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Slow Oscillations of Free Intracellular Calcium Ion Concentration in Human Fibroblasts Responding to Mechanical Stretch

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Calcium transients in single, human gingival fibroblasts were studied after mechanical stretching of flexible culture substrates. A model system was developed to reproducibly stretch and rapidly (<1 sec) refocus cells in the same focal plane so that changes in the concentration of free intracellular calcium ions ($[Ca^{2+}]_i$) were monitored without delay. Attached cells were grown on flexible bottom Petriperm dishes, loaded with fura-2/AM, and stretched by 1% or 2.8% of substrate area. The stretch caused no significant cell detachment or membrane lesions. A 1% stretch induced no calcium response, but a 2.8% stretch stimulated an initial calcium transient and the subsequent generation of $[Ca^{2+}]_i$ oscillations of up to 2,000 sec. At 1% stretch, there was no calcium response. Cell shape and plating time were important determinants in the calcium response to mechanical stimulation: the responder cells were small and round without long processes. Major calcium transients were inhibited completely by 5 mM EGTA or by 10 μM gadolinium ions, by 50 μM nifedipine, or 250 μM verapamil, suggesting an influx of calcium through stretch-activated (SA) channels and L-type calcium channels. Depolarization by high KCl (144 mM) in the extracellular medium enhanced the amplitude of calcium transients by 54%. Calcium oscillations were not inhibited by preincubation with thapsigargin, caffeine, cholera toxin, staurosporine or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), indicating that IP_3 sensitive pools, IP_3 insensitive pools, $G_{s\alpha}$ subunits, and protein kinase C, respectively, were not involved in the generation of calcium oscillations. Pretreatment with genistein, a specific tyrosine kinase inhibitor or cytochalasin D, an inhibitor of actin polymerization, or pertussis toxin, an inhibitor of $G_{i\alpha}$ and $G_{0\alpha}$ subunits, completely abolished calcium transients and oscillations. These results indicate that Ca^{2+} flux due to mechanical stretching is likely mediated through SA ion channels and is dependent on tyrosine kinases, pertussis toxin-sensitive subunits of G-proteins, and actin filaments.

A number of different cell types exhibit transduction systems that convert externally applied forces to signals that regulate cellular metabolism. For example, applied mechanical force leads to more rapid bone remodelling in vivo (Rubin and Lanyon, 1985). Mechanical loading in vitro stimulates cell division (Curtis and Seehar, 1978; Brunette, 1984), alters collagen synthesis (Leung et al., 1976; Jones et al., 1991), promotes collagenase activity (Lambert et al., 1992), and increases prostaglandin release (Somjen et al., 1980; Yeh and Rodan, 1984). These metabolic responses are mediated in part by the generation of second messengers including intracellular calcium ions (Jones et al., 1991; Naruse and Sokabe, 1993). Calcium may in turn regulate IP_3 (Suzuki et al., 1990) or protein kinase C (Jones et al., 1991), implying a fundamental role for $[Ca^{2+}]_i$ in the modulation of downstream intracellular events.

Calcium transients and elevation of IP_3 are early responses to shear forces (Berthiaume and Frangos, 1993) and strain applied to endothelial cells (Naruse and Sokabe, 1993). A direct role for stretch-activated (SA) ion channels in mediating the calcium flux due to membrane stretch has been implied in patch clamp studies of osteoblasts (Davidson, 1990) and fibroblasts (Stockbridge and French, 1988). However because of the very high force levels and large-scale deformations of cell membranes that have been used in the investigation of mechanosensitive ion channels, the physiological significance of these findings is unclear (Morris, 1990). Little definitive information is available about the involvement of SA ion channels in Ca^{2+} mobilization and biochemical signal transduction in cells responding to low levels of mechanical deformation.

Activation of calcium signalling can result in the generation of $[Ca^{2+}]_i$ oscillations (Woods et al. 1987; Berridge and Irvine, 1989). For example, cytosolic Ca^{2+} oscillations are induced in depolarized REF52 fibroblasts after stimulation by vasopressin (Harootunian et al. 1988), an agonist that stimulates IP_3 formation

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through phosphoinositide breakdown. Current models for calcium oscillations are based on various combinations of Ca^{2+} feedback, IP_3 oscillations, protein kinase C-dependent pathways, and Ca^{2+} stimulation of phospholipase C (Berridge, 1990; Dupont et al. 1991; Harootunian et al. 1991). However, there are no available data that describe regulation of mechanically induced calcium oscillations in non-excitable cells.

Cells in periodontal tissues are subjected to compressive and tensile forces during normal function. These cells exhibit calcium dependent actin assembly in response to low levels of mechanical stretch (Pender and McCulloch, 1991). Consequently periodontal cells provide a sensitive model to study physiological calcium responses induced by relatively low levels of whole cell deformation. As it is very difficult to perform patch clamp studies of calcium permeable channels in attached cells after whole cell stretch (e.g., Naruse and Sokabe 1993), instead we monitored $[Ca^{2+}]_i$ in fibroblasts after stretch. Small, precise deformations were applied to flexible substrates. We demonstrate the generation of whole cell $[Ca^{2+}]_i$ oscillations in single fibroblasts after stretching, without depolarization or treatment with hormonal agonists.

MATERIALS AND METHODS

Cell culture

Human gingival fibroblasts obtained as described (Arora and McCulloch, 1994) were grown in T-75 flasks (Costar, Mississauga, ON) containing minimal essential medium (α-MEM) supplemented with antibiotics (0.17% penicillin V, 0.1% gentamycin sulphate and 0.01 μg/ml amphotericin, Sigma, St. Louis, MO) and 15% fetal bovine serum (ICN, Flow Labs, Mississauga, ON). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide and passaged with 0.01% trypsin (Gibco BRL, Burlington, ON). Prior to each experiment, cells were detached and 10^5 cells were plated in 60 mm dishes with a flexible hydrophilic plastic growth surface (Petriperm GmbH, Reutlingen, Germany). Cells between the 3rd and 10th passage were used.

Intracellular Ca^{2+}

Cells were loaded with fura-2 by incubation with fura-2/AM (3 μM) for 30–45 minutes at 25°C, conditions in which compartmentalization was minimal and more uniform loading was obtained than at 37°C. The buffer consisted of a bicarbonate-free medium containing 150 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM $MgSO_4$, 1 mM $NaHPO_4$, and 20 mM HEPES at pH = 7.4 with an osmolarity of 291 mOs. For experiments requiring external calcium, 1 mM $CaCl_2$ was added to the buffer. The cells were plated at 37°C ± 0.2°C in a temperature-regulated chamber and allowed to attach for 60 minutes before stretching. Whole cell $[Ca^{2+}]_i$ measurements were obtained with a Nikon Diaphot II inverted microscope optically interfaced to a Deltascan 4000, dual beam, epifluorescence spectrofluorimeter and analysis system (Photon Technology Int., London, ON) operating on a 386 SX personal computer. Emitted fluorescence was collected with a 40× quartz 1.32 NA oil immersion Nikon Fluor objective and passed through a 520/30 nm barrier filter (Omega Optical, Brattleboro, VT). A variable aperture, intrabeam mask was used to restrict measurements to single cells (15 μm diameter). Signals from the photomultiplier tube (D104, PTI) were recorded at a minimum of 5 points/second.

