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Mechanism of calcium oscillations in migrating human astrocytoma cells

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Abstract

Numerous studies show that intracellular calcium controls the migration rate of different mobile cell types. We studied migrating astrocytoma cells from two human cell lines, U-87MG and A172, in order to clarify the mechanisms by which calcium potentially influences cell migration. Using the wound-healing model to assay migration, we showed that four distinct components of migration could be distinguished: (i) a Ca^{2+}/serum-dependent process; (ii) a Ca^{2+}-dependent/serum-independent process; (iii) a Ca^{2+}/serum-independent process; (iv) a Ca^{2+}-independent/serum-dependent process. In U-87MG cells which lack a Ca^{2+}-dependent/serum-independent component, we found that intracellular Ca^{2+} oscillations are involved in Ca^{2+}-dependent migration. Removing extracellular Ca^{2+} greatly decreased the frequency of migration-associated Ca^{2+} oscillations. Furthermore, non-selective inhibition of Ca^{2+} channels by heavy metals such as Cd^{2+} or La^{3+} almost completely abolished changes in intracellular Ca^{2+} observed during migration, indicating an essential role for Ca^{2+} channels in the generation of these Ca^{2+} oscillations. However, specific blockers of voltage-gated Ca^{2+} channels, including nitrendipine, ω-conotoxin GVIA, ω-conotoxin MVIIC or low concentrations of Ni^{2+} were without effect on Ca^{2+} oscillations. We examined the role of internal Ca^{2+} stores, showing that thapsigargin-sensitive Ca^{2+} stores and $InsP_3$ receptors are involved in Ca^{2+} oscillations, unlike ryanodine-sensitive Ca^{2+} stores. Detailed analysis of the spatio-temporal aspect of the Ca^{2+} oscillations revealed the existence of Ca^{2+} waves initiated at the leading cell edge which propagate throughout the cell. Previously, we have shown that the frequency of Ca^{2+} oscillations was reduced in the presence of inhibitory antibodies directed against β3 integrin subunits. A simple model of a Ca^{2+} oscillator is proposed, which may explain how the generation of Ca^{2+} oscillations is linked to cell migration.

Keywords: Migration; Calcium oscillation; Astrocytoma; Thapsigargin; Ryanodine; Integrin

1. Introduction

Transmembrane integrins bind to extracellular matrix proteins and generate signals that regulate cell proliferation and migration [1]. Several signaling pathways are implicated in the modulation of cell migration, including integrin-mediated intracellular Ca^{2+} signaling [2]. Crosstalk between integrins and growth factor receptors may occur via direct proximal clustering [3–5] or activation of common signaling pathways [6,7]. The mechanisms coupling integrin ligation to intracellular Ca^{2+} signaling have proved difficult to fully characterize [2]. Integrin-mediated Ca^{2+} signaling has been linked to tyrosine kinase activity and PLC-γ activation [8]. Integrin-dependent modulation of plasma membrane ionic

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channel activity has also been reported [9,10]. The complex spatio-temporal aspects of Ca^{2+} signaling are well suited for the modulation of a highly coordinated process such as migration which requires an asymmetric regulation of cell adhesion, with formation/strengthening at the front and disassembly/weakening at the rear of the cell. Oscillations of intracellular Ca^{2+} modulate neuronal migration [11]. Notably, changes in internal Ca^{2+} appear to be responsible for persistent forward migration of neutrophils [12]. Migrating fibroblasts have an increasing gradient of Ca^{2+} from the front to the rear of the cell [13]. In fish epithelial keratinocytes, activation of stretch-activated Ca^{2+}-permeable channels is involved in the detachment of the cell rear [14].

