Changes in cell shape, cytoskeletal proteins and adhesion sites of cultured cells after extracellular Ca²⁺ chelation

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Abstract

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Although much is known about the molecules involved in extracellular Ca²⁺ regulation, the relationship of the ion with overall cell morphology is not understood. The objective of the present study was to determine the effect of the Ca²⁺ chelator EGTA on the major cytoskeleton components, at integrin-containing adhesion sites, and their consequences on cell shape. Control mouse cell line C2C12 has a well-spread morphology with long stress fibers running in many different directions, as detected by fluorescence microscopy using rhodamine-phalloidin. In contrast, cells treated with EGTA (1.75 mM in culture medium) for 24 h became bipolar and showed less stress fibers running in one major direction. The adhesion plaque protein α_5-integrin was detected by immunofluorescence microscopy at fibrillar adhesion sites in both control and treated cells, whereas a dense labeling was seen only inside treated cells. Microtubules shifted from a radial arrangement in control cells to a longitudinal distribution in EGTA-treated cells, as analyzed by immunofluorescence microscopy. Desmin intermediate filaments were detected by immunofluorescence microscopy in a fragmented network dispersed within the entire cytoplasm in EGTA-treated cells, whereas a dense network was seen in the whole cytoplasm of control cells. The present results suggest that the role of extracellular Ca²⁺ in the regulation of C2C12 cell shape can be mediated by actin-containing stress fibers and microtubules and by intermediate filament reorganization, which may involve integrin adhesion sites.

Probably one of the most studied and yet less understood processes in cell biology is the generation and maintenance of cell shape. Cell shape changes are mediated by proteins of the cytoskeleton (microfilaments, microtubules and intermediate filaments), cell surface receptors and molecules of the extracellular matrix. Many extracellular factors, such as extracellular Ca²⁺, can initiate a cascade of events that will lead to changes in the membrane-cytoskeleton interface.

The main actin-containing structures present in cultured cells are stress fibers, which are periodical cytoskeleton cables composed

mainly of long bundles of filamentous actin, which often extend the length of a cultured cell, giving it tension and modifying its shape (1). These fibers interact at focal adhesion contact, sites of cell attachment to the extracellular matrix (2) via specific cell surface receptors, most of which belong to the integrin family of glycoproteins (3).

Although the involvement of microfilaments in the connections between cytoskeleton and extracellular matrix has been intensively studied, microtubules are also implicated in the process of cell adhesion to a substrate and in the maintenance of cell shape (4). The organization of microtubules in cells varies with cell type, but they are usually radially distributed from a microtubule-organizing center localized near the nucleus. Microtubules support cell elongation and the formation and maintenance of long processes from the cell surface (5).

Intermediate filaments are the main structural element of the cytoskeleton (6), therefore involved in the generation and maintenance of the overall cell shape. Intermediate filaments of different cell types are composed of distinct proteins, e.g., desmin filaments are found predominantly in all types of muscle cells.

Most of the studies about extracellular Ca^{2+} have focused on the regulation of Ca^{2+}-dependent adhesion proteins such as cadherins, and their role in the development and maintenance of tissues. There is a lack of data concerning the relation between extracellular Ca^{2+}-dependent systems and cell shape determination, particularly the crosstalk between membrane components and the cytoskeleton.

In the present study we examined the effects of extracellular Ca^{2+} withdrawal on the generation and maintenance of cell shape and on cytoskeletal and adhesion site organization in the cell line C2C12 grown in culture. We analyzed the distribution of microfilaments, microtubules, intermediate filaments and integrin-containing adhesion sites using immuno-

fluorescence microscopy, and the overall cell shape using phase contrast microscopy.

The mouse skeletal muscle cell line C2C12 was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil). Cells were routinely maintained in Dulbecco's modified Eagle's medium with 20% fetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin (all from Sigma, St. Louis, MO, USA), under a humidified 5% CO_2 atmosphere at 37ºC. Cells were grown at a low density of 5 x 10^5 cells/35 mm culture dish (Nunc, Copenhagen, Denmark) onto rat collagen-coated Aclar coverslips (Pro-Plastics Inc., Linden, NJ, USA). EGTA (Sigma) was added to the medium of subconfluent cultures at a final concentration of 1.75 mM (7). Both control and EGTA-treated cultures received fresh medium with or without EGTA daily. After 24 h of EGTA exposure, cultures were observed by phase contrast microscopy or submitted to fluorescence microscopy.

