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Fibroblasts Contracting Collagen Matrices Form Transient Plasma Membrane Passages through which the Cells Take Up Fluorescein Isothiocyanate-Dextran and Ca^{2+}

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When fibroblasts contract collagen matrices, the cells activate a Ca^{2+}-dependent cyclic AMP signaling pathway. We have found that contraction also stimulates uptake of fluorescein isothiocyanate-dextran molecules from the medium. Our results indicate that fluorescein isothiocyanate-dextran enters directly into the cell cytoplasm through 3- to 5-nm plasma membrane passages. These passages, which reseal in less than 5 s in the presence of divalent cations, also are likely sites of Ca^{2+} uptake during contraction and the first step in contraction-activated cyclic AMP signaling. The formation of plasma membrane passages during fibroblast contraction may reflect a general cellular response to rapid mechanical changes.

INTRODUCTION

Fibroblasts cultured in collagen matrices can be used as an in vitro model for tissue morphogenesis such as occurs during wound repair (Grinnell, 1994). In this model, cells reorganize the extracellular matrix through migratory activity that has been called "tractional remodeling" (Harris *et al.*, 1981). Contractile forces are propagated throughout the continuous collagen network, resulting in proximal-to-distal compaction of collagen fibrils around the cells (Grinnell and Lamke, 1984; Yamato *et al.*, 1995). This matrix reorganization process, which results in formation of a dense tissue-like structure (Elsdale and Bard, 1972; Bell *et al.*, 1979), has been referred to interchangeably by a variety of terms including matrix compaction, contraction, and retraction.

The fibroblast phenotype that develops as a result of tractional remodeling differs dramatically depending upon whether collagen matrices are floating or anchored in place. In floating matrices, cells remain in mechanical equilibrium indicated by isometric (stellate) cell morphology and cytoskeletal meshwork (Bell *et al.*, 1979; Bellows *et al.*, 1981). In anchored matrices, on the other hand, the matrix resists contractile forces exerted by the fibroblasts, and the cells become stressed as indicated by development of polarized cell morphology and prominent stress fibers (Mochitate *et al.*, 1991; Tomasek *et al.*, 1992). The force exerted by fibroblasts under the latter conditions was found to be comparable to that generated in skin wounds undergoing contraction (Delvoye *et al.*, 1991; Kolodney and Wysolmerski, 1992).

Contractile force required for matrix reorganization requires an intact actin cytoskeleton (Bell *et al.*, 1979; Bellows *et al.*, 1982; Guidry and Grinnell, 1985), myosin light chain kinase activity (Ehrlich *et al.*, 1991; Kolodney and Elson, 1993), and attachment of cells to collagen fibrils through $\alpha 2\beta 1$ integrins (Schiro *et al.*, 1991; Klein *et al.*, 1991). Also, the reorganization process is tightly regulated. That is, it does not occur in the absence of serum (Steinberg *et al.*, 1980; Guidry and Grinnell, 1985) or other factors such as lysophosphatidic acid (Kolodney and Elson, 1993), platelet-derived growth factor (PDGF; Clark *et al.*, 1989; Gullberg *et al.*, 1990), transforming growth factor β (Montesano and Orci, 1988), endothelin (Guidry and Hook, 1991), or thrombin (Kolodney and Wysolmerski, 1992).

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Extensive research has shown that mechanical force can regulate cell function in diverse mammalian tissues (e.g., Watson, 1991; Sadoshima *et al.*, 1992; Davies and Tripathi, 1992; Komuro and Yazaki, 1993; Simpson *et al.*, 1994; Duncan and Turner, 1995; Liu *et al.*, 1995; Osol, 1995; Yamazaki *et al.*, 1995). In general, increased mechanical stress (for example, tension or shear) results in increased cell proliferation—giving rise to the so-called "tensegrity" (Ingber and Folkman, 1989), "mechanogenetic" (Erdos *et al.*, 1991), and "mechanogenic" (Vandenburgh, 1992) models of cell growth regulation. Even the well-known dependence of cell growth on cell shape (Folkman and Moscona, 1978) may turn out to be an effect of cell tension (Curtis and Seehar, 1978; O'Neill *et al.*, 1990).

Signaling mechanisms that regulate the cellular response to mechanical force are not well understood. Recently, we have been studying mechanoregulated signaling in fibroblasts by using the anchored and floating collagen matrix models in combination. First, fibroblasts are cultured in anchored matrices and allowed to develop mechanical stress. Then, these stressed matrices are released from their anchorage sites and allowed to float. In the absence of resistance, the fibroblasts undergo rapid and synchronous contraction during which actin stress fibers disappear and the cells withdraw their pseudopodia (Mochitate *et al.*, 1991; Tomasek *et al.*, 1992; Lee *et al.*, 1993). Contraction and disassembly of the actin cytoskeleton are normal features of nonmuscle cell motility (Stossel, 1993; Giuliano and Taylor, 1995).

Like tractional remodeling, contraction of stressed fibroblasts is promoted by serum, lysophosphatidic acid, or thrombin (but not by PDGF or transforming growth factor β; Tomasek *et al.*, 1992; Pilcher *et al.*, 1994; Lin and Grinnell, 1995). In addition, however, contraction of stressed fibroblasts exhibits a spontaneous component that does not require serum and cannot be inhibited by cytochalasin (Tomasek *et al.*, 1992; He and Grinnell, 1994). Spontaneous and regulated contraction of fibroblasts in collagen matrices resemble the fast (recoil) and slow (metabolic energy-dependent) phases of fibroblast tail retraction (Chen, 1981).

Within minutes after fibroblast contraction begins, activation of adenylyl cyclase leads to a 10- to 20-fold increase in cellular cyclic AMP levels and activation of protein kinase A. The first step of this contraction-activated signal transduction pathway was found to be uptake of extracellular Ca^{2+} followed by a burst of phosphatidic acid synthesis (He and Grinnell, 1994, 1995). Within hours, PDGF and epidermal growth factor receptors become desensitized (Lin and Grinnell, 1993), possibly by losing their ability to aggregate (Tingström *et al.*, 1992). In addition, cellular DNA synthesis declines and the cells become quiescent (Nakagawa *et al.*, 1989, Mochitate *et al.*, 1991).

A small amount of actin can be detected in the medium after fibroblasts contract collagen matrices. Initially, we thought this actin was derived from breakdown of actin-containing ectocytotic vesicles released during contraction (Lee *et al.*, 1993). More recently, we studied another possible explanation for the actin in the medium based on the work of McNeil *et al.* (1984; McNeil, 1993) who reported that cells become permeable to small proteins and dextrans as a consequence of cell wounding in vitro (e.g., scrape loading) or normal mechanical stresses in vivo. We found that fibroblasts can take up extracellular fluorescein isothiocyanate (FITC)-dextran through plasma membrane passages that form during contraction. These passages were in the range of 3–5 nm and resealed in less than 5 s in the presence of divalent cations. Uptake of extracellular Ca^{2+} required for cyclic AMP signaling also appeared to occur at these sites. Details are reported herein.

MATERIALS AND METHODS

Cell Culture

Fibroblasts from human foreskin specimens (<10 passages) were maintained in Falcon 75-cm^2 tissue culture flasks in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY). Hydrated collagen matrices were prepared from Vitrogen 100 collagen (Collagen Corp., Palo Alto, CA). Neutralized collagen solutions (1.5 mg/ml) contained fibroblasts in DMEM but no serum. Aliquots (0.2 ml; 2×10^5 cells) of the cell/collagen mixtures were prewarmed to 37°C for 3 to 4 min and then placed in Costar 24-well culture plates. Each aliquot occupied an area outlined by a 12 mm in diameter circular score within a well.