Recent studies from our laboratory show that U-87MG astrocytoma cells present a Ca^{2+}/serum-dependent migration [15]. In this study, using two different astrocytoma cell lines, we show that at least four cell migration processes exist, namely: (i) a Ca^{2+}/serum-dependent process; (ii) a Ca^{2+}-dependent/serum-independent process; (iii) a Ca^{2+}/serum-independent process; (iv) a Ca^{2+}-independent/serum-dependent process. As the U-87MG cell line exhibits only a Ca^{2+}-dependent cell migration process that is serum-dependent, we focus on this cell line to analyze the molecular mechanisms of Ca^{2+} in cell migration. We have previously shown that Ca^{2+} oscillations are probably involved in this process. Here, we identify the Ca^{2+} pools involved in the generation of Ca^{2+} oscillations and discuss the possible role of $InsP_3$.

2. Materials and methods

2.1. Materials

Cell culture medium (EMEM), fetal calf serum (FCS), HEPES, L-glutamine, penicillin, streptomycin, gentamicin and trypsin-EDTA solution (0.5 g/l trypsin/0.2 g/l EDTA) were from Gibco. Thapsigargin, ryanodine, cadmium, lanthanum, nickel and nitrendipine were from Sigma. ω-Conotoxin GVIA and ω-conotoxin MVIIC were from Alamone. Oregon green 488 BAPTA-1 acetoxylmethylester, BAPTA-acetoxylmethylester (BAPTA/AM) and pluronic acid F-127 were from Molecular Probes.

2.2. Cell culture

The human astrocytoma cell lines U-87MG and A172 were obtained from the American Type Culture Collection. Cells were maintained in 5% CO_2 in air at 37°C in a humidified incubator on type I collagen (0.06 mg/ml) coated plastic dishes in EMEM supplemented with 10% heat-inactivated FCS, 0.6 mg/ml glutamine, 200 IU/ml penicillin, 200 IU/ml streptomycin and 0.1 mg/ml gentamicin.

2.3. Migration assay

U-87MG or A172 cells were seeded onto 35 mm diameter Petri dishes coated with Matrigel (178 μg/ml) and grown to confluence in a 37°C incubator gassed with 5% CO_2 in air. After 24 h of serum starvation, a rectangular lesion was created using a cell scraper [16] and cells were rinsed three times with culture medium containing or not 10% FCS. The cells were then incubated with the respective experimental medium supplemented or not with the compound to be tested. After 24 h of migration, three randomly selected fields at the lesion border were acquired using a 10× phase objective on an inverted microscope (Olympus IMT2) 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 ten most mobile cells. Analysis was made using Image Tool software (University of Texas Health Science Center at San Antonio; available by FTP from maxrad6.uthscsa.edu). For experiments with BAPTA/AM, cells were loaded for 45 min with 20 μM BAPTA/AM and 0.03% pluronic acid F-127 in a 37°C incubator gassed with 5% CO_2 in air prior to the creation of lesions.

2.4. Cytosolic free calcium measurements during migration

For intracellular Ca^{2+} measurements during migration, cells were cultured at subconfluence on 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 coated with Matrigel. Experiments were performed 48 h or 72 h after plating. Cells were incubated for 45 min with the fluorescent Ca^{2+} indica-

tor Oregon green 488 BAPTA-1 acetoxylmethylester (5 μM) in culture medium containing 0.03% pluronic acid F-127 in a 37°C incubator gassed with 5% CO_2 in air. Cells were then washed twice with an external solution (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 11 glucose, pH 7.4) before Ca^{2+} measurements. Imaging was done at 30°C in external solution, with or without the compounds to be tested, using a Bio-Rad MRC-1024 laser-scanning confocal system and an inverted microscope (Nikon Eclipse) using a 40× oil immersion epifluorescence objective (n.a. 1.4, Nikon). Emitted fluorescence was measured at 535 ± 10 nm in response to 488 nm excitation from a krypton/argon laser, with images being usually acquired at 1 s intervals during a 15 min period.

