^{2+}]_i$ measurements during VSMC adhesion on 100 µg/ml type I collagen-coated coverslips (representative of *n*=40 cells) or on 100 µg/ml fibronectin-coated coverslips (*n*=16) in the presence of 10% serum (upper panels). Lower panels: no $[Ca^{2+}]_i$ transients were observed during adhesion without serum (*n*=10), adhesion onto uncoated glass with serum (*n*=5), and adhesion on collagen in the presence of 5 µg/ml P4C10 with serum (*n*=6).

(yellow arrow) but high amounts of β_1 integrins were also detected at rear end of the cell (white arrows). We measured the intensity of fluorescence at the front and tail ends of the cells. The ratio of tail over front fluorescence is approximately doubled in BAPTA-loaded cells. Moreover, an increased labeling was observed in areas surrounding the BAPTA-loaded cells (arrowheads), indicating the presence of β_1 integrins that may have been released by the cells during migration.

DISCUSSION

The rationale for this study arose from our observation that migrating, Fura-2 loaded VSMCs display repeated transient increases in cytosolic free calcium (Fig. 1). While the frequency of these transients was quite variable for different cells, for any given cell, the frequency of such transients was relatively stable, allowing us to perform a precise phenotyping of the cells (Fig.

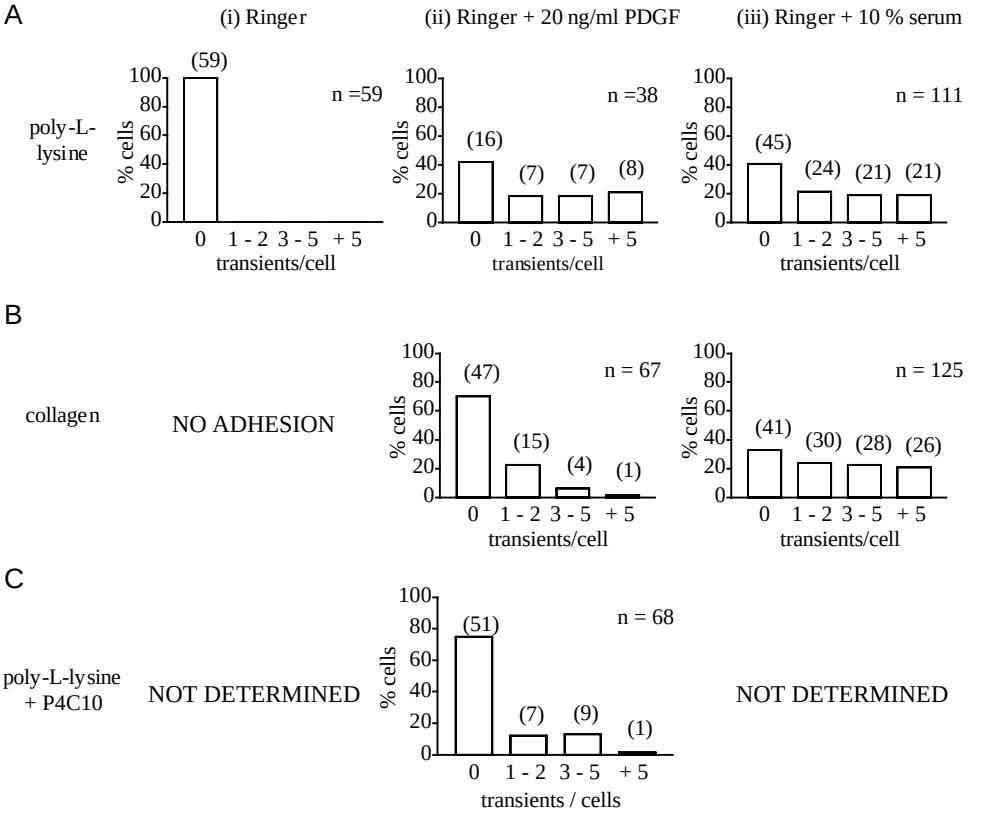

Fig. 10. Effect of PDGF-BB, serum and substratum on $[Ca^{2+}]_i$ transients during adhesion. Distributions of cells according to the number of $[Ca^{2+}]_i$ transients displayed during a 15 minute acquisition period of adhesion and spreading of VSMC on either (A) 5 µg/ml poly-L-lysine, (B) 60 µg/ml collagen-coated coverslips, or (C) 5 µg/ml poly-L-lysine after a 30 minute pretreatment of cells with 3 µg/ml P4C10. Adhesion was assessed in either (i) Ringer solution, (ii) Ringer solution + 20 ng/ml PDGF-BB or (iii) Ringer solution with 10% serum. Differences between distributions Aii and Aiii and between distributions Aiii and Biii are not significant (χ^2-test), while distributions Bii and Biii are significantly different (P<0.05).