Polymerization of collagen matrices required 60 min at 37°C, after which the cells were distributed uniformly throughout the matrix. Subsequently, culture medium (1.0 ml of DMEM/10% FBS and 50 μg/ml ascorbic acid) was added to each well and the cultures were incubated for 2 days. During the culture period, fibroblasts reorganized the anchored matrices and developed mechanical stress indicated by the appearance of actin stress fibers (Mochitate *et al.*, 1991; Tomasek *et al.*, 1992; Lee *et al.*, 1993). To initiate fibroblast contraction, anchored matrices were released from the underlying culture dishes by inserting a thin spatula between the collagen matrix and dish surface.

FITC-Dextran Uptake

FITC-dextran (4 kDa to 150 kDa) was obtained from Sigma (St. Louis, MO). Data on FITC content (0.004–0.008 mol of FITC/mol of glucose), Stokes' radii (1.4–9.0 nm), and free FITC in the samples (0.1% in 4-kDa FITC-dextran to undetectable in 150-kDa FITC-dextran) were provided by the manufacturer. No difference in the pattern of results was observed if FITC-dextran samples were dialzyed before use. Cells containing FITC-dextran to be examined by immunofluorescence microscopy were washed (three times) with Dulbecco's phosphate-buffered saline (DPBS; 150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2) and counterstained with rhodamine-conjugated wheat germ agglutinin (RITC-WGA; EY Laboratories, San Mateo, CA) to detect outlines of the plasma membranes as described previously (Mochitate *et al.*, 1991; Lee *et al.*, 1993). Fixation was not used because after fixation the cells were permeable to 4-kDa FITC-dextran. Preparations were examined and photographed with a Zeiss IIIRS fluorescence microscope.

To quantify FITC-dextran uptake, collagen matrices were washed (four times) with DMEM followed by PBS (150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.2, twice). The cells were harvested from the matrix by using 0.25% trypsin/1 mM EDTA (0.1 ml; Life Technologies) for 10 min at 37°C followed by 5 mg/ml collagenase (0.4 ml, Sigma type I) in 130 mM NaCl, 10 mM calcium acetate, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), pH 7.2, for an additional 30 min. Proteinase activity was stopped by addition of 50 μl of FBS, the cells were collected by centrifugation at 11,000 × *g* for 4 min. To release FITC-dextran, pellets were extracted in DPBS containing 0.2% Nonidet P-40 (NP40). Supernatants were clarified by centrifugation at 11,000 × *g* for 10 min, and fluorescence was measured with a fluorometer (LS-5 Spectrometer, Perkin Elmer-Cetus, Norwork, CT) using emission and excitation wavelengths of 520 nm and 490 nm, respectively.

Observations on Actin Organization Using Confocal Microscopy

Cultures were fixed for 10 min at 22°C with 3% paraformaldehyde in DPBS. To block nonspecific staining, the samples were treated with 1% glycine and 1% bovine serum albumin (Fraction V, ICN Biomedicals, Costa Mesa, CA) for 30 min. Subsequently, the cultures were permeabilized with 0.2% NP40 in DPBS for 10 min and incubated for 30 min at 37°C in DPBS containing 1% bovine serum albumin and 1 U of FITC-conjugated phalloidin (Molecular Probes, Eugene, OR). After washing, the samples were mounted with glycerol/PBS (9:1, vol/vol) containing 0.001% phenylenediamine and examined and photographed with a Leica CLSM confocal laser microscope.

DNA Synthesis

DNA synthesis was measured by thymidine incorporation. Cultures were radiolabeled metabolically for 2 h with 5 μCi/ml [$_3$H]thymidine (20 Ci/mmol, New England Nuclear, Boston, MA). At the end of the incubations, cultures were rinsed twice with DPBS and DNA was precipitated with 10% trichloroacetic acid. The precipitates were dissolved in 0.25 M NaOH/1% SDS. Radioactivity was measured as below.

Lactate Dehydrogenase (LDH) Activity

After contraction, medium was collected and matrices containing fibroblasts were homogenized (1-ml Dounce homogenizer, 50 strokes) in DPBS containing 0.2% NP40 and proteinase inhibitors [1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride]. Supernatants were separated from collagen fibrils and cell debris by centrifugation at 11,000 × *g* for 4 min. The amount of LDH in aliquots of the medium and supernatant samples was determined by using the LD diagnostic kit (Sigma).

Extracellular Ca^{2+} Uptake

Extracellular Ca_{2+} uptake during contraction was measured using $^{45}CaCl_2$ (Zhao and Muallem, 1990). Cultures were incubated with HEPES medium (25 mM HEPES, 150 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, pH 7.4) containing 10% FBS (exhaustively dialyzed against HEPES buffer) and 0.2 μCi/ml $^{45}CaCl_2$ (13.6 Ci/g, ICN Biomedicals). Uptake was stopped by switching the cultures to HEPES medium containing 1 mM $LaCl_3$ to block calcium pumps. Three washes of the fibroblast-collagen matrices with HEPES medium containing 1 mM $LaCl_3$ reduced extracellular ^{45}Ca to background levels. Intracellular $_{45}Ca$ was released by incubating the cells for 10 min at 37°C with HEPES medium containing 1% NP40. Samples of the extracts were mixed with 10 ml of BudgetSolve (Research Products International Corp., St. Laurent, Canada) and radioactivity was measured in a scintillation counter (Beckman LS6000, Beckman, Fullerton, CA).

Cyclic AMP Synthesis

Cyclic AMP levels were measured using the two-column method (Salomon, 1991) as described previously (He and Grinnell, 1994, 1995). Attached collagen matrix cultures were incubated for 2 h in 0.5 ml of culture medium containing 10 μCi/ml [^{3}H]adenine (specific activity, 36 Ci/mmol, ICN). Subsequently, cultures were washed with two changes of serum-free DMEM (0.5 ml, 10 min) after which the matrices were released to initiate contraction. The incubations were terminated by adding 0.5 ml of ice-cold 10% trichloroacetic acid containing 0.2 mM cyclic AMP (carrier) to the cultures, and the samples were incubated on ice for 1 h to allow complete extraction of nucleotides. Acid extracts from two matrices were combined and aliquots (800 μl) were applied to 1-ml Dowex 50W columns (mesh size 200–400, Sigma). The columns were washed twice with 1 ml of H_2O and then eluted with an additional 4 ml of H_2O. The eluted Dowex columns were drained completely, and the eluates were applied to a 0.75-g alumina columns (Sigma). [^{3}H]cyclic AMP was eluted from the alumina columns with 3 ml of 100 mM imidazole buffer (pH 7.3). Eluates were mixed with 10 ml of BudgetSolve and radioactivity was determined as above. Efficiency of cyclic AMP recovery was ≈50% based on OD_{260} measurements of carrier cyclic AMP.

RESULTS

Uptake of FITC-Dextran during Fibroblast Contraction

FITC-dextran was added to the incubation medium, after which anchored collagen matrices were released to initiate contraction. Figure 1C shows that under these conditions, essentially all of the cells in the matrix became fluorescently labeled. Figure 1D shows cell outlines identified by counterstaining with rhodamine-conjugated WGA. The FITC staining pattern of the cells was typically diffuse and uniform, suggesting that FITC-dextran was distributed throughout the cytoplasm rather than in a vesicular compartment such as endosomes. In marked contrast to contracting fibroblasts, only a small amount of FITC-dextran was taken up by cells in anchored matrices (Figure 1, A and B), and the fluorescence appeared to be in small clusters.