3. Results and discussion

3.1. Role of Ca^{2+} and serum in cell migration of two astrocytoma cell lines

We investigated whether the speed of migration is dependent upon changes in intracellular Ca^{2+} in two well-defined human astrocytoma cell lines, U-87MG and A172. Migration was assessed using the wound-healing model. Cells were allowed to migrate for a 24 h period after making a lesion, with or without preloading the cells with the permeant Ca^{2+} chelator BAPTA/AM. As shown in Fig. 1, the rate of migration of U-87MG cells on Matrigel-coated dishes was 482 ± 3 μm/24 h in the presence of 10% serum and 238 ± 4 μm/24 h in the absence of serum whereas the rate of migration of A172 cells was 463 ± 4 μm/24 h and 347 ± 6 μm/24 h in the presence and absence of serum respectively. This serum-dependent stimulation of migration may involve changes in intracellular levels of Ca^{2+}. Indeed, in U-87MG cells, buffering the intracellular Ca^{2+} level with BAPTA inhibited serum-dependent migration by 55% but was without effect on the serum-independent component (Fig. 1). On the contrary, in A172 cells, in the presence of BAPTA, both the serum-dependent and serum-independent components of migration were inhibited by 30% and 39% respectively (Fig. 1). Taken together, this suggests that migration in these astrocytoma cell lines is controlled by four distinct molecular mechanisms: (i) a Ca^{2+}- and serum-dependent process, (ii) a Ca^{2+}-dependent/serum-independent process, (iii) a Ca^{2+}- and serum-independent

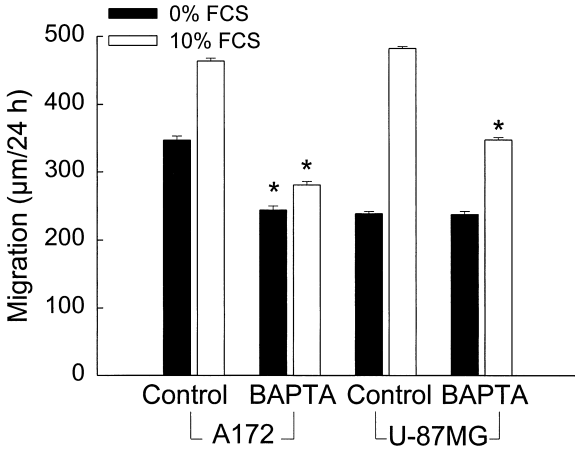

Fig. 1. Effects of serum and chelating intracellular Ca^{2+} on the rate of migration of U-87MG and A172 astrocytoma cells. Migration was assessed 24 h after lesion using the wound-healing cell culture model in the presence or absence of 10% fetal calf serum (FCS) in the culture medium. To test for an effect of intracellular Ca^{2+}, cells were loaded for 30 min with 20 μM of the intracellular Ca^{2+} chelator BAPTA/AM. The results are expressed as the mean ± S.E.M. of 220–485 cells from at least three independent experiments. $*P < 0.01$, Student's *t*-test.

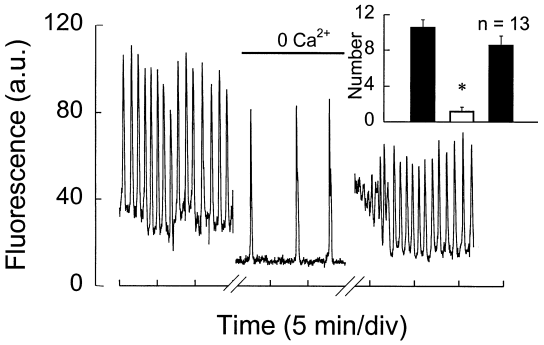

Fig. 2. Migration-associated spontaneous intracellular Ca^{2+} transients are inhibited by removal of extracellular Ca^{2+}. Calcium imaging of migrating U-87MG cells plated on Matrigel-coated glass coverslips and loaded with Oregon green (5 μM) was done on a confocal microscope. After 15 min in normal external solution, a solution containing no Ca^{2+} (inter alia 2 mM EGTA) was perfused for 30 min. Histograms represent the average number of Ca^{2+} spikes before, during and after removal of external Ca^{2+}. The results are expressed as the mean ± S.E.M. of 13 cells from three independent experiments. Fluorescence intensities are given in arbitrary units (a.u.). $*P < 0.01$, Student's *t*-test.

process and (iv) a Ca^{2+}-independent/serum-dependent process.

