Essential role for calcium waves in migration of human vascular smooth muscle cells

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Submitted 7 April 2010; accepted in final form 9 May 2011

Essential role for calcium waves in migration of human vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 301: H315–H323, 2011. First published May 13, 2011; doi:10.1152/ajpheart.00355.2010.—Vascular smooth muscle cells (SMCs) play a critical role in cardiovascular physiology and have been extensively characterized, including establishing their genome-wide expression profile (8). They express the hallmark contractile apparatus proteins to the front of cells (18). However, the role of calcium signaling in migrating SMCs and can encode cell polarity.

Calcium waves are essential for migration of human vascular SMCs and can encode cell polarity.

Calcium signaling; motility; retraction; calcium oscillations

Cell migration is a complex multistep cellular process that is involved in many physiological phenomena including embryogenesis, organogenesis, immune surveillance, tissue vascularization, and tissue repair (17, 33). Migration of vascular smooth muscle cells (SMCs) in the vessel wall is an important component of vascular remodeling during atherosclerosis (36) and after injury resulting from stent implantation, angioplasty, and organ transplantation (5, 22, 35). An emerging view is that, even in the absence of disease or overt injury, the vessel wall is continually being remodeled, an adaptive process that involves the constant repositioning of SMCs (28).

The cell migration cycle involves polarization of cells and assembly of cytoskeletal and regulatory elements to serve distinct processes (10, 33). The leading edge, once established, is the site of actin filament assembly, with dynamic regulation for protrusion of lamellipodia, and traction on the substrate (32). In concert, the rear of the cell (the uropod) must undergo detachment from the substrate, allowing retraction and net forward movement. Retraction of the uropod involves several processes, including degradation of extracellular matrix substrate (23), endocytosis of adhesion components (3), generation of force by activation of myosin light chain kinase (10), and proteolysis of talin (7).

Rapidly moving cells such as eosinophils, keratocytes, and neutrophils, which tend to move smoothly along surfaces, exhibit a gradient of intracellular cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in coordinating these distinct activities of migrating SMCs. The objective of our study was to determine whether regional changes of Ca\(^{2+}\) orchestrate the migratory cycle in human vascular SMCs. We carried out Ca\(^{2+}\) imaging using digital fluorescence microscopy of fura-2 loaded human smooth muscle cells. We found that motile SMCs exhibited Ca\(^{2+}\) waves that characteristically swept from the rear of polarized cells toward the leading edge. Ca\(^{2+}\) waves were less evident in nonpolarized, stationary cells, although acute stimulation of these SMCs with the agonists platelet-derived growth factor-BB or histamine could elicit transient rise of [Ca\(^{2+}\)]. To investigate a role for Ca\(^{2+}\) waves in the migratory cycle, we loaded cells with the Ca\(^{2+}\) chelator BAPTA, which abolished Ca\(^{2+}\) waves and significantly reduced retraction, supporting a causal role for Ca\(^{2+}\) in initiation of retraction. However, lamellipodial motility was still evident in BAPTA-loaded cells. The incidence of Ca\(^{2+}\) oscillations was reduced when Ca\(^{2+}\) release from intracellular stores was disrupted with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin or by treatment with the inositol 1,4,5-trisphosphate receptor blocker 2-aminophenyl-diphenyl borate or xestospongin C, implicating Ca\(^{2+}\) stores in generation of waves. We conclude that Ca\(^{2+}\) waves are essential for migration of human vascular SMCs and can encode cell polarity.

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cell motility. We demonstrate that Ca\(^{2+}\) waves characteristically sweep from the rear of polarized cells toward the leading edge. Abolition of Ca\(^{2+}\) waves reduces cell retraction and migration. We propose that Ca\(^{2+}\) waves are important in the regulation of smooth muscle motility and encode cell polarity.