Subsequent harvesting of fibroblasts from the matrix, which requires approximately 100 min for washing and treatment of the matrices with trypsin followed by collagenase, did not result in release from the cells of intracellular FITC-dextran. Figure 2 shows the appearance of cells that had been incubated with FITC-dextran in anchored or contracting matrices and then harvested and cultured overnight. Cells from the anchored matrices (Figure 2A) contained little fluorescence (compare Figures 2A and 1A), whereas cells from contracting matrices (Figure 2B) were brightly fluorescent (compare Figures 2B and 1C).

Fibroblasts did not appear to be damaged as a consequence of contraction or the subsequent procedure for harvesting the cells from the matrices. Similar numbers of fibroblasts were recovered from anchored or contracting matrices, and the harvested cells attached and spread normally on culture dishes (Figure 2). As an additional control, we measured cellular

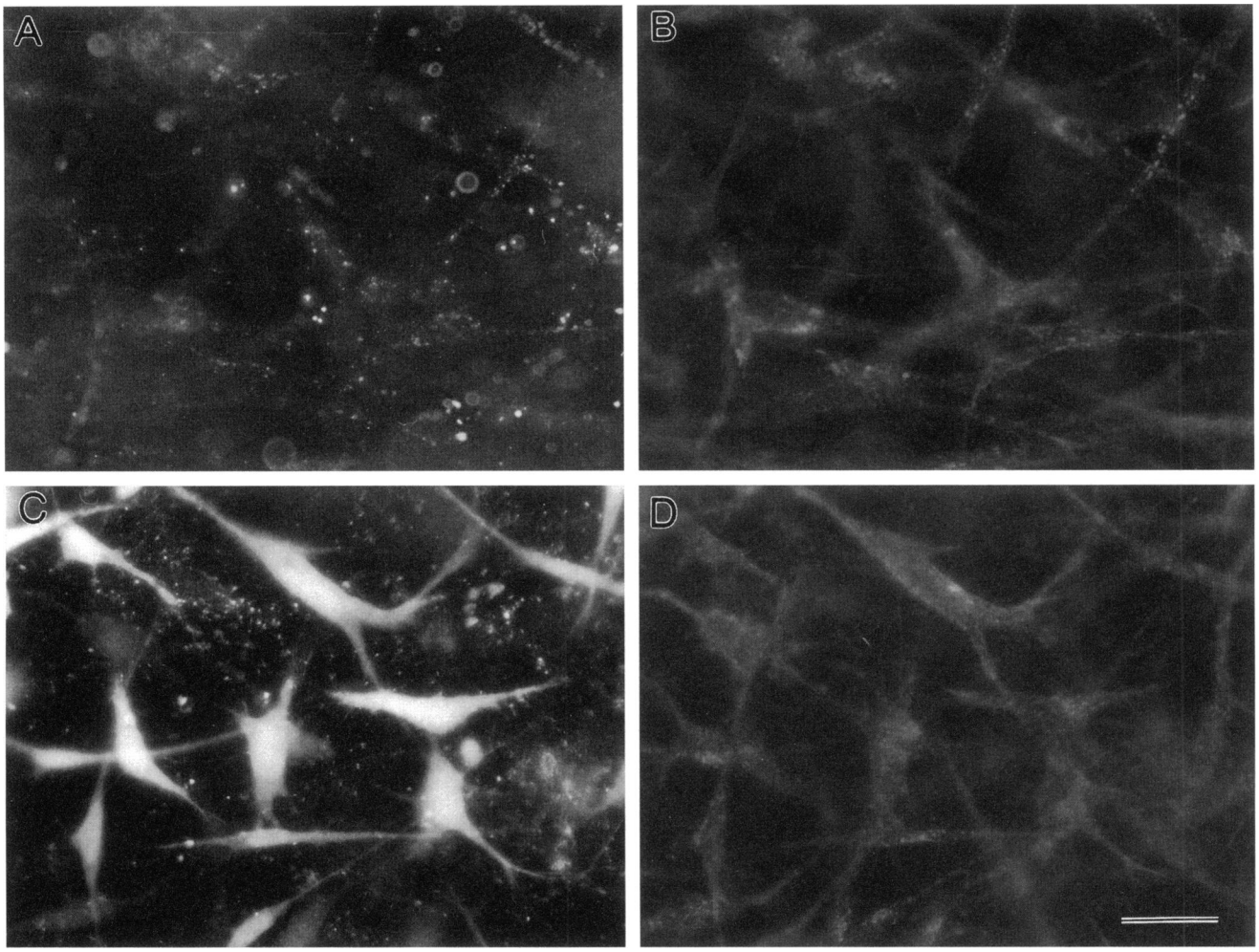

Figure 1. FITC-dextran uptake during fibroblast contraction. Fibroblasts in anchored collagen matrix cultures were incubated for 10 min with 10 mg/ml 4-kDa FITC-dextran and one-half of the matrices were released to initiate contraction. After 10 min, the samples were washed and counterstained with RITC-WGA. (A and B) Cells in anchored matrices. (C and D) Cells undergoing contraction. (A and C) FITC-dextran. (B and D) RITC-WGA. Bar, 50 μm. Other details are described in MATERIALS AND METHODS.

DNA synthesis. Figure 3 shows that fibroblasts harvested from anchored or contracting matrices and replated on culture dishes showed similar rates of [^{3}H]thymidine incorporation.

Quantification of FITC-Dextran Uptake

Because fibroblasts retained intracellular FITC-dextran through the washing and harvesting procedures, it was possible to complement morphological observations with quantitative measurements. We chose to harvest the cells from the matrices beginning after 10 min of contraction because this time was consistent with the time used in our previous studies on contraction-activated signaling (He and Grinnell, 1994, 1995). Uptake of 4-kDa FITC-dextran was linear between 0 and 20 mg/ml, and most experiments were carried out at 10 mg/ml. Table 1 shows that contraction stimulated an ≈10-fold increase in 4-kDa FITC-dextran uptake. Uptake by contracting cells was ≈0.01% of the total FITC-dextran added to the medium. In molecular terms, this averaged 4.5×10^8 molecules/cell.

Figure 4 shows that FITC-dextran had to be present in the cultures at the time when contraction was initiated for uptake to be observed. Some uptake was evident even if FITC-dextran was added only 5 s before contraction (−5 s). If, on the other hand, FITC-dextran was added to the cultures 5 s or later after initiating contraction, then levels of cell-associated fluorescence were no greater than with fibroblasts in anchored matrices. These results suggested that the plasma membrane sites permeable to FITC-dextran were open for less than 5 s.