For immunofluorescence microscopy, cultures were fixed with 4% formaldehyde in PBS for 3 min at room temperature and permeabilized with 0.5% Triton X-100 (Sigma) in PBS (8). For some experiments, cells were simultaneously incubated with a mouse anti-α-tubulin monoclonal antibody and a rabbit polyclonal antibody against desmin (both from Sigma). Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-goat anti-mouse IgG antibody and a tetramethylrhodamine isothiocyanate-goat anti-rabbit IgG antibody (both from Jackson Immunoresearch Labs., West Grove, PA, USA). In other experiments, cells were incubated with a rat anti-$α_5$-integrin monoclonal antibody (Pharmingen, San Diego, CA, USA), washed and incubated with a FITC-goat anti-rat IgG antibody (Sigma), followed by the filamentous actin-specific probe rhodamine-phalloidin (3.3 μM; Molecular Probes Inc., Eugene, OR, USA). Some cells were stained only with rhodamine-phalloi-

din. The nuclei were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Molecular Probes Inc.) at 0.1 µg/ml in 0.9% NaCl. Cells were mounted in glycerol containing, by weight, 5% n-propyl gallate (Sigma), 0.25% 1,4-diazabicyclo (2,2,2) octane (Sigma), and 0.0025% para-phenylenediamine (Sigma). Cells were examined with an epifluorescence Axiovert 100 light microscope (Carl Zeiss, Oberkochen, Germany), using appropriate filter sets. Images were acquired with a C2400i integrated CCD camera (Hamamatsu Photonics, Shizuoka, Japan) using an Argus 20 image processor (Hamamatsu Photonics). Digitalized images were transferred to a Dell OptiPlex GL 575 computer (Dell Corporate, Round Rock, TX, USA) and plates were mounted using Adobe

Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

Control experiments with no primary antibody showed only a faint background staining (data not shown). Live C2C12 cells grown on collagen-coated Aclar coverslips were examined and images were acquired by phase contrast microscopy using the same microscope and digital system as described above.