METHODS

Cell culture and reagents. Experiments were performed on human vascular smooth muscle cells obtained from explant outgrowth of segments of internal thoracic artery retrieved during coronary artery bypass surgery (24, 25). Tissue collection was carried out in accordance with guidelines of the review board for research involving human subjects at the University of Western Ontario. Cells obtained this way show distinct phenotypes, with clone HITC6 (human internal thoracic C6) expressing smooth muscle molecular markers, responding to contractile agonists by elevation of [Ca\(^{2+}\)], and converting to a contractile phenotype with serum deprivation (8, 24, 25). HITC6 cells exhibit an epithelioid appearance in the presence of serum and are responsive to the migratory effect of PDGF-BB (24). Cells from passages 23 to 33, a period of phenotypic stability and before the onset of senescence (39), were grown in M199 media supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA) and plated on substrate of rat-tail collagen (0.1 ng/ml). PDGF-BB, and dishes were mounted on an inverted fluorescence microscope (Nikon). Cells were imaged using a Plan Fluor 20x/0.45 NA for phase contrast or Plan Fluor 20X/0.75 oil/water immersion objective for fluorescence imaging. Fura-2 was excited with alternating wavelengths of 340/380 nm (16). Images were acquired at 6- to 8-s intervals over 30 min, with cells maintained at 35°C. To minimize evaporation of media, plates were covered with mineral oil or solution was refreshed every 10 min. In some experiments, where indicated, we recorded whole-cell Ca\(^{2+}\) levels in SMCs using a fluorimeter-based ratiometric system, as described in detail elsewhere (16, 24, 25).

Measurements of [Ca\(^{2+}\)], pose technical challenges due to the thin and variable profile of cells and changing shape. The ratio of fluorescence intensity at 340/380 nm gives an index of [Ca\(^{2+}\)]i, and reliably conveys the frequency and time course of Ca\(^{2+}\) transients. However, we did not attempt to calibrate this due to factors such as inhomogeneity of dye and Ca\(^{2+}\) within cells.

Statistics. Results are expressed as means ± SE with error bars in the figures representing SE and n indicating the number of cells, or independent preparations studied, as indicated. Statistical comparisons were made using either ANOVA or Student’s t-test, as indicated in the figure legends, with P < 0.05 taken to indicate significance.

RESULTS

Phases of human vascular SMC migration cycle. HITC6 smooth muscle cells maintained with 10% FBS exhibit a proliferative phenotype characterized by synthesis of matrix proteins and cell motility (24). The SMCs studied here varied between 50 and 200 μm in length and up to 50 μm in width. We focused our attention on polarized SMCs, bathing cells in...
M199 supplemented with FBS and PDGF-BB to enhance motility (25). We used digital time-lapse phase contrast microscopy and characterized HITC6 cells based on stellate appearance and the presence of active extension of lamellipodia at leading edges, coupled with retraction of trailing uropodia at trailing edges (Fig. 1A; Supplemental Movie S1). Polarized SMCs showed ongoing lamellipod ruffling, which gave rise to extension of the leading edge at a rate of ~15 μm/30 min (Fig. 1B). In contrast, retraction of the uropod occurred with abrupt transitions (Fig. 1A). This process is representative of at least three polarized cells per microscope field that retracted during a 30-min recording period, repeated on more than 11 days with different cell preparations, collectively amounting to studying more than 45 cells in bright field microscopy. These distinct, although linked, processes contributed to cell translocation, and we proceeded to use fluorescence microscopy to examine Ca^{2+}.

**Ca^{2+} oscillations and waves in HITC6 cells.** To define the relationship between patterns of Ca^{2+} and the cell migration cycle we carried out ratiometric Ca^{2+} imaging of cells. Diversity in patterns of Ca^{2+} was apparent, with some cells having stable, unchanging [Ca^{2+}]i, and others exhibiting prominent Ca^{2+} oscillations, similar to that reported (37). Figure 2 illustrates a representative cell that was polarized, but where [Ca^{2+}]i was initially steady over time, with minor regional differences apparent. To test responsiveness, the chemotaxin PDGF-BB was applied focally near the leading edge of the cell.
from an application pipette (location shown schematically in Fig. 2B). After a latency of ~60 s, which is longer than required for diffusion of the PDGF across the entire cell, PDGF-BB elicited a brief spike of [Ca²⁺] (Fig. 2A). This indicated that, despite an initial constant level of resting [Ca²⁺], the SMCs remained highly responsive. Closer inspection of the Ca²⁺ response revealed that the rise of [Ca²⁺] was initiated at the uropod of the cell, a site farthest from the point of application, and propagated toward the leading edge of the cell (Fig. 2C). Similar waves of [Ca²⁺] were elicited by PDGF in 17 cells tested, and the polarity of the Ca²⁺ waves is considered in further detail below.