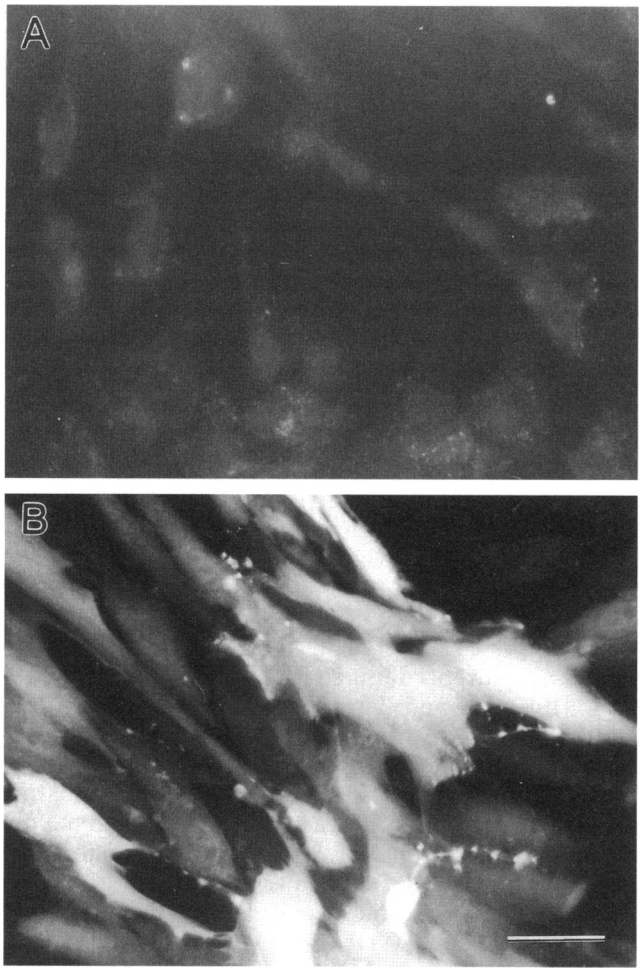

Figure 2. Distribution of fluorescence in fibroblasts harvested from collagen matrices. Fibroblasts in anchored collagen matrix cultures were incubated for 10 min with 10 mg/ml 4-kDa FITC-dextran and one-half of the matrices were released to initiate contraction. After 10 min, the cells were washed and harvested from the collagen matrices and cultured overnight on glass coverslips in DMEM containing 10% FBS. (A) Cells from anchored collagen matrices. (B) Cells from contracting matrices. Bar, 50 μm.

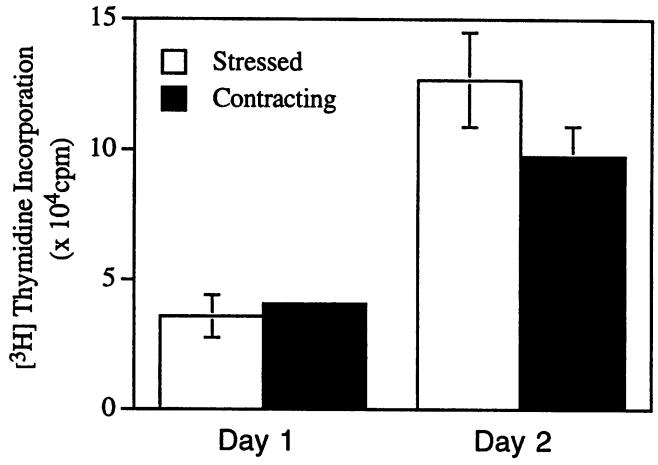

Figure 3. DNA synthesis by fibroblasts harvested from collagen matrices. Fibroblasts were incubated as described in Figure 2. On the days shown the cultures were incubated with [^{3}H]thymidine to determine DNA synthesis. Data are averages ± SD from triplicate samples.

Spontaneous Contraction Is Sufficient for FITC-Dextran Uptake

As mentioned in the INTRODUCTION, two processes play a role in fibroblast contraction: one spontaneous (i.e., serum-independent) and the other regulated (by serum or growth factors). Figure 5 shows morphological features of fibroblasts undergoing spontaneous and regulated contraction. Initially, prominent actin stress fibers were observed in fibroblasts in anchored matrices (Figure 5A, arrows). These stress fibers collapsed and actin aggregates were observed after 10 min of spontaneous contraction (Figure 5B, arrows) or regulated contraction. After 60 min, overall cell shape and actin cytoskeleton organization of cells undergoing spontaneous contraction did not change further (Figure 5, C versus B). In cells undergoing regulated contraction, however, the actin cytoskeleton retracted into a condensed perinuclear distribution (Figure 5D, arrows).

Experiments were carried out to determine whether uptake of FITC-dextran required regulated contraction or whether mechanical changes in the cells associated with spontaneous contraction were sufficient for opening of FITC-permeable plasma membrane passages. Figure 6A shows that FITC-dextran uptake during contraction did not require serum in the me-

Table 1. Uptake of 4-kDa FITC-dextran during fibroblast contraction

	Matrix	
Parameter	Anchored	Contracting
Fluorescence intensity	7.2 ± 6.6	65.9 ± 29.9
Total FITC-dextran uptake (ng)	80	747
FITC-dextran molecules (no./cell)	0.48×10^8	4.5×10^8

Fibroblasts in anchored collagen matrix cultures were incubated for 10 min with 10 mg/ml FITC-dextran at which time one-half of the cultures were released to initiate contraction. After 10 min of contraction, samples were washed, cells were harvested, and FITC-dextran uptake was measured by spectrofluorometry. Other details are described in MATERIALS AND METHODS. Data are averages ± SD from 17 experiments carried out over an 8-month period. Total uptake determined from a standard curve of fluorescence reading vs. FITC-dextran (0–2 μg/ml). Molecules/cell were calculated based on 2×10^5 cells.

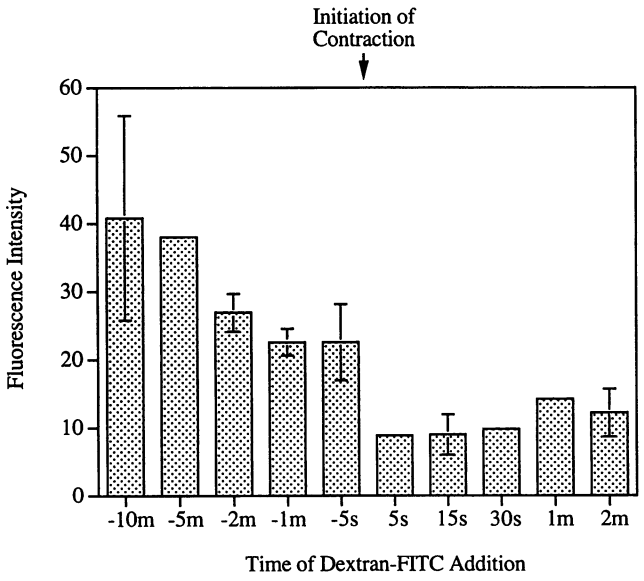

Figure 4. Timing of FITC-dextran uptake by contracting fibroblasts. Fibroblasts were incubated with 10 mg/ml 4-kDa FITC-dextran beginning at the times indicated before (−) or after (+) matrix contraction was initiated. After 10 min of contraction, samples were washed, cells were harvested, and FITC-dextran uptake was measured by spectrofluorometry. Other details are described in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

dium, consistent with the idea that FITC-permeable plasma membrane sites opened during spontaneous contraction. Furthermore, as shown in Figure 6B, FITC-dextran uptake by contracting fibroblasts was not inhibited by cytochalasin D. On the other hand, FITC-dextran uptake by cells in anchored matrices was not enhanced by cytochalasin D, indicating that disruption of the cytoskeleton per se without reorganization of the cell surface did not lead to formation of the plasma membrane passages.