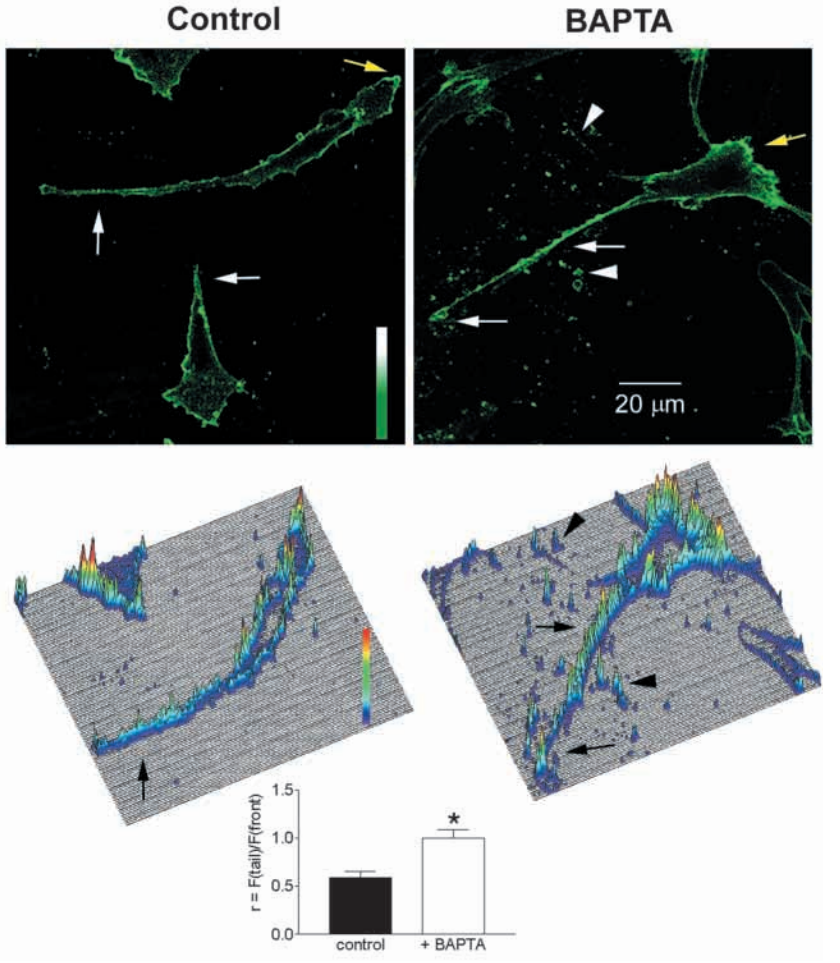

Fig. 11. Effect of BAPTA on surface distribution of β_1 integrin in migrating VSMCs. Cells, loaded or not for 30 minutes with 20 μM BAPTA-AM, were allowed to migrate for 24 hours on type I collagen-coated coverslips in the presence of 10% serum. They were then immunostained with the P4C10 anti-β_1 integrin antibody. Yellow arrows indicate the migration front of the cells, white arrows the trailing end, and arrowheads the patches of β_1 integrins present on the substratum (upper panels). Fluorescence intensities were quantified using a pseudo-colour scale (lower panels). Arrows indicate fluorescence intensities at the tails of the migrating VSMCs and arrowheads indicate fluorescence observed on the substratum around the BAPTA-loaded cell. Similar results were observed in four separate experiments. Quantification of fluorescence was determined on four cells in each condition. The ratio of the fluorescence intensities (tail/front) is presented in the lower part of the figure. Differences are significant (P<0.05) according to Student's *t*-test.

3). The parameter used for this classification was the number of $[Ca^{2+}]_i$ transients observed during the 15 minute acquisition time. When performing longer experiments (80 minutes), more than 90% of the cells displayed $[Ca^{2+}]_i$ transients, although only during limited time periods (Fig. 2). This shows that apparent heterogeneity of cell responses for shorter acquisition times is not the result of the presence of various cell subtypes. It rather suggests that transients are only seen during specific, discrete stages of the migration process. Unlike the amplitude of these transients which was highly variable, the duration (30-35 seconds for return to baseline) was similar in all VSMCs and close to the duration of $[Ca^{2+}]_i$ transients observed in migrating neutrophils (Marks and Maxfield, 1990). As proposed by Maxfield and his colleagues, we believe it more appropriate that these relatively slow, repeated increases and decreases in $[Ca^{2+}]_i$ be called transients rather than oscillations. No consistent subcellular compartimentalization of calcium gradients was detected, although we did observe larger amplitude $[Ca^{2+}]_i$ transients at the trailing edge of several migrating VSMCs. A more precise study of the origin and eventual subcellular localization of $[Ca^{2+}]_i$ transients should be carried out using confocal microscopy.