A representative ratio image (340/380 nm) of a larger field of view is illustrated in Fig. 3A, with more than six SMCs in the field, each one evident by a characteristically dark nucleus (low 340-to-380 ratio) in a resting cell. To quantify responses, regions of interest (numbered circles in Fig. 3A) were chosen near the center of cells, avoiding nuclei. Diversity in patterns was apparent in any given image field, with various cells oscillating at different frequencies, as well as some quiescent cells (Fig. 3B). Ca²⁺ transients in neighboring SMCs were not synchronous, suggesting negligible intercellular coupling. The Ca²⁺ transients in Fig. 3B are representative of responses recorded in 187 polarized cells studied on 7 different days and five separate cell preparations. The percentage of cells exhibiting Ca²⁺ transients (at least 1 transient over a 30-min period) was 85 ± 3% (n = 15) for cells bathed with M199 with 10% FBS and PDGF. Omission of PDGF reduced the percentage of cells exhibiting Ca²⁺ transients to 73 ± 6% (n = 13), and reduction of FBS to 1% further reduced the incidence to 44 ± 2% (n = 14; Fig. 3C). Thus the presence of PDGF and FBS influences initiation of Ca²⁺ oscillations.

To test responsiveness of quiescent, nonpolarized cells, agonists were administered and did cause acute increases of [Ca²⁺] in response to histamine (10 μM, n = 7), PDGF-BB (1–5 ng/ml, n = 11), and FGF-2 (25 ng/ml, n = 8). Notably, the agonist-evoked increases in [Ca²⁺] in nonpolarized cells inevitably occurred as global changes, arising simultaneously across the entire cell.
cell (Fig. 3D), a pattern that was distinct from the waves that are the focus of the remainder of this report. When tested in six independent cell preparations over 14 days of recording, PDGF-BB evoked Ca\textsuperscript{2+} waves in 17 of 129 responsive cells, with the remaining 112 cells exhibiting global rise of [Ca\textsuperscript{2+}]. Such responses were comparable with cells plated on collagen coated coverslips, as with uncoated coverslips, suggesting the matrix did not play an essential role in determining the pattern of [Ca\textsuperscript{2+}], transients in HITC6 SMCs.

**Ca\textsuperscript{2+} waves encode polarity in vascular SMCs.** To explore the role of Ca\textsuperscript{2+} in migration, we focused our attention on Ca\textsuperscript{2+} events in individual polarized cells. Basal [Ca\textsuperscript{2+}], as indicated by the 340-to-380 fluorescence ratio, was typically higher in the rear of SMCs (Fig. 4, A–C). When regional patterns were investigated, the oscillations of [Ca\textsuperscript{2+}], occurred as waves, evident as the shift of Ca\textsuperscript{2+} traces in the expanded regions of Fig. 4, B and C. Inspection of 148 cells from 15 experiments, six independent cell preparations, revealed that Ca\textsuperscript{2+} waves inevitably propagated from the rear of polarized cells toward the leading edge (Fig. 4; see also Supplemental Movie S2). The frequency of Ca\textsuperscript{2+} waves varied from 1 to 22 for each 30-min observation period. The differences in Ca\textsuperscript{2+} wave frequency were due in part to the bathing medium, with least variability encountered when cells were maintained in 10% FBS with 10 ng/ml PDGF. Under these conditions, 3–5 Ca\textsuperscript{2+} waves were evident over 30 min in 38% of cells (61 of 163 cells examined, 8 experiments from 4 different cell passages), resulting in an overall frequency of 0.13 Ca\textsuperscript{2+} waves/min.