Size of Plasma Membrane Passages

To learn more about the plasma membrane passages responsible for FITC-dextran uptake, experiments were carried out by using a series of FITC-dextran molecules from 4 kDa to 150 kDa. Figure 7 shows that relative FITC-dextran uptake was similar in the range of 4–20 kDa, indicating that the plasma membrane passages freely accommodated transport of these molecules (Stokes' radii, 1.4–3.2 nm). With 40-kDa FITC-dextran (Stokes' radius, 4.3 nm) and larger molecules, uptake decreased markedly, indicating that transport was retarded. Thus, these data indicated that the plasma membrane passages were in the range of 3–5 nm.

It should be noted that size-dependent differences in uptake were not a result of differences in diffusion of FITC-dextran through the collagen matrix. For instance, uptake of 40-kDa FITC-dextran was the same regardless whether the matrices were preincubated with FITC-dextran for 30 min, 60 min, or 2 h before initiating contraction. Moreover, as shown in Figure 8, most of the small amount of the LDH (Stokes' radius, ≈3 nm) released from fibroblasts during contraction diffused through the matrix into the culture medium within 5 min. We could not tell from these experiments whether the LDH observed in the medium came through the FITC-permeable plasma membrane passages of intact cells or was released from a few disrupted cells.

Resealing of the Plasma Membrane Requires Extracellular Divalent Cations

Experiments also were carried out to learn whether divalent cations were important for closing of the plasma membrane passages. Extracellular Ca^{2+} ions have been reported to be required for resealing of plasma membranes of erythrocyte ghosts (Bodemann and Passow, 1972; Johnson and Kirkwood, 1978), as well as repair of damaged tissue cells (Yawo and Kuno, 1983; Xie and Barrett, 1991). Figure 9 compares uptake of FITC-dextran in the presence and absence of 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) in the incubation medium. FITC-dextran was added at 2 min before contraction (−2 m) or at 0–5 min after contraction (0–5 m). In the absence of EGTA, FITC-dextran added after contraction did not enter the cells (see also Figure 4). In the presence of EGTA, however, the plasma membrane remained at least partially open judging from the ability of cells to take up some FITC-dextran that was added as late as 5 min after contraction was initiated. In control experiments, we found that addition of EGTA did not affect FITC-dextran uptake by fibroblasts in anchored matrices. Figure 10 shows that 3 mM EGTA was required to keep the plasma membrane open. This concentration of EGTA slightly exceeds the amount of extracellular Ca^{2+} in serum-containing medium and does not inhibit fibroblast contraction of collagen matrices (He and Grinnell, 1994).

Extracellular Ca^{2+} Uptake during Contraction

Finally, we tested the possibility that FITC-dextran–permeable plasma membrane passages that formed during contraction also were sites of Ca^{2+} uptake. Unlike cells in monolayer culture, Ca^{2+} uptake by fibroblasts contracting collagen matrices could not be imaged by using fluorescent dyes because the visual field moves (millimeter distances) during contraction. Consequently, Ca^{2+} uptake measurements were carried out by using ^{45}Ca. Figure 11A shows that if $^{45}CaCl_2$ was added to the incubation medium before

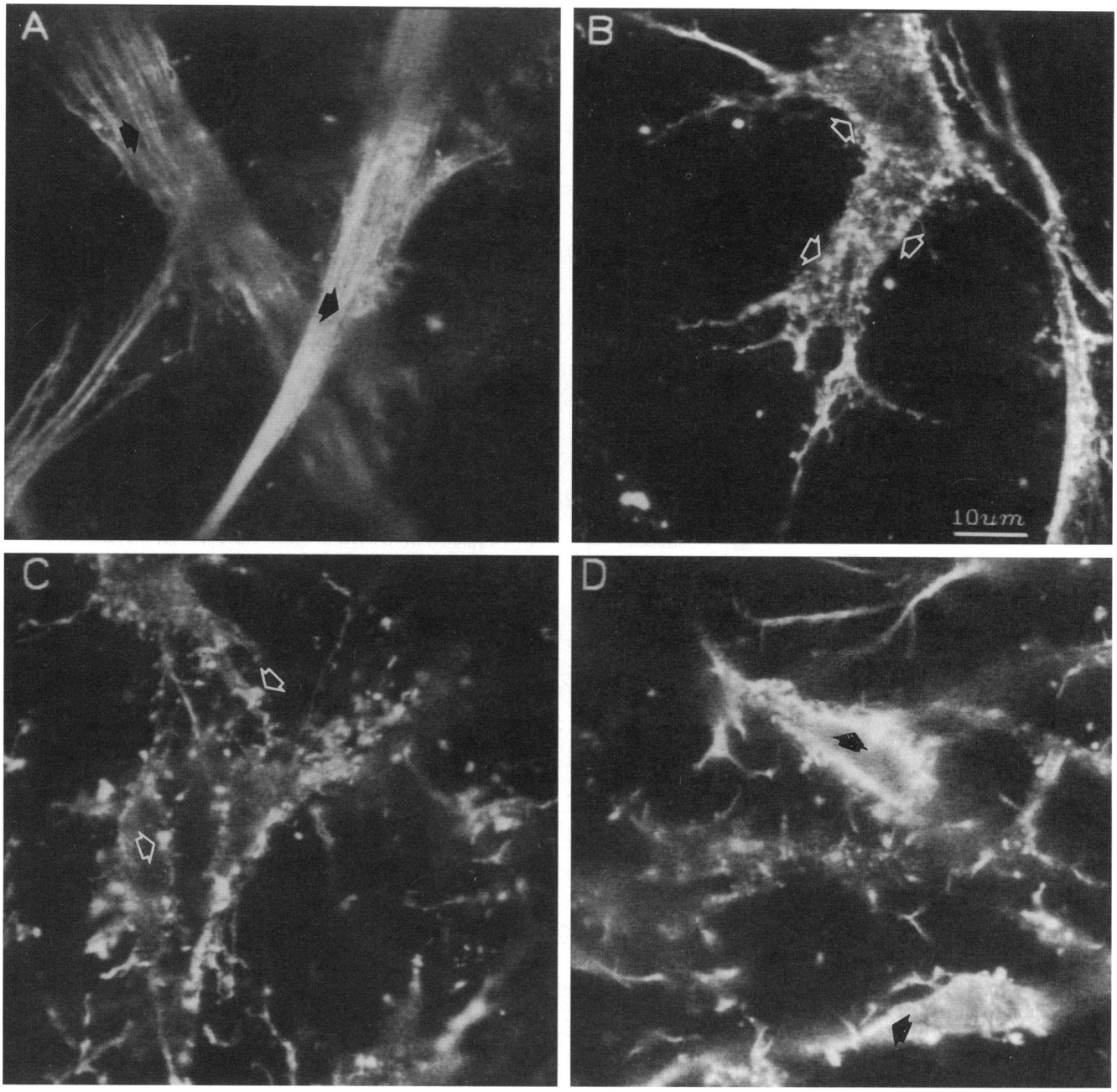

Figure 5. Changes in actin organization in fibroblasts undergoing spontaneous and regulated contraction. Fibroblasts in anchored collagen matrix cultures were released to initiate spontaneous contraction (serum-free medium) or regulated contraction (serum-containing medium). At the times indicated, samples were fixed, permeabilized, and stained with FITC-phalloidin to determine actin distribution. (A) Cells in anchored matrices show prominent stress fibers (arrows). (B and C) Cells contracting collagen matrices in serum-free DMEM for 10 min or 60 min show disrupted actin cytoskeleton and actin aggregates (arrows). (D) Cells contracting collagen matrices in DMEM containing 10% FBS for 60 min show the actin cytoskeleton retracted and condensed in the perinuclear cell region (arrows). Other details are in MATERIALS AND METHODS.

releasing the anchored matrices, then contraction stimulated uptake of ^{45}Ca. If, on the other hand, ^{45}Ca was added to the medium 10 min after releasing anchored matrices, then as shown in Figure 11B, ^{45}Ca uptake was no greater in contracting fibroblasts than in cells within anchored matrices. The amount of ^{45}Ca taken up by the cells was ≈0.025% of the total added to the medium.