C2C12 cells grown in normal medium without EGTA (control cells) were well spread and showed many contacts with the substrate (Figure 1A). In contrast, most of the cells grown in the presence of EGTA for 24 h changed to a bipolar morphology, presumably with fewer contacts with the substrate (Figure 1B).
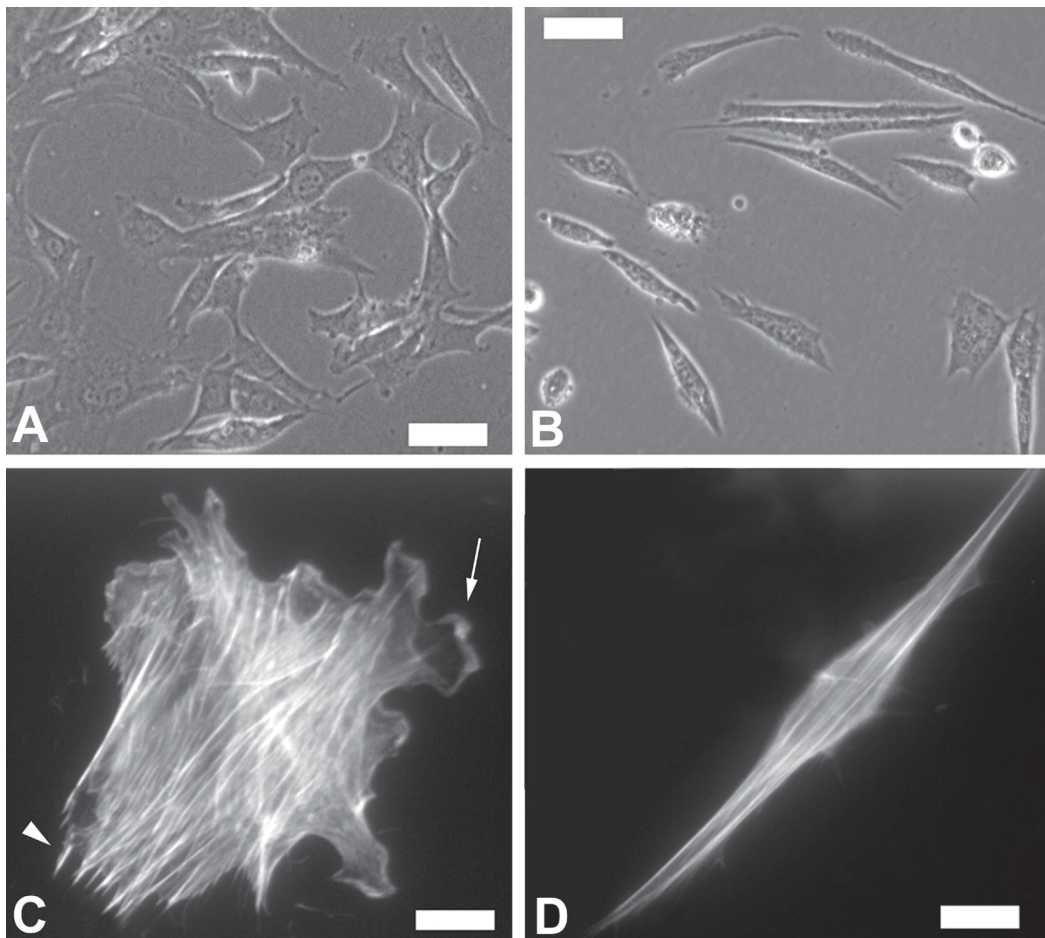
Control C2C12 cells stained with the

Figure 1. Phase contrast microscopy of C2C12 cells grown in the absence (A) of EGTA (control cells) or in the presence (B) of 1.75 mM EGTA for 24 h. Fluorescence microscopy of C2C12 cells grown in the absence of EGTA (control cells, C) or in the presence of 1.75 mM EGTA for 24 h (D) and stained with rhodamine-phalloidin. Arrow in *C* points to ruffled membrane and arrowhead in *C* points to adhesion sites. Bars in *A* and *B* = 50 µm. Bars in *C* and *D* = 10 µm.