Ca\textsuperscript{2+} waves originated in the uropod and swept forward smoothly, traversing the nucleus unimpeded. Interestingly, in many cases Ca\textsuperscript{2+} waves did not propagate into the lamellipodia at the leading edge of cells (Fig. 5A, region of interest No. 1 at top at leading edge and ratio No. 1; Fig. 5B, representative of responses in 12 cells), suggesting that compartmentalization of Ca\textsuperscript{2+} can occur. Sequential retraction of the uropodia complicated the presentation of regional changes of [Ca\textsuperscript{2+}], because the regions of interest were moving (uropodia marked in Fig. 5A). Moreover, despite ongoing retraction of the uropodia, Ca\textsuperscript{2+} waves persisted as the cells maintained their polarity and continued their migration (Fig. 5B).

**Essential role for Ca\textsuperscript{2+} waves and oscillations in retraction.** The tight association between Ca\textsuperscript{2+} waves and migratory events led us to hypothesize that Ca\textsuperscript{2+} waves were essential for retraction of uropodia and thus critical elements for encoding cell polarity and cell migration. To test this we first compared the incidence of retraction in cells with and without PDGF-BB, which, as shown above in Fig. 3C, was found to increase the incidence of Ca\textsuperscript{2+} oscillations. A significantly greater percentage of cells retracted during 30-min recordnings in the presence of PDGF (Fig. 6A), supporting a relationship between the two events. Essentially every SMC that retracted also showed Ca\textsuperscript{2+} waves (Fig. 6B). This relationship was maintained irrespective of whether PDGF was present (36 of 36 cells with PDGF and 9 of 12 cells without PDGF). In contrast, cells that did not show Ca\textsuperscript{2+} oscillations showed significantly fewer cases of retraction (<25% of cells; Fig. 6B at right).

To investigate whether Ca\textsuperscript{2+} waves played a causal role in initiating retraction, we loaded SMCs with the Ca\textsuperscript{2+} chelator BAPTA. BAPTA acts as a mobile buffer to attenuate changes in [Ca\textsuperscript{2+}]. When applied to cells (in the membrane-permeant form BAPTA-AM), BAPTA abolished Ca\textsuperscript{2+} oscillations (representative traces, Fig. 6, C and D; quantification, Fig. 6E), which was accompanied by a reduction in the basal calcium levels. Notably, this resulted in significant reduction in the incidence of retraction (Fig. 6F; P < 0.05), supporting the model that Ca\textsuperscript{2+} elevation is an important element mediating cell retraction.

To test for sources of Ca\textsuperscript{2+}, we added the Ca\textsuperscript{2+}-channel blocker nifedipine (1 \mu M), but did not detect delay or inhibition of uropod retraction (84 cells from 4 different plates and 2 cell passages; 30%, 25 cells of 84 retracted; values similar to control in Fig. 6A). This suggested that Ca\textsuperscript{2+} waves were mediated by release from intracellular stores or Ca\textsuperscript{2+} entry through pathways distinct from L-type Ca\textsuperscript{2+} channels (19). Interestingly, in the course of imaging we found that lamellipod motility persisted at the leading edges after BAPTA loading or addition of nifedipine.