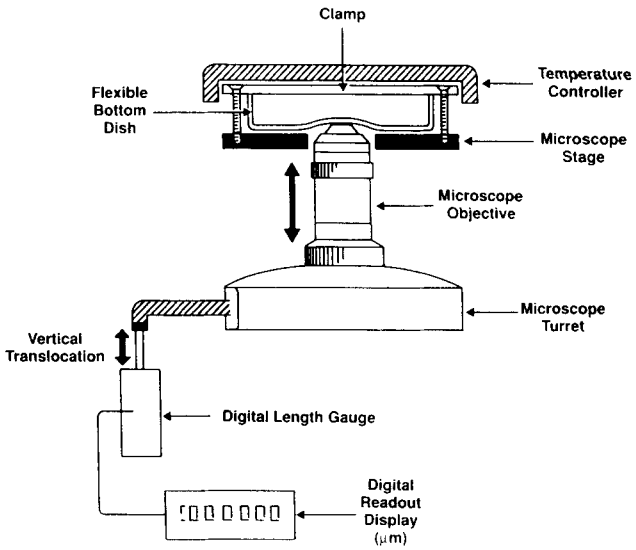

Fig. 1. Diagram of the inverted fluorescence microscrope and the mechanism for precisely deforming the bottom surface of the flexible membrane of the culture dish immediately under the cell to be measured. Note that the region of membrane deformation is within the middle one third of the culture dish but stretching occurs across the whole area of the membrane.

Estimates of $[Ca^{2+}]_i$ independent of the precise intracellular concentration of fura-2 were calculated from dual-excitation emitted fluorescence according to the equation of Grynkiewicz et al. (1985) where $[Ca^{2+}]_i$ (in nM) = $K_d \times Sf2/Sb2 \times (R - R_{min})/(R_{max} - R)$. The K_d (224 nM) and Sf2/Sb2 ratio (12) were calculated from eleven excitation wavelength scans of 1 μM fura2-free acid (Molecular Probes, Eugene, OR) in buffers with $[Ca^{2+}]$ ranging from 0–39.8 μM (calcium calibration kit, Molecular Probes). The maximal 346/380 ratio (R_{max}) was measured after saturation of intracellular fura-2 with Ca^{2+} by adding 3 μM ionomycin (Calbiochem, La Jolla, CA) to allow equilibration with extracellular calcium ions. The minimal 346/380 ratio (R_{min}) measured during complete dissociation of fura-2 from Ca^{2+} was obtained by adding 2mM EGTA to the ionomycin-treated cells. Background autofluorescence was estimated by incubating cells with buffer containing 1 mM Mn^{2+} and the photon count was subtracted from the cellular fluorescence values before $[Ca^{2+}]_i$ was estimated.

Cell stretching

A modified, inverted fluorescence microscope (Nikon Diaphot) with fixed stage and moveable objective lens/turret assembly was used to stretch cells and then rapidly measure $[Ca^{2+}]_i$. The turret of the microscope was attached to a pressure sensitive, digital length gauge (Heidenhain, Traunreut, Germany). Any vertical displacement of the turret was precisely measured as the distance travelled by the objective in an upward or downward direction (Fig. 1). Defined vertical deforma-

tion (accuracy = ±1 μM) of the membrane by the upper face of the objective was achieved by rigid attachment of the Petriperm dish to the microscope stage and by upward movement of the objective to deform the flexible membrane. Two levels of stretch (3 mm and 5 mm of vertical deformation) were applied to evaluate if different stretch levels induced variations of $[Ca^{2+}]_i$ responses. Stretches >7 mm produced irreversible deformation of the membrane of Petriperm dishes and were not used. After a single stretch (1 sec duration), the objective and the turret were returned to the baseline reading on the digital length gauge that had originally been established for optimal focus. This approach provided a system for very rapid and accurate refocusing (<1 sec) after stretching. The proportional (%) stretch of the membrane was calculated by first measuring the height of the membrane displaced (h) with the digital length gauge. The stretch induced a localized, vertical deformation of the membrane in the middle one-third of the dish (Fig. 1), but the actual stretch of the membrane was delivered across the whole dish, albeit not uniformly.

We estimated the overall expanded membrane area during stretch by assuming that the entire membrane area of the dish was stretched. This appears to be a reasonable assumption as planimetric measurements of stretched membranes (Pender and McCulloch, 1991) showed stretching right up to the junction of the membrane with the dish. The unstretched (control) area of the membrane was πr^2 where r = 30 mm. The expanded membrane area was $\pi(r^2 + h^2)$, a term derived by integrating the equation: expanded membrane area = $\int_0^{\Theta_r} 2\pi R \sin \Theta R \, d\Theta$ (Naruse and Sokabe, 1993). In this equation, Θ_r was one-half of the central angle of the expanded membrane, R was the radius of curvature induced by the deformation and the equation can be reduced to: % expanded area = $100 \times (h/r)^2$. Immediately above the lens, there was an estimated 1% increase of surface area for 3 mm of vertical deformation and a 2.8% increased surface area for 5 mm of vertical deformation.

Cell size

Estimates of cell length and area, during and after stretch were made with three different methods. The actual dimensional change of the cells during stretching was assessed by, first, loading cells with the cytoplasmic space filling dye Calcein/AM (2 μM; Molecular Probes). Then, an image analyser (Leica, Wetzlar, Germany) was used to estimate the difference in the maximum length of live cells before and during the stretching period. Cells were measured in control conditions and then stretched. Cells were held in the stretched mode with plastic templates (3 or 5 mm high) that mimicked the shape of the microscope lens. The cells were brought back into focus and cells were measured again. Second, cells were fixed with formaldehyde, stained with FITC-phalloidin (5×10^{-5} M; Pender and McCulloch, 1991), and the maximum length of cells before and during stretching was assessed. These two methods provided data to compare dimensional changes of live and dead cells (i.e., active versus passive responses) before and during stretching. In the third method, cells were loaded with Calcein/AM, measured, stretched, and then returned to the original, unstretched condition and measured again. These data were used to assess if cells exhibited any rebound after stretching.

Possible cell damage due to membrane stretch was assessed by measuring the intensity of fura-2 emission at the isosbestic point (356 nm excitation) before and after stretching. The emission intensity at this wavelength is independent of changes in $[Ca^{2+}]_i$ but is dependent on the amount of intracellular fura-2, thereby providing a measure of membrane integrity. We also assessed if stretching disrupted cell attachment to the substrate. Calcein/AM loaded cells were imaged with a laser scanning confocal microscope (Leica) and transverse, optical sections (nominal thickness = 0.5 μm) were obtained near the cell-substrate interface.

Calcium pathways

Specific inhibitors were used to identify the different pathways that may mediate transmembranous Ca^{2+} fluxes and the mobilization of intracellular calcium in response to stretch. Gadolinium chloride (Aldrich, Milwaukee, WI; Yang and Sachs, 1989) and Mn^{2+} were used to assess conductance through putative stretch-activated channels, whereas EGTA incubations were used to examine the source of the calcium. Verapamil and nifedipine (Sigma) were used to examine the contribution of L-type calcium channels to stretch. The contribution of internal calcium release to the cell stretch response was studied by incubating cells with thapsigargin (Tg; Sigma), whereas the role of caffeine-releaseable stores was examined by incubation with caffeine (Sigma). Tetra-phorbol 12-myristate 13-acetate (TPA; Sigma) or 1-(5-isoquinolinesulfonyl)-2-methylpiperzine (H-7; Toronto Research Chemicals, Mississauga, ON) or staurosporine (Sigma) were used as inhibitors of protein kinase C. The role of tyrosine kinases in the generation of $[Ca^{2+}]_i$ transients was examined by the use of genistein to inhibit kinase activity (BRL, Life Technologies, Gaithersburg, MD) or its inactive analogue genistin (Extrasynthase, France). The contribution of actin polymerization in elevating $[Ca^{2+}]_i$ in response to cell stretch was assessed by incubating cells with cytochalasin D (Sigma). For all experiments involving blockade of putative regulatory pathways of the calcium permeable channels, data from previous studies of mechanical stretching of fibroblasts (Pender and McCulloch, 1991) or of cell signalling in fibroblasts (Knowles et al., 1991; McCulloch and Knowles, 1993) were used to determine drug dosages. For drugs that had not been previously evaluated (H-7, genistein), dose-response experiments were conducted on cells in suspension (10^6 cells/ml). To establish maximal inhibitory concentrations within a physiological range, PGE_2 at 10^{-5} M was used as an agonist.