Figure 2. Immunofluorescence microscopy of C2C12 cells grown in the absence (A,B,C) of EGTA (control cells) or in the presence (D,E,F) of 1.75 mM EGTA for 24 h and an anti-α-tubulin monoclonal antibody (A,D), with an anti-desmin polyclonal antibody (B,E) and with the nuclear stain DAPI (C,F). Arrows in *A* and *D* point to microtubule-organizing centers. Bars = 10 µm for all panels.
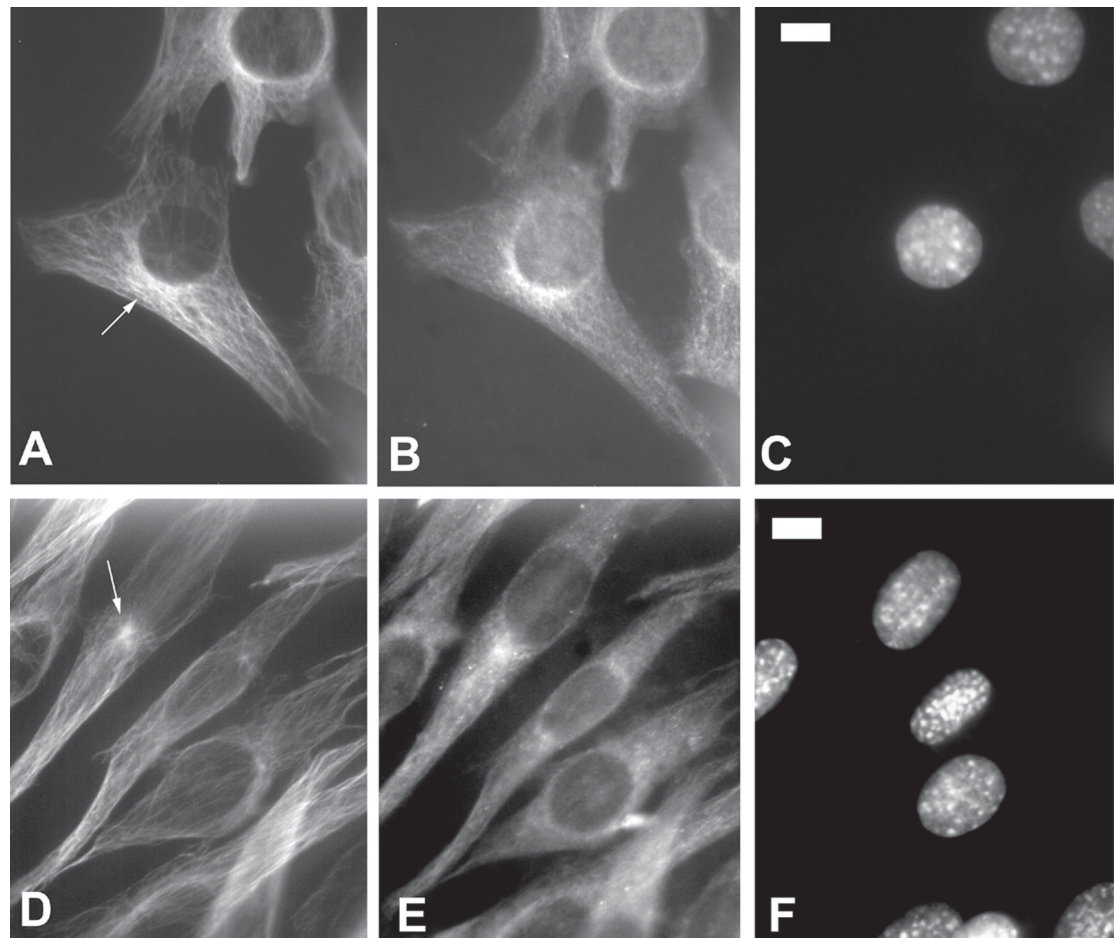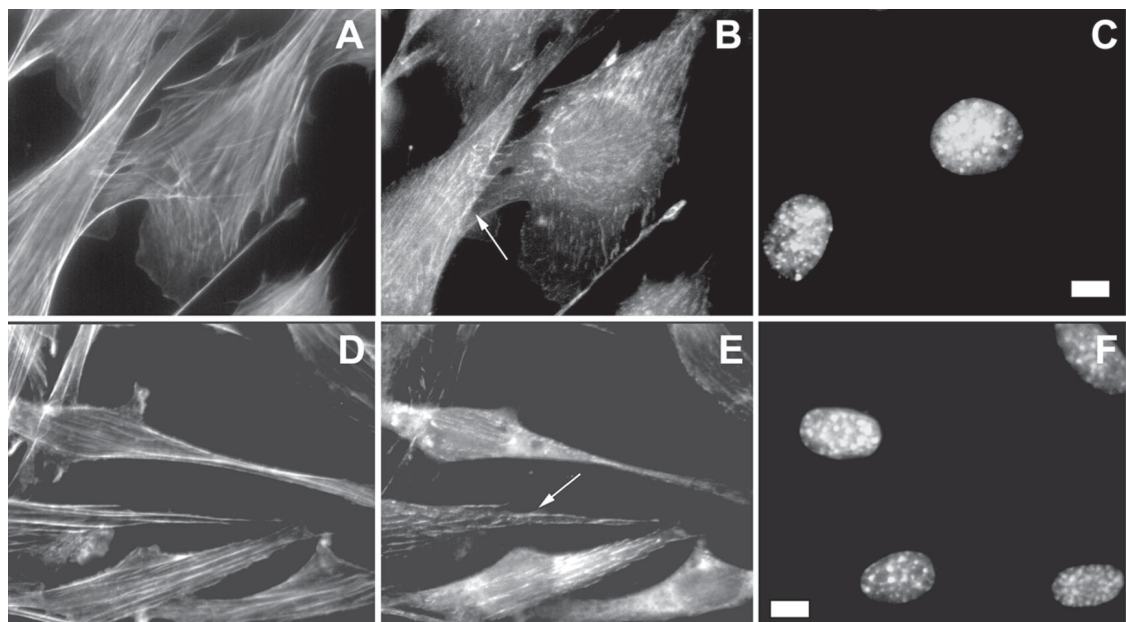