3.2. Role of serum in the generation of Ca^{2+} oscillations

We next focused on the U-87MG cell line to analyze how changes in internal Ca^{2+} are involved in serum-dependent migration, as in these cells, the absence of a Ca^{2+}-dependent/serum independent component facilitates the analysis of the underlying molecular processes. Using confocal microscopy, Ca^{2+} imaging with the fluorescent probe Oregon green was carried out during migration. We have previously found [15] that in migrating U-87MG cells, Ca^{2+} transients were observed only in the presence of serum (Fig. 2). It is known that such Ca^{2+} oscillations occur during migration in a wide variety of cell types including granule cells, neutrophils, smooth muscle cells and growth cones of neurons [11,12,16,17]. However, the relationship between Ca^{2+} oscillations and the rate of migration appears to be both context- and cell type-specific. For example, in cerebellar granule cells, the speed of migration was positively correlated with both the amplitude and the frequency of the Ca^{2+} spikes [11] whereas during axon outgrowth, normal growth cone motility is reduced during phases of high frequency Ca^{2+} oscillations.

Previously, we also demonstrated that the generation of Ca^{2+} transients in migrating U-87MG cells was inhibited by preincubating the cells with tyrosine kinase inhibitors such as tyrphostin 23 and tyrphostin 47 [15]. Taken together with the serum dependence of migration (Fig. 1), we hypothesized that growth factors contained in serum might play a role in the generation of Ca^{2+} transients. This has already been demonstrated in other systems where growth factors stimulate Ca^{2+}-dependent signaling pathways [16,18]. Thus, we tested several growth factors in serum-free external solution to analyze whether they were able to mimic the effect of serum with respect to the generation of Ca^{2+} transients. Application of none of epidermal growth factor (EGF), platelet-derived growth factor (PDGF-BB) and insulin reproduced the Ca^{2+} signaling observed in the presence of serum in U-87MG cells; this was also the case for EGF+PDGF and EGF+insulin. While spontaneous changes in Ca^{2+} were observed for all three compounds and the two combinations tested, the frequency of these Ca^{2+} transients was clearly reduced, by approx. 75% as compared to serum (Table 1). Although other growth factors such as nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) might be involved in both serum-dependent phenomena (migration, Ca^{2+} signaling), alternative pathways might exist. For example, recently it has been suggested that the Ca^{2+}-binding protein S100A6 which is present in a subpopulation of astrocytes, might alter Ca^{2+} homeostasis [19]. Moreover, addition of S100A4 proteins to the culture medium was shown to stimulate the migration rate of astrocytic tumor cells [20]. These findings suggest that proteins of the S100 family might participate in the Ca^{2+}-dependent migration process observed in our system and warrant further investigations.

3.3. External Ca^{2+} is involved in the generation of Ca^{2+} oscillations

We next investigated the sources of Ca^{2+} responsible for the Ca^{2+} oscillations observed in migrating U-87MG cells. When the normal external solution was replaced by a Ca^{2+}-free solution (containing 2 mM EGTA), the frequency of Ca^{2+} transients was greatly reduced in all cells tested (Fig. 2). In

Table 1
Effects of various compounds on spontaneous Ca^{2+} oscillations in migrating U-87MG astrocytoma cells

Substance	Number of Ca^{2+} spikes
No FCS	0
10% FCS	++++
EGF (0.1–50 ng/ml)	+
PDGF (0.1–50 ng/ml)	+
Insulin (0.1–10 nM)	+
EGF (50 ng/ml)+PDGF (50 ng/ml)	+
EGF (50 ng/ml)+insulin (10 nM)	+

After 24 h of serum starvation, Ca^{2+} imaging was done (see legend to Fig. 2). Measurements of changes in $[Ca^{2+}]_i$ were made in the presence of the indicated compounds and the number of Ca^{2+} transients analyzed over a 15 min period. No Ca^{2+} oscillations were observed in the absence of serum (FCS). In the presence of EGF, PDGF and insulin, a decrease of 75% in the number of Ca^{2+} spikes was observed, compared to serum. A combination of EGF+PDGF or insulin was also ineffective. At least 50 cells were measured for each experimental condition.