Analysis of data

Although the amplitude and frequency of the calcium oscillations varied from cell to cell, they were reasonably constant for individual cells responding to repeated stretching. We restricted observations only to responding cells by first establishing that each cell would indeed exhibit a calcium response to the stretch. Next, cells were treated with the appropriate blocker

TABLE 1. Assessment of cell damage by stretching[1]

	3 mm stretch	5 mm stretch
% change of fura-2 fluorescence at isosbestic point	−0.30% ± 0.12%	−0.20% ± 0.25%
% change of cell area at substrate	n.d.	+2.30% ± 0.7%

[1]Mean % change (± s.e.m.) of fura-2 fluorescence measured by fluorimeter on single attached fibroblasts at either 3 mm or 5 mm of vertical deformation, before and after stretch. No significant loss of dye ($P > 0.2$) at 5 mm stretch. (n = 10 for each of 3 mm and 5 mm stretch). Mean % change (± s.e.m.) of cell area measured by confocal microscopic optical sections in Calcein-loaded cells. Cells exhibited slightly increased area after stretching.

and the cell was stretched. The pre- and posttreatment calcium responses after stretching were recorded. Thus each cell provided its own internal control for each series of trials within an experiment, an approach previously described as fingerprint analysis (Bird et al., 1993).

Means and standard errors of the mean were calculated for the cell dimensions and for the variables associated with intracellular $[Ca^{2+}]$ measurements. Calcium measurements were restricted to: (1) baseline $[Ca^{2+}]_i$, (2) percentage change of the transient $[Ca^{2+}]_i$ above baseline levels, (3) net change in $[Ca^{2+}]_i$, (4) time between oscillations of $[Ca^{2+}]_i$, and (5) time width of the half maximal $[Ca^{2+}]_i$ transient. From preliminary observations of the calcium transients and oscillations generated by cell stretching, we adopted the following analytical approach for assessing the calcium response: calcium transients that exceeded 100% of the resting basal Ca^{2+} level were described as transients, whereas rhythmic increases of Ca^{2+} were described as oscillations (see Fig. 5 for representative traces).

RESULTS

Cell stretching

After a single 3 mm or 5 mm vertical applied stretch, cells were refocussed and the change in fura-2 fluorescence intensity at the isosbestic point was compared to the unstretched control conditions. There was no significant change in fluorescence after stretching of 5 mm (Table 1), indicating that dye loss and focal plane changes were negligible as a result of the two different levels of applied experimental stretch. Attached cell areas before and after 5 mm of vertical deformation were assessed by confocal microscopy. Cell area was increased slightly as a result of the stretch (Table 1, Fig. 2) but these changes were detectable only by computer-assisted measurement and were not obvious by visual inspection.

Live cells loaded with Calcein/AM were held in a stretched (5 mm vertical deformation) configuration and assessed by image analysis. These cells also exhibited a small reduction in maximum cell length compared to their prestretch length (i.e., rebound; Table 2). In contrast, formaldehyde-fixed (dead) cells stained with FITC-phalloidin and stretched (5 mm of vertical deformation) showed an increase of maximum length compared to the prestretched length and thus did not exhibit rebound. Thus the stretch of the flexible substrate did indeed deliver measureable stretching of the attached cells, but the return to pre-stretch cell length was dependent on cell viability. Next, we determined if the cell rebound after stretching was dependent on actin assembly. Calcein-loaded cells were stretched with and without cytochalasin D (500 ng/ml; 30 min) in the buffer and measured 60 seconds after stretch. Compared to cytochalasin-treated cells, controls exhibited sevenfold more rebound after stretching (Table 2).

Calcium responses

When single fibroblasts loaded with fura-2 were stretched, vertical deformations of the membrane of ≤3 mm were never found to stimulate a Ca^{2+} response (n = 30), whereas stretches of 5 mm induced calcium transients. Cell shape and time of plating were important factors in eliciting a $[Ca^{2+}]_i$ response. The responder cells were always small and round without long processes (Fig. 3) and exhibited calcium fluxes on repeated stretches (Fig. 4). In preliminary experiments using 5 mm stretch, two of 30 cells (7%) responded to the mechanical stretch when plated 24 hours before the experiment, whereas five out of 35 cells (14%) plated 2 hours before stretching exhibited responses. Subsequently, out of 280 trials to examine the proportional response of cells to stretch after 2-hour plating times, 35 cells exhibited calcium responses (12.5%).

The basal intracellular calcium level was 95.8 ± 7.33 nM. Although each fibroblast displayed a unique pattern of intracellular calcium responses to the same level of stretch, the application of stretch in responding cells always resulted in rapid calcium transients and subsequent calcium oscillations that were interrupted by a second Ca^{2+} transient of high amplitude (see examples in Fig. 5A; statistical analysis in Table 3). In some cells, oscillations were observed up to 2,000 seconds prior to return to baseline values (Fig. 5B) or there were oscillations that were superimposed on the decaying tail of an existing oscillation. These oscillations were not included in the statistical analyses (Table 3). In all instances, the oscillations exhibited the "sawtooth" shape transients described by Berridge (1990) and in which Ca^{2+} returned to or near to basal Ca^{2+} levels.

Role of extracellular calcium

To establish if the increase in intracellular calcium in response to stretching was due to calcium influx or due to internal mobilization of calcium, fura-2 loaded cells were incubated in 5 mM EGTA immediately prior to stretching. These cells (n = 5) exhibited no rise in cytosolic calcium nor any oscillations, suggesting that the primary and secondary calcium transients and the generation of oscillations were absolutely dependent on calcium influx from external bathing calcium (Fig. 6).

Stretch-activated calcium channels

The opening of calcium-conducting SA channels in response to mechanical stimulation has been studied previously in a variety of cell types by patch-clamp techniques (Stockbridge and French, 1988; Davidson et al., 1990; Davidson 1993). To evaluate the contribution of stretch-activated, calcium-permeable channels to the increase of Ca^{2+} after mechanical stretch, we used the putative SA channel blocker gadolinium chloride (Yang and Sachs, 1989). Nonstretched cells treated with 10 μM $GdCl_3$ exhibited a very slow rise in intracellular calcium. After 1 minute incubation with 10 μM

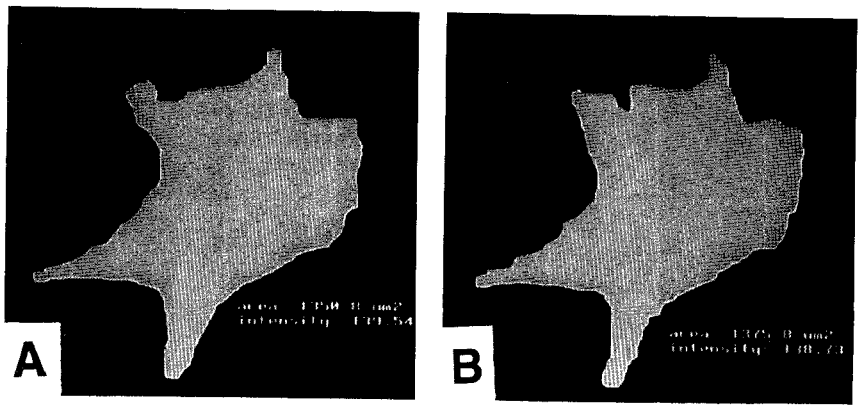

Fig. 2. Paired micrographs of the same cell before (**A**) and after (**B**) stretching, showing only very small changes in cell area at the substrate-cell interface. Cells were loaded with Calcein/AM and imaged by confocal microscopy. ×540