Our study is the first to describe $[Ca^{2+}]_i$ transients in migrating VSMCs and $[Ca^{2+}]_i$ transients in cells migrating on type I collagen-coated surfaces. Similar repeated calcium transients have been reported and extensively studied in migrating neutrophils (Marks and Maxfield, 1990; Mandeville et al., 1995), where $\alpha_v\beta_3$ integrins and the adhesive proteins fibronectin and vitronectin play key roles. In migrating neutrophils, intracellular calcium levels correlate with speed of migration and persistent forward motion (Mandeville et al., 1995). Calcium transients were also observed during the migration of MDCK-F cells (Wojnowski et al., 1994). In granule cells of cerebellar microexplant cultures, a positive correlation was found between rate of cell movement and the amplitude and frequency of Ca^{2+} fluctuations (Komuro and Rakic, 1996). Lee and colleagues demonstrated that $[Ca^{2+}]_i$ transients occur more frequently in cells 'stuck' to the substratum or when subjected to mechanical stretching (Lee et al., 1999). A recent report shows that growth cones generate transient elevations of $[Ca^{2+}]_i$ as they migrate within the embryonic spinal cord but that the rate of axon outgrowth is, in this case, inversely proportional to the frequency of transients (Gomez and Spitzer, 1999). However, $[Ca^{2+}]_i$ transients do not seem to be a ubiquitous characteristic of migrating cells. Indeed, human umbilical vein endothelial cells migrating on type I collagen do not display calcium transients, while on the other hand, migration of these cells on vitronectin is associated with a sustained (2 hour) elevation in calcium, with no transients (Leavesley et al., 1993).

Cell polarization, which is the first step underlying migration, is stimulated by chemoattractants and growth

factors. We thus tested the serum-dependence of both migration and $[Ca^{2+}]_i$ transients in our VSMCs. Serum starvation during the 24 hour migration period led to an almost complete arrest of calcium signaling, but only to a 22% decrease in migration speed (Fig. 3). It has been proposed that molecular interventions in migration can be examined specifically in terms of changes in speed of migration, persistence of motion, or both (Lauffenburger and Horwitz, 1996). For instance, some alterations in integrin-cytoskeleton interaction influence migration speed but not the persistence of motion (Schmidt et al., 1995). It was also shown that the requirement for $[Ca^{2+}]_i$ transients in neutrophil migration is not absolute (Marks et al., 1991). In our wound-healing model of VSMC migration, we were able to define a rapid, serum-dependent migration, and a slower, serum-independent motion (Fig. 3B). Serum-independent slow migration appears to be independent of the $[Ca^{2+}]_i$ transients since serum-starved cells continue to migrate in the almost complete absence of $[Ca^{2+}]_i$ transients (Fig. 3). Furthermore, in BAPTA-loaded cells, rapid motion is abolished and the speed of migration is comparable to that of serum-starved cells (Fig. 7). BAPTA seems however to affect the overall rate of migration in the absence of serum. Although not statistically significant, this effect could suggest that calcium is also involved in other steps of the regulation of migration, independently of transients. Taken together, this strongly suggests that rapid motion is a direct consequence of serum-related $[Ca^{2+}]_i$ transients. To our knowledge, this effect of serum on $[Ca^{2+}]_i$ transients has not been described previously in any cell type.

We also found that addition of serum to serum-starved cells caused a rapid (within 10 minutes), essentially complete reactivation of calcium signalling (Fig. 3A). This effect is thus unlikely to be dependent on transcriptional changes. Our results with PDGF-BB (Fig. 8) suggest that this is a likely candidate for one of the serum components responsible. However, other growth factors as well as extracellular matrix proteins may well contribute to the observed effect of serum. The difference in the frequency of transients obtained for adhesion of VSMCs on collagen in the presence of 20 ng/ml PDGF-BB versus 10% serum (Fig. 10Bii versus iii) seems to confirm a complementary role for other serum compounds in the generation of $[Ca^{2+}]_i$ transients. Further studies are necessary to determine the exact identity of such compounds.