Figure 3. Immunofluorescence microscopy of C2C12 cells grown in the absence (A,B,C) of EGTA (control cells) or in the presence (D,E,F) of 1.75 mM EGTA for 24 h and stained with rhodamine-phalloidin (A,D), with an anti-α_5-integrin monoclonal antibody (B,E) and with the nuclear stain DAPI (C,F). Arrows in *B* and *E* point to adhesion sites. Bars = 10 µm for all panels.

filamentous actin-specific probe rhodamine-phalloidin showed characteristic long stress fibers running in many different directions (Figure 1C), focal adhesions at the termination of stress fibers close to the cell membrane (Figure 1C, arrowhead) and cortical actin in ruffled membranes (Figure 1C, arrow). Treated cells showed a smaller number of stress fibers within the cells, running mainly in the longitudinal direction of the cell, and no focal adhesions or ruffle membranes were visualized (Figure 1D).

Tubulin staining showed a radial arrangement of microtubules in control C2C12 cells (Figure 2A), in contrast with a longitudinal distribution of microtubules in cells treated with EGTA (Figure 2D). In addition, a single and distinctive microtubule-organizing center was observed close to the nuclear membrane in both control and EGTA-treated C2C12 cells (arrows in Figure 2A,D). The desmin staining showed a filament network dispersed within the whole cytoplasm, and especially concentrated in the perinuclear region of both control and treated cells (Figure 2B,E, respectively), correlated with the microtubule network. EGTA-treated cells appeared to have a more fragmented desmin network compared to control cells (Figure 2E,B, respectively). Nuclear staining with DAPI showed a normal nuclear pattern, with no nuclear fragmentation in both control cells (Figures 2C and 3C) and in cells treated with EGTA (Figures 2F and 3F), suggesting that treated cells were still viable.

Both control and EGTA-treated C2C12 cells, double-stained with phalloidin and anti-α_5-integrin antibody, showed integrin-containing fibrillar adhesions (Figure 3B and E, arrows) at the termination of stress fibers (Figure 3A and D). Besides, a dense α_5-integrin labeling was seen inside most treated cells, which does not correspond to the stress fiber distribution (Figure 3E).

The change in cell shape observed after extracellular Ca^{2+} withdrawal was correlated with the change in the number and distribution of stress fibers, and with the change in the organization of α_5-integrin fibrillar adhesions (2). It is possible that the dense intracellular α_5-integrin staining observed in EGTA-treated cells corresponds to the internalization of the protein, as described by others (9). Furthermore, the elongation of EGTA-treated cells may be microtubule dependent (10) since microtubules shifted from the radial arrangement they display in round and well-spread cells to a longitudinal distribution, which is unique for bipolar cells (11).

The fragmented desmin filaments observed in EGTA-treated cells could be related to the activation of the calpain protease system, which has been reported to cause a rapid cleavage of desmin (12). Changes in the distribution of intermediate filament networks have been described in several cell types that undergo differentiation and cell shape changes (13).

It has been proposed that extracellular Ca^{2+}-binding proteins could regulate the availability of free Ca^{2+} outside the cell, influencing several cellular functions, such as cell adhesion and migration (14). We showed here that extracellular Ca^{2+} withdrawal caused changes in cell shape and in the organization of cytoskeleton and adhesion sites. Although such major changes in the cell could be attributed to changes in isolated molecules only, it is more reasonable that such changes are complex events that involve cytoskeletal structures and their membrane connections.

Acknowledgments

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