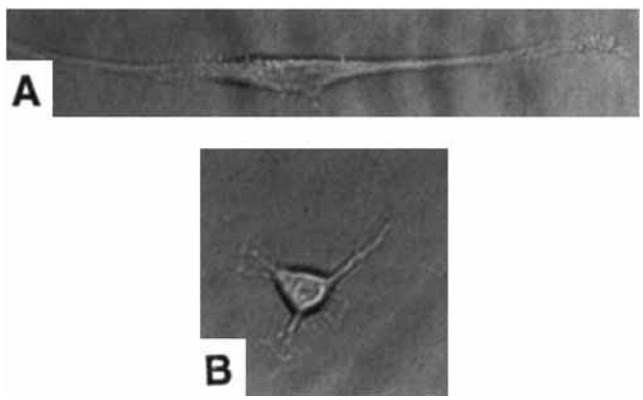

Fig. 3. Brightfield micrographs of a fibroblast that exhibited no calcium flux to whole cell mechanical stretch (**A**) and an example of a typical calcium-responsive cell (**B**). Note the small and round morphology of the responder cell and the relatively short cell processes (×500). Responder cells exhibited $[Ca^{2+}]_i$ response to stretch in ~12% of trials.

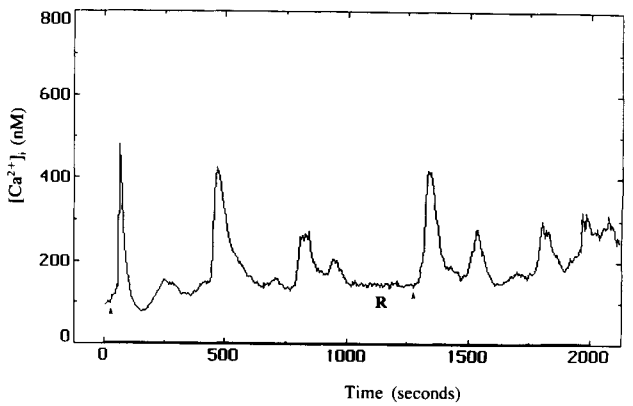

Fig. 4. Sample tracing of $[Ca^{2+}]_i$ demonstrating responses of single fibroblast to stretching with 5 mm vertical deformation of membrane (or 2.8% increased surface area). Ratio fluorimetry of fura-2-loaded single fibroblasts was used to measure $[Ca^{2+}]_i$. Arrow shows the time point when stretch was applied. First stretch (arrow) followed by recovery (R) after stretch and induction of a second response after a second stretch (arrow). Note the variation of amplitude but relative conservation of period between the stretches in the same cell.

TABLE 2. Changes of cell length after stretching[1]

	Live cells	Fixed cells
Held in stretched configuration	−2.83% ± 2.0%	+1.33% ± 0.34%
Stretched and then relaxed in normal buffer	−1.0% ± 3.6%	n.d.
Stretched and then relaxed in cytochalasin	+5.0% ± 5.1%	n.d.

[1]Live cells were loaded with Calcein. Fixed (dead) cells were stained with FITC-phalloidin. Cells were stretched by 5 mm vertical deformation of membrane and held in stretched configuration or relaxed to control conditions. Data are mean % difference (± s.e.m.) in length of single cells (before stretch-after stretch). (n = 20 for each of live and dead cells).

$GdCl_3$, cells exhibited no detectable calcium transients or oscillations (n = 5). When stretched after 30 minutes of incubation with 10 μM $GdCl_3$, the two major calcium transients that we observed in control cells were blocked, although the cells continued to exhibit very low amplitude calcium oscillations after the stretch (Fig. 7; n = 5). As the apparent blockade of calcium could be due to $GdCl_3$ quenching of intracellular fura-2 after endocytosis over long periods, we evaluated whether $GdCl_3$ could quench fura-2 fluorescence. Incubation of fura-2 free acid (3 μm) with $GdCl_3$ (10 μm) demonstrated no quenching (Fig. 7A inset).

Although Gd^{3+} at 10 μm is a strong blocker of SA channels, some other types of Ca^{2+} channels (e.g., L-type) are also blocked by Gd^{3+} (Biagi and Enyeart, 1990). Consequently in the next experiment we sought to minimize the potential blockade of non-SA channels and evaluated single fibroblasts after incubation with low concentrations of gadolinium ions (1μM) for 80 minutes and then stretched the cells. In these experiments, there were no primary transients or oscillations. However, a secondary transient of reduced amplitude (~66%) was still present (Fig. 7B; n = 8).

Mn^{2+} is one of the most permeable cations of mechanosensitive ion channels (Ohmori, 1985; Ito et al., 1990). Quenching of fura-2 fluorescence by Mn^{2+} permits detection of its entry into the cytoplasm. When 1

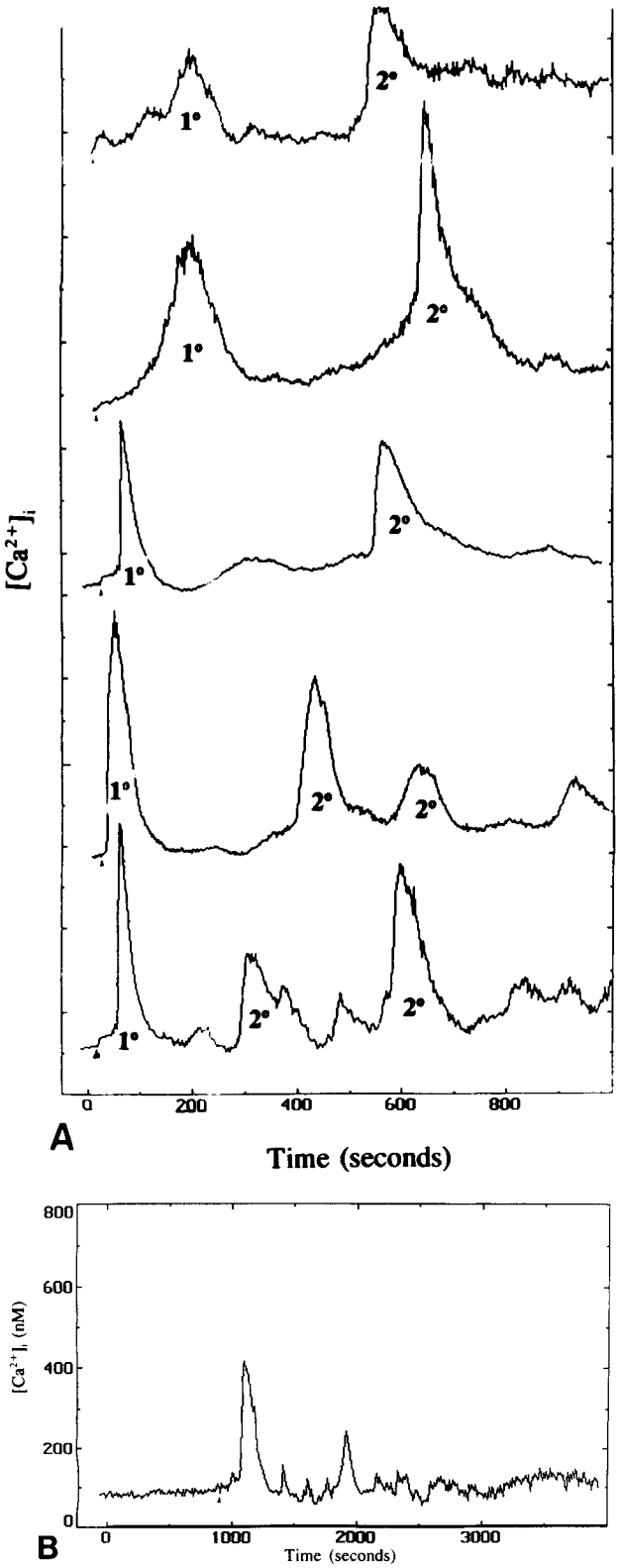

Fig. 5. **A.** $[Ca^{2+}]_i$ tracings of 5 different attached fibroblasts after stretching. The 1° and 2° transients were defined operationally if increases of $[Ca^{2+}]_i$ exceeded 100% of the basal $[Ca^{2+}]_i$. Oscillations were defined as rhythmic transients <100% of basal $[Ca^{2+}]_i$ levels. Note the wide variation of amplitude and period between different cells. The illustrated cells were chosen as those exhibiting the most extreme examples of the various types of calcium transients and oscillations. The traces have been offset for clarity. **B.** $[Ca^{2+}]_i$ tracing of a single fibroblast demonstrating stable $[Ca^{2+}]_i$ prior to stretching (arrow) and the prolonged oscillations that are detectable for up to 3,000 seconds before a stable baseline is established again.