To explore the role of intracellular sources of Ca\textsuperscript{2+}, we recorded the frequency of Ca\textsuperscript{2+} oscillations in four to six image fields, first as a control and then 2 to 5 min after addition of test
agents. Treatment of SMCs with thapsigargin (2 μM), to block the sarcoplasmic reticulum Ca\(^{2+}\) ATPase, abolished Ca\(^{2+}\) oscillations (frequency of oscillations reduced from 55% of cells in control fields to 0%; \(P < 0.001\); repeated on 4 days). Furthermore, the membrane-permeant IP\(_3\) receptor blocker 2-aminoethoxydiphenyl borate (2-APB; 75 μM) also caused significant suppression of the incidence of Ca\(^{2+}\) oscillations (frequency of oscillations reduced from 57% in control to 14%; \(P < 0.001\); repeated on 3 days), supporting a role for IP\(_3\)-regulated Ca\(^{2+}\) stores in migration. However, 2-APB is reported to block a number of other channel types in vascular smooth muscle (e.g., Ref. 26), so we further examined the effects of the more selective IP\(_3\) receptor blocker xestospongin C (9). We first carried out control experiments to validate the effect of xestospongin C on global rise of calcium elicited by PDGF. For these experiments we used photometry-based fluorescence recording, which enabled longer-term recordings. Cells reproducibly responded to PDGF with a transient rise of [Ca\(^{2+}\)], as described above, which was abolished with focal application of xestospongin C (25 μM) from a micropipette (new Fig. 7A). These effects were reversible (Fig. 7A at right; replicated in 13 cells). Having established that this protocol effectively blocked IP\(_3\) receptor-mediated events, we next used fluorescence imaging to examine SMCs exhibiting spontaneous Ca\(^{2+}\) oscillations and waves. Addition of xestospongin C caused reduction or abolition of the Ca\(^{2+}\) events in 26 of 29 cells studied (Fig. 7B; 4 different cell preparations), supporting the involvement of intracellular Ca\(^{2+}\) stores in the oscillations. Gene expression profiling of HITC6 SMCs has been carried out using Affymetrix U133 human arrays, and analysis of those data using MAS 5.0 (Affymetrix) and GeneSpring (Silicon Genetics) reveals that these cells express the type 1 and type 2 IP\(_3\) receptors (supplemental material accompanying Ref. 8). Taken together, our studies reveal that Ca\(^{2+}\) has specific roles in controlling SMC polarity and motility, controlling uropod retraction but not lamellipodial extension.

**DISCUSSION**

We have found that: 1) Ca\(^{2+}\) waves are an important element of human vascular SMC migration; 2) Ca\(^{2+}\) waves...
have directionality, sweeping along the migrating cell from uropod toward the leading edge; 3) Ca\(^{2+}\) waves may be excluded from the lamellipodia of the leading edge; and 4) Ca\(^{2+}\) required for SMC migration comes from IP3-sensitive Ca\(^{2+}\) stores. We conclude that regional patterns of Ca\(^{2+}\) encode SMC polarity and motility, a level of control not previously recognized in smooth muscle.

The migratory cycle requires polarization of cells, protrusion of lamellipodia, and retraction of uropodia (17, 33), steps that are not always distinct in rapidly migrating cells such as neutrophils and eosinophils (2, 18). However, vascular SMCs are larger and are considered slow migrators (23), so the different components are readily distinguishable in time and space. This has allowed characterization of distinct processes contributing to migration of HIT SMCs, including signaling contributing to lamellipodial protrusion (6) as well as detachment of uropodia from substrate (23).

The presence of Ca\(^{2+}\) oscillations in cultured SMCs has been reported. Maintaining the HITC6 SMC in FBS with PDGF-BB increased the frequency with which we observed Ca\(^{2+}\) oscillations, conditions similar to that reported earlier (37), emphasizing the importance of growth factors in initiating the oscillations. Indeed, the correlation between the presence of Ca\(^{2+}\) oscillations and migration of cells is reported (11, 27). Al-Although blocking of Ca\(^{2+}\) oscillations with BAPTA and/or Ca\(^{2+}\)-free solutions diminished or abolished migration (27), no imaging of the cells was carried out to confirm the presence of Ca\(^{2+}\) waves, as demonstrated here. A limitation of the BAPTA studies was that basal Ca\(^{2+}\) levels also declined with abolition of oscillations, supporting a critical role for Ca\(^{2+}\) but making it difficult to establish unequivocally the cause of the reduced motility we observed.