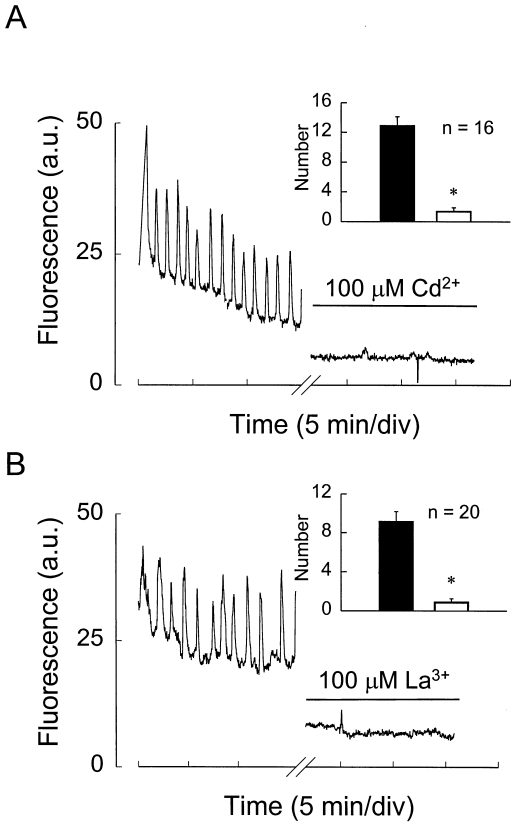

Fig. 3. The inorganic Ca^{2+} channel blockers Cd^{2+} and La^{3+} inhibit spontaneous Ca^{2+} transients in migrating U-87MG cells. Cells were labeled with Oregon green as described in the legend of Fig. 2. At the times indicated, Cd^{2+}- or La^{3+}-containing solutions were bath-perfused. Histograms represent the average number of Ca^{2+} spikes before and after addition of heavy metals and are expressed as the mean ± S.E.M. of 16–20 cells from at least three independent experiments. Fluorescence intensities are given in arbitrary units (a.u.). $*P < 0.01$, Student's *t*-test.

54% of the cells analyzed, Ca^{2+} transients were completely abolished, and in the remaining 46%, the number of Ca^{2+} transients was reduced by 70–90%, without a change in Ca^{2+} spike amplitude. Remarkably, upon re-introduction of normal external solution containing 2 mM Ca^{2+}, the frequency of Ca^{2+} transients was re-established in a quasi-instantaneous manner. This result clearly demonstrates that external Ca^{2+} and very likely, Ca^{2+} influx are necessary for high frequency Ca^{2+} oscillations and suggests the involvement of Ca^{2+} channels. Indeed, application of 100 μM Cd^{2+} and 100 μM La^{3+}, non-selective inorganic Ca^{2+} channel blockers, produced almost complete block of Ca^{2+} transients. Both compounds were equipotent, with the number of calcium spikes being reduced by 90% (Fig. 3). In an attempt to define more precisely the type of Ca^{2+} channel responsible for Ca^{2+} influx and the associated oscillations in intracellular Ca^{2+}, we tested several selective inhibitors of different types of voltage-gated Ca^{2+} channels. Neither the frequency nor the amplitude of Ca^{2+} transients in migrating U-87MG cells was affected (data not shown) by application of 10 μM nitrendipine (L-type Ca^{2+} channel blocker), 100 μM ω-conotoxin GVIA (N-type Ca^{2+} channel blocker), 5 μM