mM $MnCl_2$ was added to the normal external bathing solution, a single stretch induced a single intracellular Ca^{2+} transient of fivefold lower amplitude than controls. This transient decayed over time and there were no further transients and oscillations (Fig. 8; n = 4). In contrast, cells without stretching exhibited no change in $[Ca^{2+}]_i$ after addition of Mn^{2+}. To determine if Mn^{2+} entry into the cytoplasm through SA channels occurred in the absence of Ca^{2+}, the medium was depleted of extracellular Ca^{2+} and on a molar basis was totally replaced with Mn^{2+} in the buffer. A 5 mm stretch caused no increase in $[Ca^{2+}]_i$ above baseline and the signal decreased slowly, reflecting fluorescence quenching of fura-2 by the entry of Mn^{2+} into the cytoplasm (n = 3). When $GdCl_3$ (10 μM for 1 minute) was added to buffer with 1 mM $MnCl_2$ containing Ca^{2+}, there was no clacium flux and a very slow reduction of $[Ca_{2+}]_i$, indicating that $GdCl_3$ inhibited entry of Mn^{2+} via calcium-permeable channels (n = 3).

L-type calcium channels

To examine if the initial rise in $[Ca^{+}]_i$ was due in part to calcium influx through L-type, voltage-dependent Ca^{2+} channels, we incubated cells with 50 μM nifedipine for 30 minutes. Nonstretched cells showed a slow elevation of basal Ca^{2+}, consistent with earlier work (Rosales and Brown, 1992), whereas when stretched, cells showed an initial rise in calcium but with ~45% of the amplitude of nontreated stretched cells. The latter oscillations were of extremely small amplitude or were undetectable (Fig. 9; n = 3). After incubation with 50 μM verapamil for 30 minutes (n = 3), fibroblasts exhibited an initial calcium transient and returned to basal levels. When stretched, the primary transient was blocked and oscillations were of low amplitude, whereas secondary transients were of the same amplitude as nontreated stretched cells. After incubation in 250 μM verapamil (n = 3), calcium transients were not seen in unstretched cells and after stretching, there was no detectable change in the $[Ca^{2+}]_i$.

Depolarization

To determine if the response of $[Ca^{2+}]_i$ to stretch would occur after depolarization, cells were incubated in depolarization buffer consisting of normal buffer with the following modifications: 5 mM NaCl, 144 mM KCl, and 1 mM $CaCl_2$. Cells were incubated in this buffer immediately prior to stretching. Under these conditions stretched fibroblasts exhibited large increases in the primary transient (505 nM; n = 4), which was 144 nM (54%) more than control cells (Fig. 10). The second transient was 268 nM above baseline levels and lower amplitude cytosolic calcium oscillations were not observed.

Internal stores

The contribution of Ins(1,4,5)P_3-induced calcium release to the stretch-activated calcium response was studied by treatment of fibroblasts with thapsigargin (Tg), a tumor-promoting sesquiterpene lactone. Tg blocks the ATPase required for Ca^{2+} reuptake into intracellular pools, and they leak until they are empty (Berridge, 1990). To ensure that the intracellular calcium stores were indeed depleted, fibroblasts were treated with 1 μM Tg for 60 minutes. After this proto-

TABLE 3. Calcium response to stretching[1]

	1° transients	2° transients	Oscillations
Mean amplitude above baseline (nM)	361 ± 36	451 ± 43	72.4 ± 8.3
Time width at half amplitude (seconds)	59 ± 7.0	85 ± 5.5	—
Time from stretching to peak of transient or to appearance of first oscillation (seconds)	70 ± 22.8	455 ± 37.3	267 ± 34.3

[1]Mean (± s.e.m.) of calcium transients and oscillations in cells after 5 mm of vertical deformation (n = 35 cells).

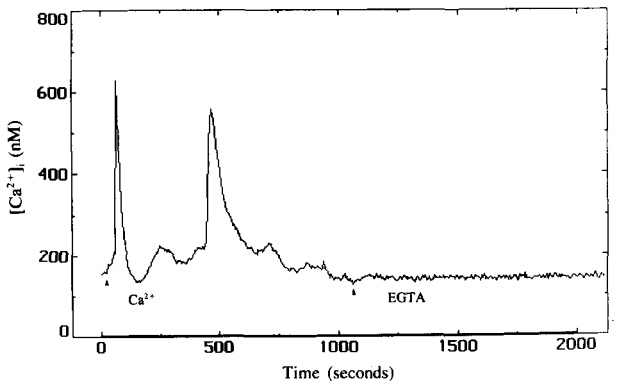

Fig. 6. Representative tracing of $[Ca^{2+}]_i$ response of a single fibroblast after stretching (first arrow) in normal buffer, followed by incubation with 5 mM EGTA and a second stretch (second arrow). Note the complete blockade of calcium transients and oscillations after depletion of bathing buffer Ca^{2+}.

col, reapplication of 1 μM Tg produced no further increase in $[Ca^{2+}]_i$. In the presence of 5 mM external EGTA, Tg induced a $[Ca^{2+}]_i$ rise that slowly decreased to basal levels, indicating the release of calcium from internal stores (Fig. 11, inset). A similar response of higher amplitude was seen in the presence of external calcium (Fig. 7a), but we never observed spontaneous oscillations as described in rat parotid acinar cells (Foskett and Wong, 1991). When stretched, Tg-treated fibroblasts exhibited calcium transients of lower amplitude and with longer period oscillations compared to cells without Tg (Fig. 11; n = 3). In the presence of EGTA, the stretched cells that had been pretreated with Tg showed no transients or oscillations (n = 3).

If mechanical stretching of cells elevates IP_3 and there is transfer of Ca^{2+} from the IP_3-sensitive internal stores to a separate IP_3-insensitive pool, then we anticipated increased $[Ca^{2+}]_i$ and repetitive dumping of calcium by Ca^{2+}-induced Ca^{2+} release (CICR). A characteristic of CICR in muscle and some neurones (Malgaroli et al., 1990) is its sensitivity to caffeine (to deplete releasable Ca^{2+} stores) or ryanodine to block the ryanodine receptor (McPherson and Campbell, 1993). Fibroblasts treated with 10 mM caffeine for 10 minutes exhibited no spontaneous oscillations as has been reported earlier for rat parotid acinar cells (Foskett and Wong, 1991). After stretching, there was no detectable alteration of the pattern of Ca^{2+} transients or oscillations compared to control cells without caffeine (n = 5; Fig. 12).