Growth factor-mediated VSMC migration on type I collagen has been shown in in vitro studies to be dependent on $\alpha_2\beta_1$ integrins (Pickering et al., 1997; Skinner et al., 1994), although $\alpha_1\beta_1$ seems to be a critical collagen receptor in vivo (Gotwals et al., 1996). The function-blocking anti-β_1 integrin mAb P4C10 inhibited VSMC migration even in the absence of 10% serum (Fig. 5B), suggesting that like rapid motion, serum-independent migration involves β_1 integrins. As α_2 is the most expressed α subunit in our VSMCs (Fig. 4), a correlation could exist between $\alpha_2\beta_1$ integrin engagement and the generation of $[Ca^{2+}]_i$ transients. It has indeed been reported that several integrins can mediate calcium signaling (for a review, see Sjaastad and Nelson, 1997). These include $\alpha_{II}\beta_3$ and $\alpha_2\beta_1$ in platelets in the presence of collagen, β_2 integrins in leukocytes, α_v in endothelial cells and MDCK cells, and $\alpha_v\beta_3$ in osteoclasts. The integrin $\alpha_2\beta_1$ thus appears likely to be implicated in the generation of $[Ca^{2+}]_i$ transients observed in VSMCs migrating on collagen in the presence of serum. From our experiments on migration with P4C10, we may conclude that $[Ca^{2+}]_i$ transients are dependent on the engagement of β_1 integrin (Fig. 5). However, one could also suggest that this effect of P4C10 on transients is only a consequence of the inhibition of migration by this antibody (Fig. 5B). To address this possibility, calcium measurements during adhesion on collagen (integrin-dependent adhesion) and poly-L-lysine (integrin independent adhesion) were made. During adhesion of VSMCs on poly-L-lysine, $[Ca^{2+}]_i$ transients were observed in the presence of PDGF-BB as well as in the presence of 10% serum (Fig. 10A), which could suggest that integrin engagement is not an absolute requirement for the generation of transients. However, although adhesion on poly-L-lysine is usually presented as integrin-independent, it remains possible that this adhesion could involve β_1 integrin binding to extracellular matrix components secreted by the cells. To preclude this possibility, we performed similar adhesion experiments on poly-L-lysine in the presence of the anti-β_1 integrin-blocking antibody P4C10. In these conditions, transients were partially inhibited (Fig. 10C). This demonstrates that integrin-independent adhesion (on poly-L-lysine in the presence of P4C10) as well as β_1 integrin engagement can both generate $[Ca^{2+}]_i$ transients. Finally, these results clearly confirm that PDGF-BB or other serum components are obligatory triggering elements for the generation of transients during adhesion and migration.

It is known that the binding affinity of some integrins for their substrate is dependent on $[Ca^{2+}]_i$ (Sjaastad et al., 1994). We hypothesize that when VSMC migration is stimulated by serum, $[Ca^{2+}]_i$ transients that are elicited could in turn regulate the affinity of integrins for extracellular matrix proteins. The presence of $[Ca^{2+}]_i$ transients would allow the rapid transition between high and low affinity states of the integrins, which is a crucial mechanism for the migration process (Hughes and Pfaff, 1998). Indeed, locking of integrins in a constitutively high-affinity state inhibits cell migration (Huttenlocher et al., 1996). It was also shown in skeletal muscle fibroblasts that a substantial fraction of integrins are left (ripping-released) on the substratum as the cell detaches and migrates (Palecek et al., 1996). In BAPTA-loaded VSMCs, β_1 integrins were found in increased amounts at the cell tail and on the extracellular matrix (Fig. 11), suggesting an increased 'ripping release' of β_1 integrins when transients are abolished. This is consistent with the observation made on neutrophils migrating on vitronectin, where calcineurin activation by $[Ca^{2+}]_i$ transients induces release of adhesion followed by endocytosis of $\alpha_v\beta_3$ integrins and recycling to the leading edge of the cell (Lawson and Maxfield, 1995).

Using DHCB, we demonstrated that migration of VSMC requires a dynamic regulation of the cytoskeleton, since DHCB dose-dependently inhibited migration (Fig. 6A). However, the rapid cell shape change induced by DHCB did not alter the frequency of $[Ca^{2+}]_i$ transients, as also found previously during migration of neutrophils (Pettit and Hallett, 1996). This would suggest that $[Ca^{2+}]_i$ transients are not merely a consequence of the spreading and forward motion of the cell, because they persist when the cell assumes a rounded shape under the effect of DHCB.

Our results suggest that $[Ca^{2+}]_i$ transients are present in adhering/migrating VSMCs when, and only when, two

events occur simultaneously: (1) β_1 integrin-dependent or -independent spreading and (2) stimulation of the cells by serum components such as PDGF-BB. Clearly, the exact origin and the role(s) of these $[Ca^{2+}]_i$ transients in migrating and adhering VSMCs merit further detailed investigation. It seems likely, however, considering the known dependence of integrin affinity and cytoskeletal organization on calcium (Stossel, 1993), that these transients could be a key regulating signal for migration of VSMCs. This concept is of obvious pathophysiological interest since VSMC migration is a key event in the formation of neointima during vascular proliferative diseases (Ross, 1993).

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