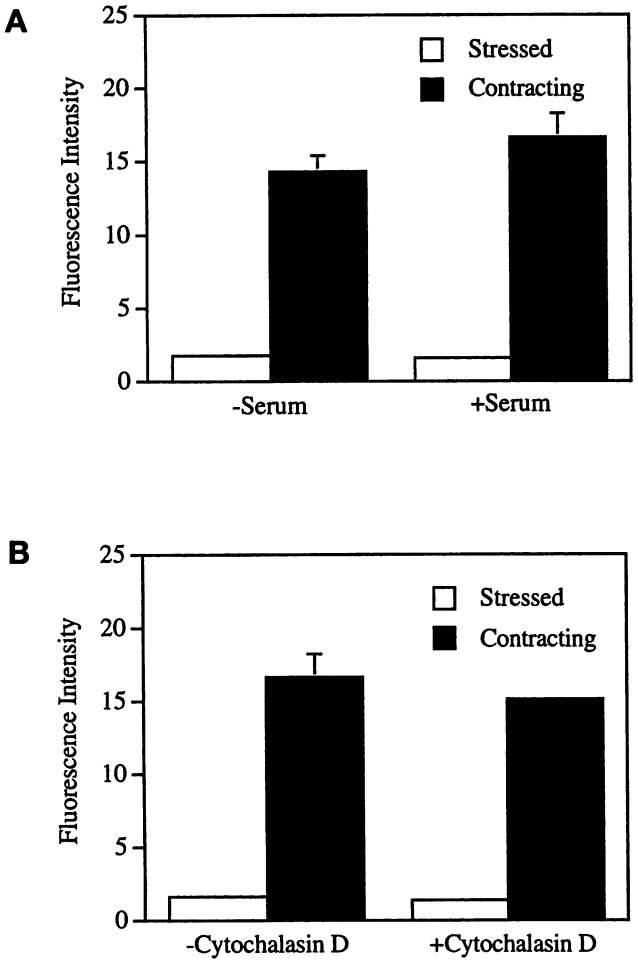

Figure 6. Effect of serum and cytochalasin D on FITC-dextran uptake. Fibroblasts were incubated with 10 mg/ml 4-kDa FITC-dextran and with or without 10% FBS (A) or in the presence or absence of 10 μM cytochalasin D (B), as indicated for 20 min, after which matrices were released to initiate contraction. After 10 min, samples were washed, cells were harvested, and FITC-dextran uptake was measured by spectrofluorometry. Other details are described in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

We also compared Ca^{2+} uptake during spontaneous and regulated contraction. Figure 12 shows that ^{45}Ca uptake was similar with or without serum in the medium and in the presence or absence of cytochalasin D. Therefore, spontaneous contraction was sufficient to trigger ^{45}Ca uptake as was the case for FITC-dextran. Since uptake of FITC-dextran and ^{45}Ca occurred at the same time, and the amount of ^{45}Ca uptake (as a percentage of total added) was comparable to FITC-dextran uptake, it seemed likely that ^{45}Ca and FITC-dextran entered the cells together.

Previously, this laboratory has reported that Ca^{2+} uptake is the first step in contraction-activated cyclic AMP signaling (He and Grinnell, 1994, 1995). It was of interest, therefore, to test whether spontaneous contraction also was sufficient to activate cyclic AMP signaling. Figure 13 shows that the major burst of cyclic AMP occurred as a result of spontaneous contraction. The secondary increase during regulated contraction was much less prominent.

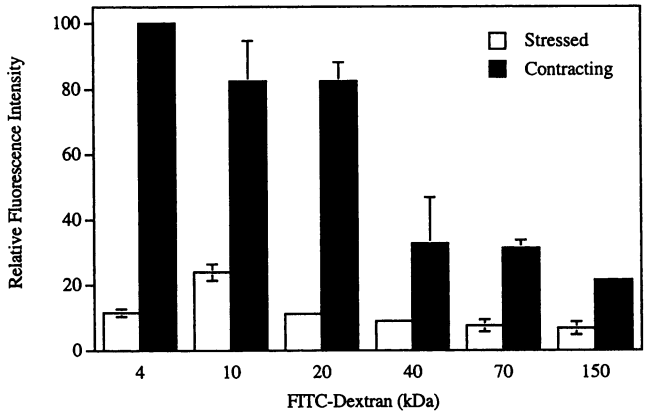

Figure 7. Effect of molecular size on FITC-dextran uptake. Fibroblasts in anchored collagen matrices were incubated for 1 h with 10 mg/ml FITC-dextran of different sizes as indicated, after which one-half of the matrices were released. Ten minutes later, samples were washed, cells were harvested from anchored matrices and contracting matrices, and FITC-dextran uptake was measured by spectrofluorometry. Fluorescence intensity measurements were normalized according to specific activity (mol of FITC/mol of glucose), which ranged from 0.004 to 0.008. The value for 4-kDa dextran was arbitrarily set to 100. Other details are described in MATERIALS AND METHODS. Data presented are averages ± SD from duplicate samples.

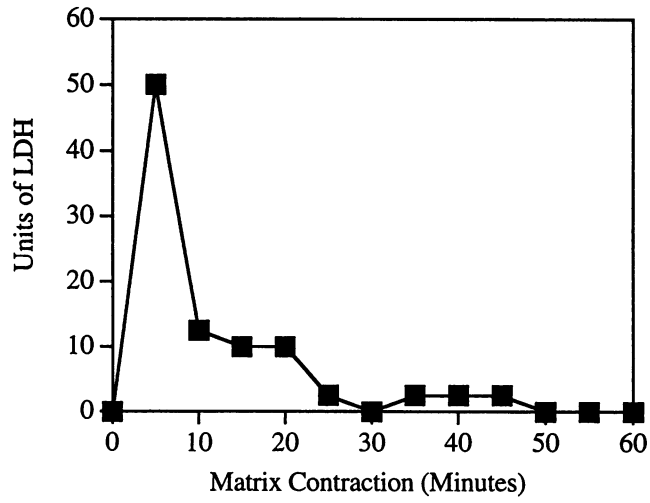

Figure 8. LDH release during fibroblast contraction. Fibroblasts in anchored collagen matrix cultures were released to initiate contraction. Samples of the medium were collected at the times indicated and LDH activity was determined. Total LDH activity/culture was 740 units. Other details are in MATERIALS AND METHODS.