Modulation of intracellular calcium resonses by tyrosine kinase

Focal adhesion-associated kinases (Schaller and Parsons, 1993) are reported to play a central role in the generation and activation of some of the molecular signals, including changes in $[Ca^{2+}]_i$. The formation of cell adhesions may induce multiple $[Ca^{2+}]_i$ transients (Jaconi et al., 1991). We used genistein as a selective inhibitor of tyrosine kinases as it does not show inhibitory activity against other kinases such as protein kinase A and protein kinase C at the concentrations used here (Catarsi and Drapeau, 1993). After 10 minutes of incubation with genistein at 100 μM, stretched fibroblasts exhibited 95% inhibition of the amplitude of major calcium transients as compared to pretreatment controls treated with genistin (100 μm) after stretching (Fig. 13, n = 5). There were no calcium oscillations after genistein treatment. Further, cells treated with PGE_2 at 10^{-5}M after preincubation with 100 μM genistein also exhibited ~90% reduction of the amplitude of the calcium transient compared to controls without genistein. Thus genistein was effective in abrogating calcium transients after two quite different forms of stimuli.

G proteins

To determine if the stretch-induced $[Ca^{2+}]_i$ response was mediated through G-proteins, cells were treated for 30 minutes with 4 μg/ml cholera toxin (CTX) or with pertussis toxin (4 μg/ml), a dosage that is known to provide a complete block of stretch-activated actin assembly in fibroblasts (Pender and McCulloch, 1991). Cells that were pretreated with cholera toxin followed by stretching exhibited no inhibition of major intracellular transients and oscillations when compared to controls (Fig. 14; n = 3). After incubation with pertussis toxin, there was a complete absence of transients and oscillations (n = 4).

Protein kinase C

Down-regulation of protein kinase C by incubation with tetra-phorbol 12-myristate 13-acetate (TPA) at 2 μM for 30 minutes, followed by mechanical stretching resulted in the abolition of the major transients. However, Ca^{2+} oscillations of reduced amplitude persisted (Fig. 15, n = 3). Incubation with a low concentration of the PKC inhibitor staurosporine (100 nM; 30 minutes), a potent, albeit nonspecific protein kinase C inhibitor induced no detectable change of resting $[Ca^{2+}]_i$ in unstretched cells. When stretched, the major transients and oscillations were also indistinguishable from controls (Fig. 15, n = 3). Fibroblasts stretched in the presence of the more specific inhibitor of PKC, 1-(5-isoquin-

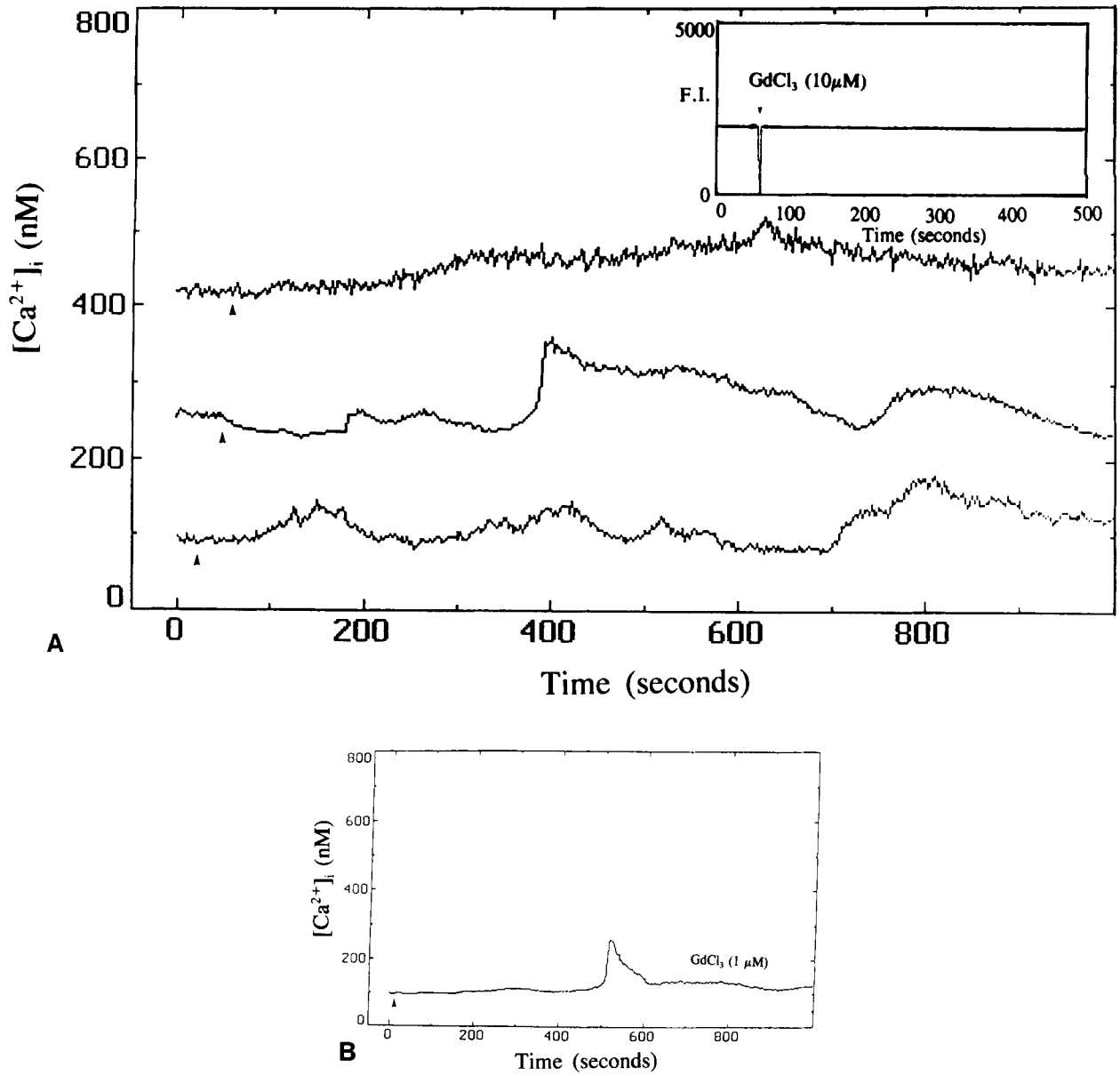

Fig. 7. **A.** $[Ca^{2+}]_i$ response of cells to stretch after gadolinium treatment. Bottom two traces show response of two individual fibroblasts pretreated with gadolinium ions (10 μM for 30 minutes). Top trace shows $[Ca^{2+}]_i$ in a cell pretreated for 1 minute with 10 μM gadolinium. Note the virtual blockade of calcium flux after 1 minute incubation. The traces have been offset for clarity. Inset shows fura-2 free acid (3 μM) in normal buffer before and after addition of 10 μM gadolinium ions. Note the absence of detectable fluorescence quenching (excitation = 340 nm, emission = 510 nm). **B.** Sample $[Ca^{2+}]_i$ tracing demonstrating response of a single fibroblast pretreated with gadolinium ions (1 μM; 80 minutes) and then stretched (arrow). Note the blockade of the 1° transient and of oscillations; only a low amplitude, 2° transient was occasionally detected as shown in this figure.

olinesulfonyl)-2-methylpiperazine (H-7; 30 minutes; Quick et al. 1992) exhibited no primary or secondary transients, but cytosolic Ca^{2+} oscillations persisted (Fig. 15, n = 3).

Actin filaments

The role of filamentous actin in the regulation of stretch-induced calcium flux was assessed by incubation of cells with cytochalasin D (500 ng/ml; 30 minutes). This treatment abolishes polymerization of cortical actin after low levels of stretch in fibroblasts (Pender and McCulloch, 1991). Fibroblasts were incubated with cytochalasin D for 60 minutes and were stretched after drug washout. Treated cells exhibited no major transients or oscillations (Fig. 16, n = 4). This finding indicated that in cells responding to mechanical stretch, membrane-anchored filamentous actin may regulate calcium flux.