The way in which Ca\(^{2+}\) oscillations encode information to determine cell polarity, and to coordinate cell migration, is not understood. Elevation of [Ca\(^{2+}\)], at the uropod is essential for disassembly of adhesion complexes and initiation of retraction, involving Ca\(^{2+}\)-dependent effectors such as myosin II, calpain, and calcineurin (14, 18, 41). Oscillations of Ca\(^{2+}\) alone could be sufficient to initiate retraction, since the tail end of the cell would only see periodic increases. However, we speculate that the antegrade sweep of [Ca\(^{2+}\)], ensures that uropod retraction proceeds in an optimally coordinated manner. This may be especially important for strongly adherent cells such as SMCs because the contractile force required to retract the tail has the capacity to rip the cell (23). By ensuring that substrate release proceeds from back to front, the Ca\(^{2+}\) wave may minimize plasma membrane tearing and facilitate forward gliding. Potential targets for Ca\(^{2+}\) include the Ca\(^{2+}\)-regulated phosphatase calcineurin or the Ca\(^{2+}\)-activated protease calpain (4) as well as activation or expression of matrix metalloproteinases (21). It is interesting that the cellular localization of type 1 IP3 receptor has recently been shown to play a crucial role in regulating arterial muscle contraction (1). It would be of interest to determine whether there was any notable spatial organization or dynamic reorganization of IP3 receptors in migrating smooth muscle cells.

Activation of PDGF receptors initiates diverse signaling elements, including phospholipase C\(\gamma\), release of Ca\(^{2+}\) from intracellular stores, and phosphatidylinositol 3-kinase (15). PDGF receptors themselves undergo translocation, with initial distribution evenly about arterial SMCs, then translocating on stimulation with PDGF to perinuclear regions, in a RhOB-dependent manner (12). Integration of the signals, as well as other downstream mediators (13), may account for the latency in the Ca\(^{2+}\) elevation we observed in response to PDGF-BB, as well as any delay from rise of Ca\(^{2+}\) to migration.

Ca\(^{2+}\) also regulates a number of actin severing proteins, such as gelsolin, which are localized to the leading lamellipodia of migrating cells (33). Although we found that Ca\(^{2+}\) waves often failed to invade the lamellipodia of SMCs, other events may coordinate remodeling at the leading edge. The recent discovery of Ca\(^{2+}\) flickers in lamellipodia of fibroblasts illustrates a way in which spatio-temporal signaling by Ca\(^{2+}\) can direct migration (40). Ca\(^{2+}\) flickers are activated in response to chemoattractant signaling, but also by increases of membrane tension, a process thought to involve activation of a Ca\(^{2+}\)-permeable channel of the transient receptor potential family (40). The local rise of [Ca\(^{2+}\)], orchestrates cell motility and turning. The discovery of spatially restricted rise of Ca\(^{2+}\) may explain how one signal can carry out so many actions, from regulating actin polymerization and other Ca\(^{2+}\)-dependent paths to initiating turning and lamellipodial extension (29). Perhaps due to the restricted sampling rate of ratio images in this study, we did not detect Ca\(^{2+}\) flickers in lamellipodia of vascular SMC, although PDGF-BB is an established chemotaxin for these cells (31). Moreover, the extremely thin profile of the SMC leading edge resulted in very low levels of fluorescent dyes, challenging the limits of Ca\(^{2+}\) imaging in the present study. However, further effort is warranted using more sensitive cameras and faster imaging rates.
In summary, we report that motile SMCs exhibit spontaneous Ca\textsuperscript{2+} waves that characteristically sweep from the rear of polarized cells toward the leading edge. Blocking Ca\textsuperscript{2+} waves with a Ca\textsuperscript{2+} chelator significantly reduced retraction, supporting a causal role for Ca\textsuperscript{2+} in initiation of retraction. We propose that Ca\textsuperscript{2+} waves are important in the regulation of smooth muscle motility and encode cell polarity.

ACKNOWLEDGMENTS

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GRANTS

We thank The Canadian Institutes of Health Research (Grants 10019 and 11715) and the Canada Foundation for Innovation (Project No. 5651) for support of these studies. R. Espinosa-Tanguma was supported by CONACYT (Grant 62220). J. G. Pickering was supported with a Career Investigator award (Grant 62220) and the Canada Foundation for Innovation (Project No. 5651) for its support. S. L. P. Mexico, 78210.

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