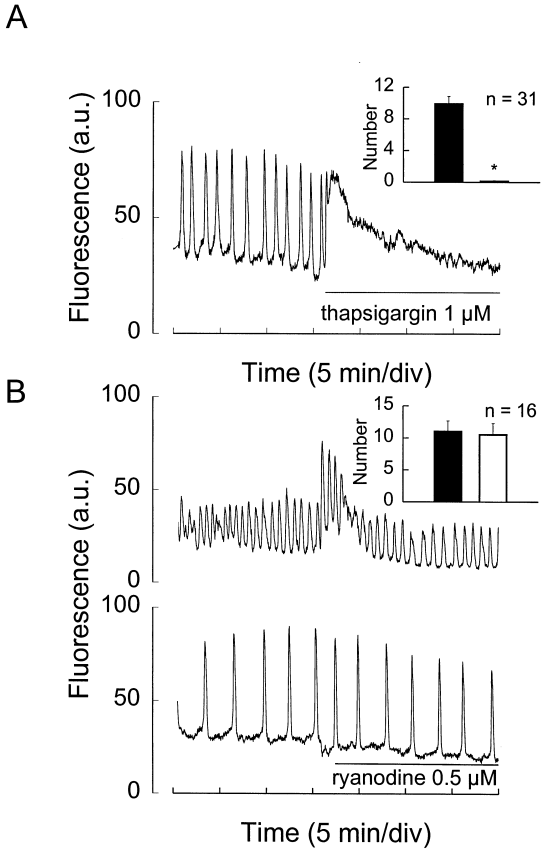

Fig. 4. Analysis of the contribution of internal Ca^{2+} stores on spontaneous Ca^{2+} transients in migrating U-87MG cells. At the times indicated, thapsigargin- or ryanodine-containing solutions were bath-perfused. (A) Thapsigargin provoked an increase in basal Ca^{2+}, in agreement with the emptying of internal Ca^{2+} stores, followed by complete block of Ca^{2+} oscillations. (B) Addition of ryanodine induced a small increase in basal Ca^{2+} in 30% of the cells (upper trace) and was without effect in 70% of the cells (lower trace). Note that Ca^{2+} oscillations were not affected by ryanodine. Histograms represent the average number of Ca^{2+} spikes before and after treatments and are expressed as the mean ± S.E.M. of 16–31 cells from at least three independent experiments. Fluorescence intensities are given in arbitrary units (a.u.). $*P < 0.01$, Student's *t*-test.