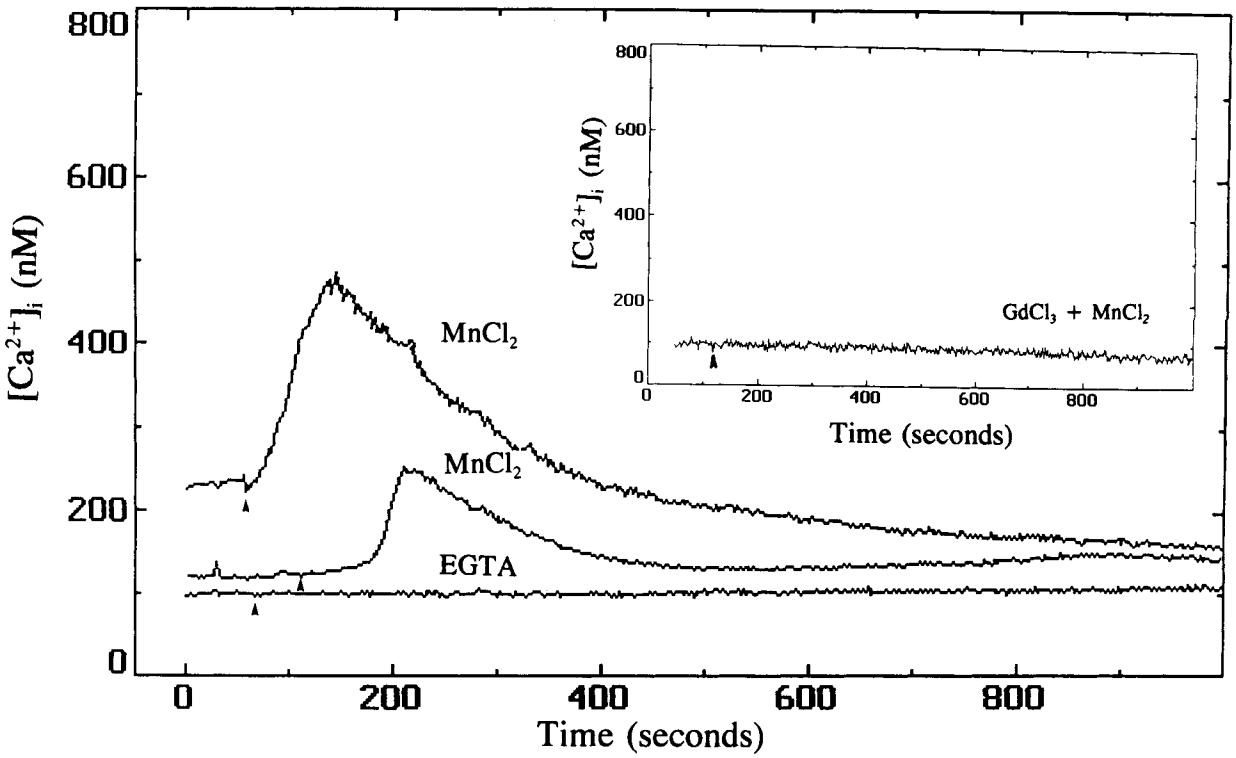

Fig. 8. Representative tracings showing $[Ca^{2+}]_i$ response of a fibroblast after stretching (arrow) when pre-incubated with 1 mM $MnCl_2$ in the normal external bathing buffer containing 1 mM Ca^{2+}. Top two traces show $[Ca^{2+}]_i$ of single fibroblasts after stretching. Bottom trace is an example of a stretched cell after Ca^{2+} was replaced with 5 mM EGTA in the external buffer. **Inset:** $[Ca^{2+}]_i$ response of a representative stretched fibroblast pretreated with 10 μM gadolinium ions for 1 minute and followed by incubation in buffer containing 1 mM $MnCl_2$. Note that Mn^{2+} quenching is minimal when SA channels are blocked by gadolinium.

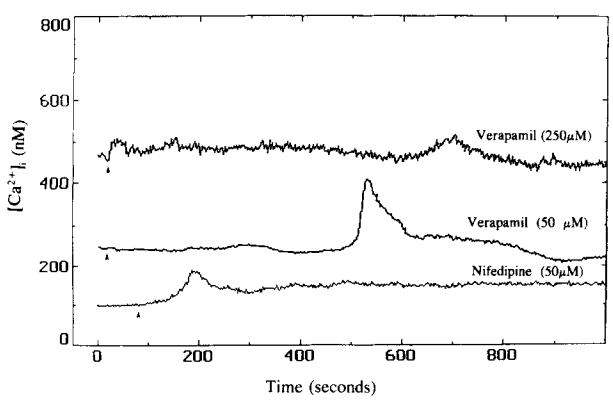

Fig. 9. Top trace demonstrates $[Ca^{2+}]_i$ response after stretching in a cell preincubated with 250 μM verapamil in normal buffer. Middle trace shows response with 50 μM verapamil and the bottom trace demonstrates response of a cell preincubated with 50 μM nifedipine in normal buffer. Arrow in all traces indicates time of stretch (5 mm vertical deformation). Note the reduced amplitude of the 1° transient and blockade of oscillations and 2° transients.

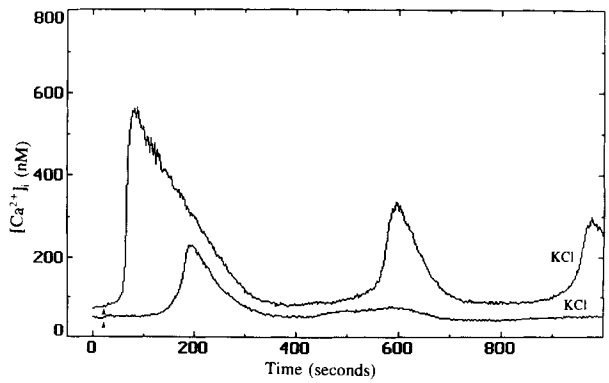

Fig. 10. Sample tracings of $[Ca^{2+}]_i$ in single fibroblasts after stretch (arrow). Cells were incubated with 144 mM KCl in normal medium to depolarize cell. Note the large amplitude transients with long periods between repeated transients and the absence of shorter period oscillations.

DISCUSSION

Cell stretching

Several methods have been used to study intracellular ion regulation after mechanical stimulation: physical prodding of cells (Xia and Ferrier, 1992), pumping of buffer solution against a cell (Morris and Horn, 1991), application of pressure through a whole cell patch clamp pipette (Gustin et al. 1988), or swelling cells with hypotonic buffer (Falke and Misler, 1987). These methods can cause artifacts such as membrane disruptions, breakdown of the patch seal, or large-scale water movement that are not likely to be physiological responses to mechanical stimulation. Indeed, Morris (1992) has em-