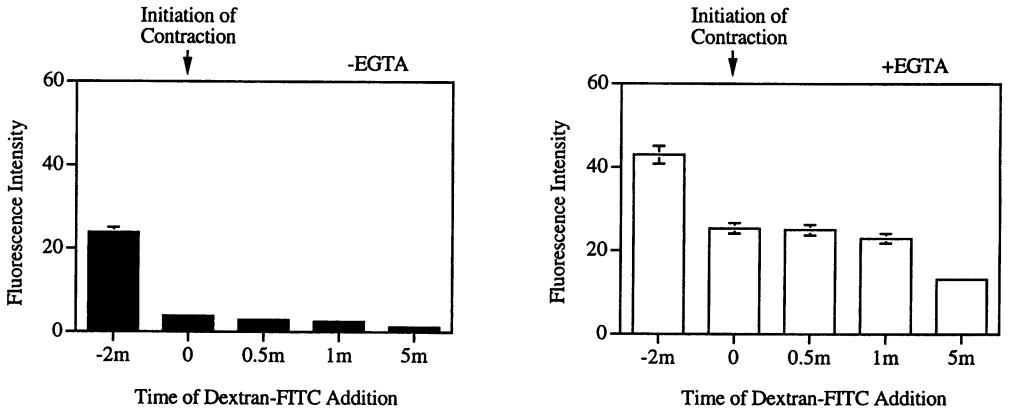

Figure 9. Effect of EGTA on plasma membrane closing. Fibroblasts in DMEM containing 10% FBS with or without 3 mM EGTA as indicated were incubated with 10 mg/ml 4-kDa FITC-dextran beginning at the times shown (−) or after (+) fibroblast contraction was initiated. After 10 min of contraction, samples were washed (which results in closing of plasma membrane passages), cells were harvested, and FITC-dextran uptake was measured by spectrofluorometry. Other details are described in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

DISCUSSION

Contracting Fibroblasts Take Up FITC-Dextran

When anchored collagen matrices are released from their anchorage sites, fibroblasts undergo rapid contraction and activate a Ca^{2+}-dependent cyclic AMP signaling pathway (He and Grinnell, 1994, 1995). In the current studies, we found that contraction also stimulates cells to take up extracellular FITC-dextran. The results indicated that FITC-dextran passes directly into the cell cytoplasm through 3- to 5-nm plasma membrane passages. These passages, which resealed in less than 5 s in the presence of divalent cations, also were likely sites of Ca^{2+} uptake during contraction and the first step in contraction-activated cyclic AMP signaling. Formation of plasma membrane passages occurred in fibroblasts throughout the matrix. That is, essentially all of the cells became fluorescent in the presence of FITC-dextran, and cells harvested from collagen matrices after contraction retained their fluorescent appearance but did not appear to be negatively affected otherwise or to have lower rates of DNA synthesis compared with cells harvested from anchored matrices.

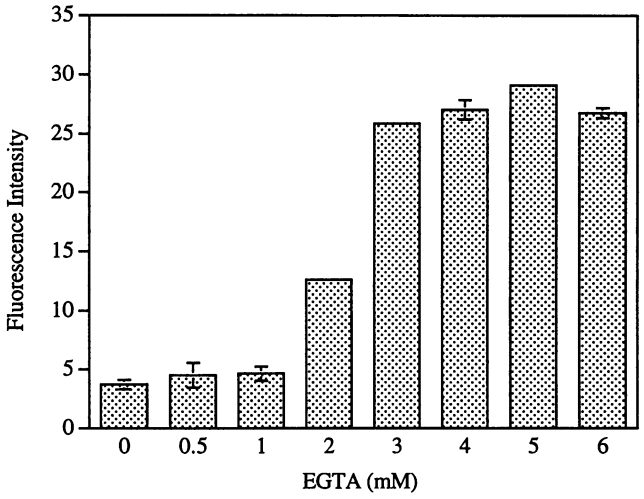

Figure 10. Dose-response relationship for EGTA inhibition of plasma membrane closing. Fibroblasts in DMEM containing 10% FBS and EGTA as indicated were released to initiate contraction. One minute later, 10 mg/ml 4-kDa FITC-dextran were added. After 10 min of contraction, samples were washed, cells were harvested, and FITC-dextran uptake was measured by spectrofluorometry. Other details are described in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

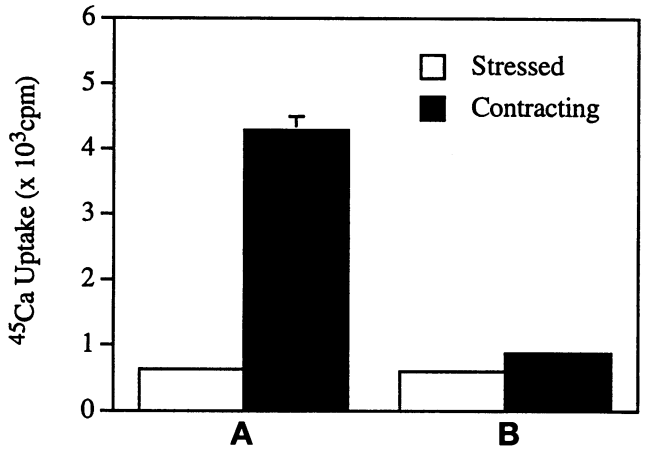

Figure 11. Uptake of ^{45}Ca during contraction. Fibroblasts in anchored collagen matrix cultures were switched to HEPES medium (+10% dialyzed FBS). $^{45}CaCl_2$ was added 10 min before contraction was initiated (A) or 10 min after contraction was initiated (B). Twenty minutes after the addition of $^{45}CaCl_2$, cell-associated ^{45}Ca was determined. Other details are in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

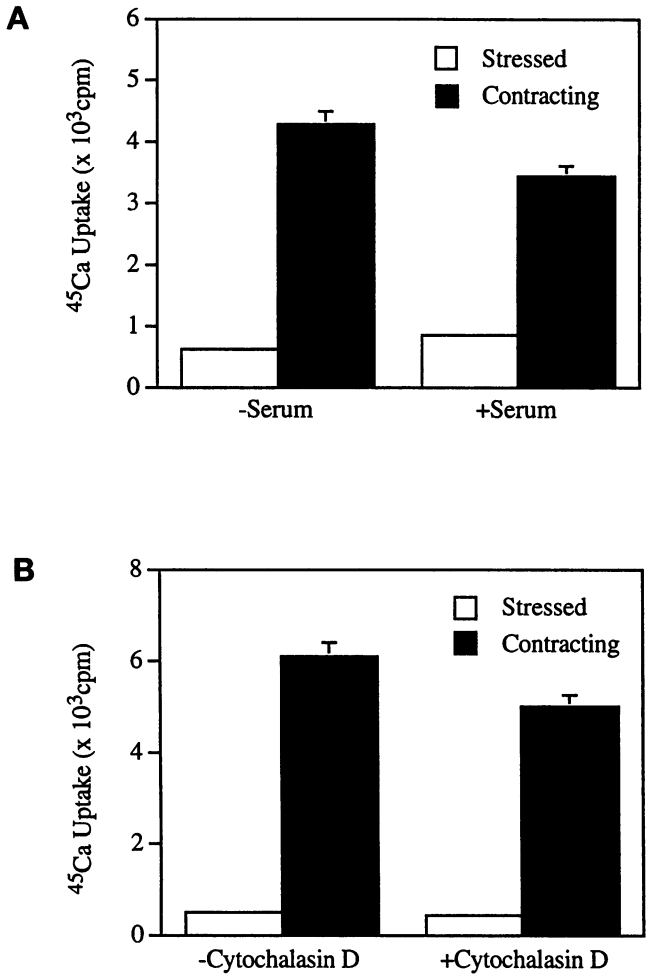

Figure 12. Effect of serum and cytochalasin D on ^{45}Ca uptake. Fibroblasts were treated the same as in Figure 11A, except that dialyzed FBS (10%) (A) or cytochalasin D (10 μM) (B) was added to HEPES medium as indicated. Data are averages ± SD from duplicate samples.