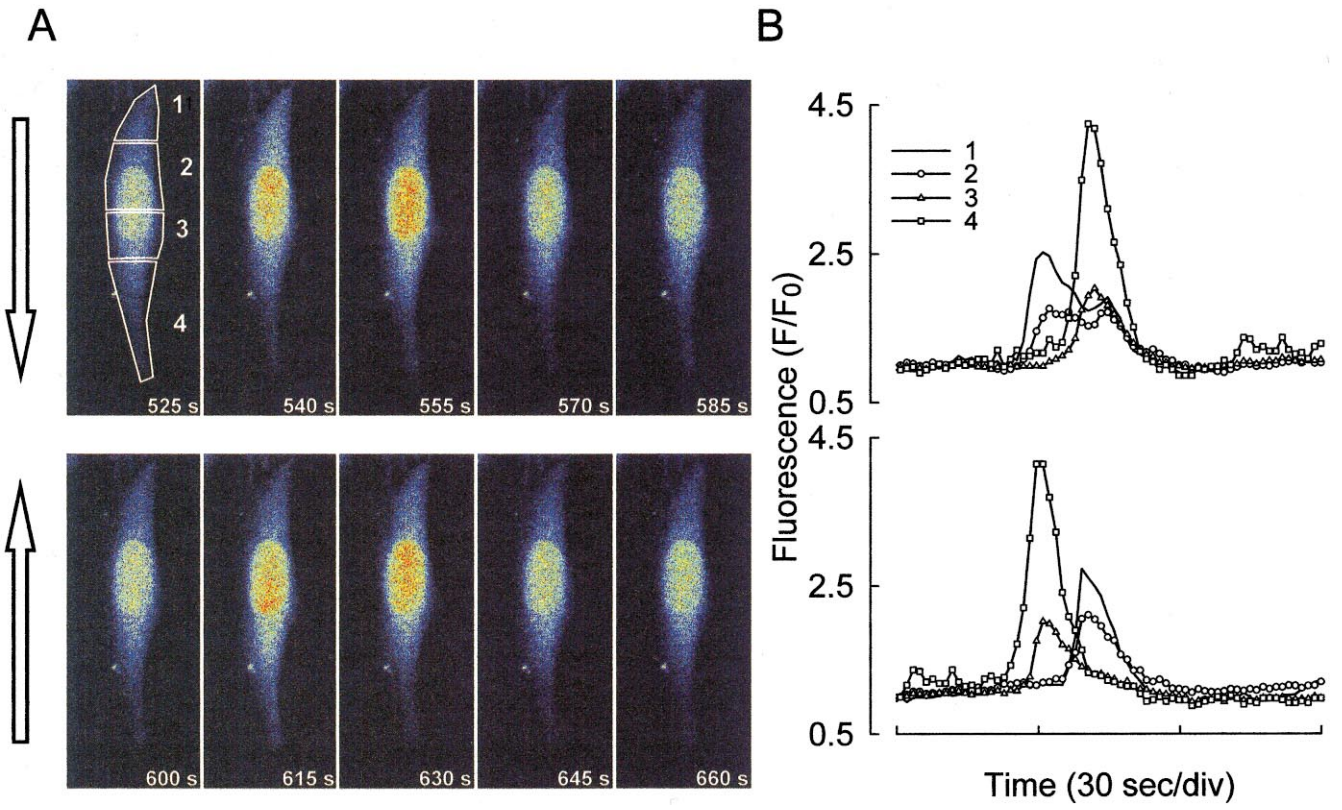

Fig. 5. Spatio-temporal analysis of spontaneous Ca^{2+} transients in migrating U-87MG cells. (A) Sequential view of images taken every 15 s show a Ca^{2+} oscillation initiated at the leading edge (region 1) of the cell which propagates throughout the whole cell (towards the rear of cell, region 4). The Ca^{2+} wave then reverses direction. Arrows indicate the direction of the Ca^{2+} wave propagation. (B) Analysis of Ca^{2+} changes in the four regions of interest defined in A. All fluorescence intensities F over time have been divided pixel-by-pixel by F_0, the basal fluorescence observed at $t=0$.

ω-conotoxin MVIIC (P-/Q-type Ca^{2+} channel blocker) or 100 μM Ni^{2+} (T-type Ca^{2+} channel blocker). Clearly additional experiments are needed to precisely identify the molecular basis underlying Ca^{2+} influx necessary for the Ca^{2+} oscillations observed in migrating U-87MG cells.

3.4. *$InsP_3$ and internal Ca^{2+} stores are involved in the generation of Ca^{2+} oscillations*

The role of the internal Ca^{2+} stores in the Ca^{2+} oscillations was explored using pharmacological compounds against ryanodine- and $InsP_3$-sensitive internal Ca^{2+} stores. Addition of 1 μM thapsigargin, a highly potent inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, induced an initial sharp increase in $[Ca^{2+}]_i$, consistent with internal stores being released, and thereafter completely abolished Ca^{2+} oscillations. In contrast, in 65% of the cells analyzed, addition of 0.5 μM ryanodine had no effect on either the amplitude or the frequency of Ca^{2+} oscillations (Fig. 4B, lower trace), suggesting that ryanodine-sensitive internal Ca^{2+} stores are not involved. In the other 35% of cells, ryanodine induced an initial increase of the basal level of $[Ca^{2+}]_i$ (maximally, 2-fold) for 4–6 min followed by a return to baseline, consistent with release of Ca^{2+} from internal stores, but subsequently was without effect on both the amplitude and frequency of Ca^{2+} oscillations (Fig. 4B, upper trace). Together with our previous finding that inhibition of phospholipase C, the enzyme responsible for the generation of $InsP_3$, by application of 10 μM U73122 reduced the number of Ca^{2+} spikes by 60% compared to control values [15], our data indicate an essential role for $InsP_3$-sensitive internal Ca^{2+} stores present in endoplasmic reticulum in the generation of spontaneous Ca^{2+} oscillations in migrating U-87MG cells.