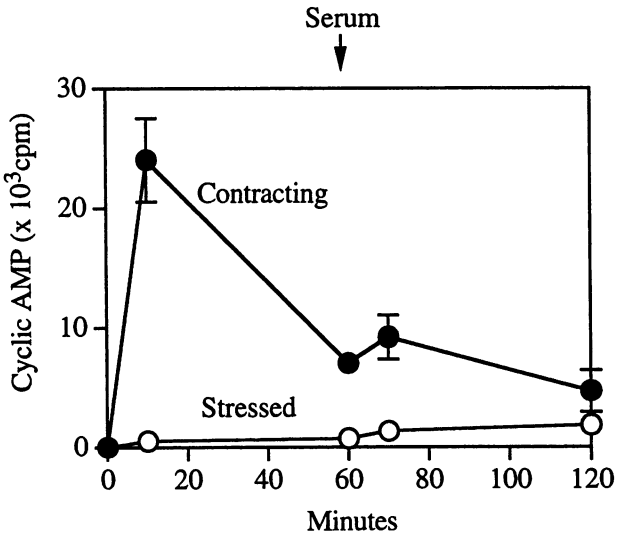

Figure 13. Activation of cyclic AMP signaling occurs during spontaneous contraction. Fibroblasts in anchored collagen matrix cultures were prelabeled overnight with [^{3}H]adenine. The cultures were switched to serum-free DMEM and one-half of the matrices were released to initiate contraction. After 60 min, FBS (10%) was added to stimulate regulated contraction. At the times shown, samples were extracted and used to measure cyclic AMP production as described in MATERIALS AND METHODS. Data are averages ± SD from duplicate samples.

Plasma Membrane Passages and Signal Transduction

Previous studies demonstrated that plasma membrane passages permeable to small proteins and dextrans occur in vivo in a variety of tissues subjected to normal mechanical stress as well as trauma (McNeil and Ito, 1990; McNeil, 1993; Clarke *et al.*, 1995). These passages were proposed to provide a potential mechanism for release of basic fibroblast growth factor or other growth factors lacking a signal sequence (McNeil *et al.*, 1989; McNeil, 1993). Our findings suggest, however, that plasma membrane passages may have a much broader significance for cell signal transduction. Uptake of FITC-dextran and ^{45}Ca occurred at the same time, and the amount of ^{45}Ca uptake was comparable to FITC-dextran uptake, i.e., ≈0.01-0.025% of the total added to the medium. It seemed likely, therefore, that ^{45}Ca and FITC-dextran entered the cells together. Since cyclic AMP levels increased under the same conditions, we believe that the contraction-dependent plasma membrane passages provide the sites of Ca^{2+} uptake required for contraction-activated cyclic AMP signaling.

Other evidence for activation of signal transduction pathways in response to formation of plasma membrane passages comes from in vitro wounding experiments. When a cell monolayer is wounded by scraping, cells along the wound edge take up FITC-dextran (Swanson and McNeil, 1987), selectively turn on c-fos and several other genes (Verrier *et al.*, 1986; Pawar *et al.*, 1995), and are activated to migrate (Gurney, 1969; Vasiliev *et al.*, 1969). Ca^{2+} entry into wounded cells also has been implicated in activation of phospholipase A2 (Yawo and Kuno, 1983, 1985), activation of calpain (Xie and Barrett, 1991), stimulation of Ca^{2+}-induced exocytosis (Steinhardt *et al.*, 1994; Bi *et al.*, 1995; Miyake and McNeil, 1995), and resealing of the plasma membrane depended on Ca^{2+} uptake in the latter studies. We also found that chelating Ca^{2+} with EGTA retarded closing of the plasma membrane passages after fibroblast contraction.

Role of Adhesion Site Shearing in Formation of Plasma Membrane Passages

How dextran-permeable passages form in the plasma membrane remains to be determined, but changes in

cell surface and actin cytoskeletal organization associated with spontaneous contraction appeared to be sufficient to induce opening. On the other hand, plasma membrane passages were not observed to form if the actin cytoskeleton was disrupted in the absence of cell contraction, i.e., when fibroblasts in anchored matrices were treated with cytochalasin D.

Rapid reorganization of cell–matrix adhesion sites may be involved in formation of the passages. The 200-nm ectocytotic vesicles released during contraction were observed to be connected to the plasma membrane by thin stalks or found in the collagen matrix, often bound to collagen fibrils. We suggested that these vesicles, which contain actin, β1 integrin, and annexins II and VI, might have been adhesion sites between cells and collagen fibrils (Lee *et al.*, 1993). An attractive hypothesis, therefore, is that shearing of adhesion sites resulted in formation of plasma membrane passages corresponding to the 3- to 5-nm FITC-dextran-permeable sites. Release of ectocytotic vesicles also has been observed in other circumstances where cells undergo rapid changes in cell–matrix interactions, e.g., during cartilage mineralization (Anderson, 1984), which is accompanied by the extracellular appearance of cytoplasmic annexins (Pfaffle *et al.*, 1988).

A shearing mechanism would be consistent with the more general observation of "membrane ripping" as a typical feature of fibroblast motility (Regen and Horwitz, 1992; Lauffenburger and Horwitz, 1996) necessary for cells to move through extracellular matrix (Hay, 1989). In addition, the force generated by fibroblast tail retraction has been shown to be sufficient to shear cells from extracellular matrix at adhesion sites leaving behind membrane fragments (Chen, 1981). Even the cell rounding response induced by trypsinization of cells may under some circumstances result in permeabilization of cells to trypsin and other small proteins (Brugmans *et al.*, 1979; Lemons *et al.*, 1988).

Role of Plasma Membrane Passages in Mechanocoupling

Extensive research has shown that mechanical force can regulate cell function in diverse mammalian tissues (for review, Watson, 1991; Sadoshima *et al.*, 1992; Davies and Tripathi, 1992; Komuro and Yazaki, 1993; Simpson *et al.*, 1994; Duncan and Turner, 1995; Liu *et al.*, 1995; Osol, 1995; Yamazaki *et al.*, 1995). The coupling mechanisms between force and response, however, are not well understood. One proposed coupling mechanism depends on direct linkage of extracellular matrix to the cell cytoskeleton through integrins, i.e., "tensegrity" (Ingber, 1991; Wang *et al.*, 1993), and a role for integrins in mechanoregulated signaling has been shown by several laboratories (Kroll *et al.*, 1993; Chen and Grinnell, 1995; Nebe *et al.*, 1995; Wilson *et al.*, 1995). In addition, mechanocoupling has been shown to involve Ca^{2+} uptake into cells (Sachs, 1988; Morris, 1990; French, 1992). Unlike sensory cells such as the vertebrate hair cell, for which there is a detailed molecular model of mechanocoupling (Hudspeth, 1989; Hudspeth and Gillespie, 1994), mechanosensitive ion channels in nonsensory cells remain poorly defined.

Although formation of plasma membrane passages during fibroblast contraction provides a mechanism for mechanoregulated Ca^{2+} entry into cells and results in a variety of downstream signaling events, this mechanism is clearly different from the traditional concept of mechanosensitive ion channels. At the present time, we have no evidence for specificity of the passages. Nor do we know whether opening of the passages is regulated other than by rapid changes in mechanical stress. The anchored collagen matrix model will be particularly useful in further studies on these problems, since contraction occurs synchronously throughout the entire cell population and does not involve macroscopic wounding of the cells. Given the widespread occurrence in vivo and in vitro of plasma membrane passages permeable to small proteins and dextran, formation of these passages may be a general cellular response to rapid changes in mechanical stress.

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