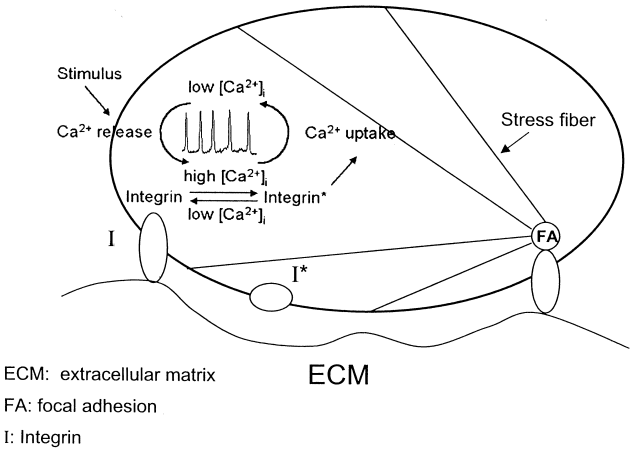

Fig. 6. A simple Ca^{2+} oscillator model of cell migration. Increases in Ca^{2+} cause integrins to switch to a low affinity state for extracellular matrix ligands, thereby promoting migration.

3.5. Ca^{2+} oscillations are propagated Ca^{2+} waves

Cytosolic oscillations in Ca^{2+} are well-known phenomena that require complex temporal co-ordination between plasmalemmal and endoplasmic Ca^{2+} channels responsible for increases in $[Ca^{2+}]_i$ and the various mechanisms underlying decreases in Ca^{2+} (uptake, extrusion, Ca^{2+}-dependent inactivation). In addition to this temporal aspect of Ca^{2+} signaling, it is known that $InsP_3$ receptors participate in the spatial propagation of Ca^{2+} signals (or Ca^{2+} waves) in many non-excitable cells [21]. We found evidence for such Ca^{2+} wave behavior in migrating U-87MG cells as illustrated in Fig. 5. Transient increases in Ca^{2+} are initiated at one edge of the cell, propagate to engulf the whole cell within 1 min and then 'back-propagate' in the opposite direction in cyclical manner. Since changes in Ca^{2+} levels can induce conformational relaxation of integrins [22], the observed Ca^{2+} wave behavior may allow spatio-temporal co-ordination of integrin affinity for extracellular matrix ligands, thereby representing a possible link between Ca^{2+} oscillations and cellular motility, as discussed below.

4. Conclusion

Previously, we have shown that inhibitory antibodies directed against the β3 integrin subunit specifically decrease serum-induced Ca^{2+} transients in migrating U-87MG cells [15]. Based on those results, we proposed a schematic model of a Ca^{2+} oscillator (Fig. 6) that might account for the Ca^{2+}-dependent component of migration. This oscillator model links changes in intracellular $[Ca^{2+}]$ to cyclic modifications of integrin conformation (I and I*) and has four components: (i) a state of elevated intracellular $[Ca^{2+}]$ resulting from Ca^{2+} release from internal stores and Ca^{2+} entry via non-voltage-dependent Ca^{2+} channels; (ii) a state of low intracellular $[Ca^{2+}]$ due to uptake, extrusion and/or binding to calciproteins; (iii and iv) a Ca^{2+}-sensitive protein which oscillates between two conformational states as a function of $[Ca^{2+}]_i$. In our case, the Ca^{2+}-sensitive oscillator is represented by integrins. We hypothesize that increases in $[Ca^{2+}]_i$ induce either directly or indirectly the conversion of integrins from a high affinity (I) to a low affinity state (I*) for extracellular matrix ligands, accompanied by a disassembly of focal adhesions. This then would promote cell motility. The low affinity state of integrins in turn would contribute to a decrease in $[Ca^{2+}]_i$, by yet undefined mechanisms. In each step of this cycling process, regulatory elements, for example $InsP_3$ concentration, could modify the frequency and/or amplitude of this oscillator.

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