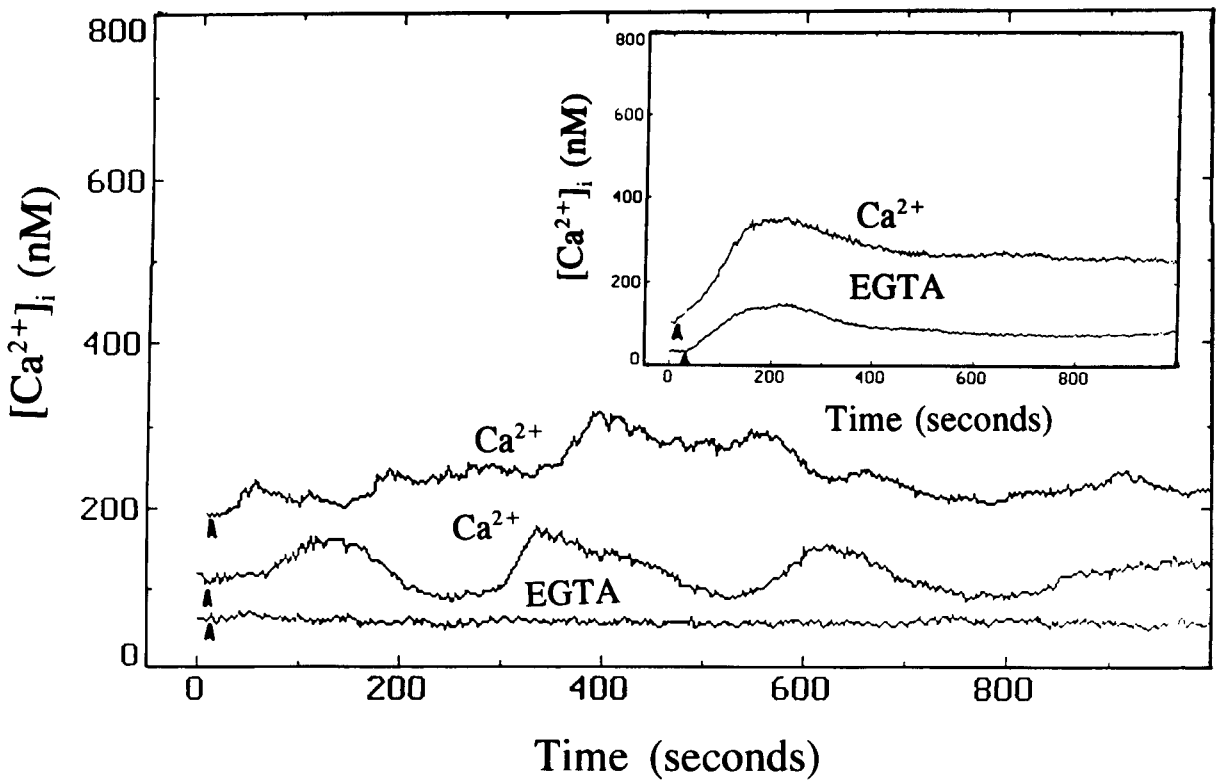

Fig. 11. $[Ca^{2+}]_i$ response in cells pretreated with thapsigargin (1 μM) for 60 minutes to deplete internal calcium stores and stretched (arrow) in medium containing Ca^{2+} (top two traces: Ca^{2+}) or in medium containing 5 mM EGTA. The traces have been offset for clarification. Note that low amplitude calcium transients are detectable after thapsigargin treatment but not when calcium is depleted from the medium. **Inset:** top trace shows response of a single, non stretched fibroblast to thapsigargin (1 μM) in buffer with calcium ions (Ca^{2+}). Bottom trace shows $[Ca^{2+}]_i$ response in a cell incubated in buffer with 5 mM EGTA and depleted of calcium ions. The bottom trace has been offset to clarify the response.

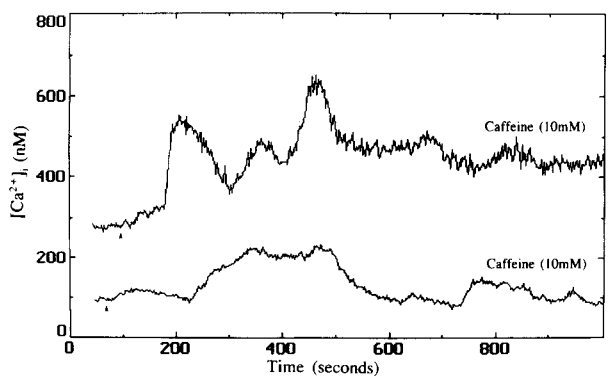

Fig. 12. Two traces of $[Ca^{2+}]_i$ in single fibroblasts pretreated with 10 mM caffeine for 30 minutes showing persistent transients and oscillations after stretch (arrow).

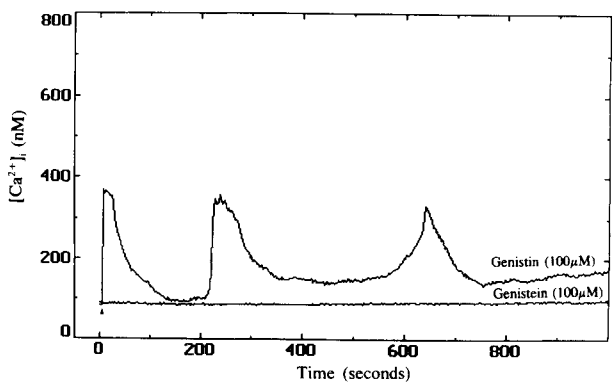

Fig. 13. Top trace demonstrates $[Ca^{2+}]_i$ response to stretching after pre-incubation with genistin (100 μM) an inactive analogue of the selective tyrosine kinase inhibitor genistein. The bottom trace shows response in a cell pre-incubated with genistein (100 μM).

phasized the importance of physiologically meaningful levels of mechanical force application to cells for study of mechanosensitive ion channels. Cognizant of the limitations imposed by the direct pulling of attached pipettes on cell membranes, or the very large scale deformations of cells that can be achieved with silicone membranes (Naruse and Sokabe, 1993), we cultured cells on flexible substrates with very low elastic deformation limits and the substrate was stretched at very low strain levels (<3%). This system appears to provide reproducible, quantitative, and nondestructive mechanical stimulation of cells, although the physical movement of the cell precludes precise, simultaneous

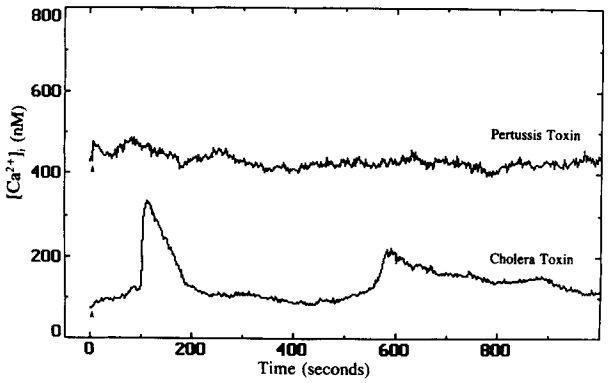

Fig. 14. Sample traces of $[Ca^{2+}]_i$ response to stretched cells following stimulation or inhibition of GTPases. Top trace shows response after pretreatment for 30 minutes with 4 μg/ml pertussis toxin. Bottom trace shows response when the cell was pretreated with cholera toxin (4 μg/ml; 30 minutes). Pertussis toxin blocks transients and oscillations completely.

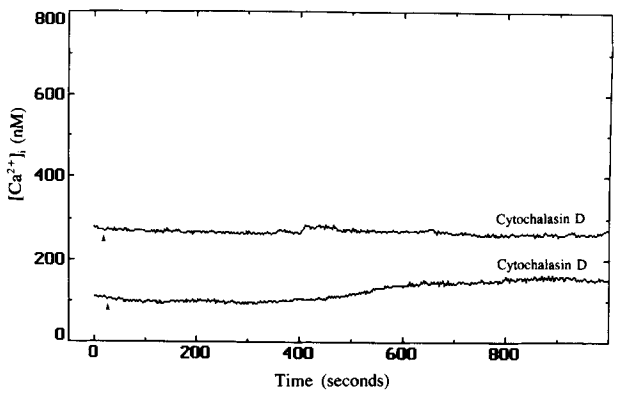

Fig. 16. Representative tracings in two fibroblasts showing $[Ca^{2+}]_i$ responses of fibroblasts after stretching (arrow) following pretreatment with cytochalasin D (500 ng/ml; 30 minutes). Note the complete blockade of transients and oscillations. Traces have been offset for clarity.

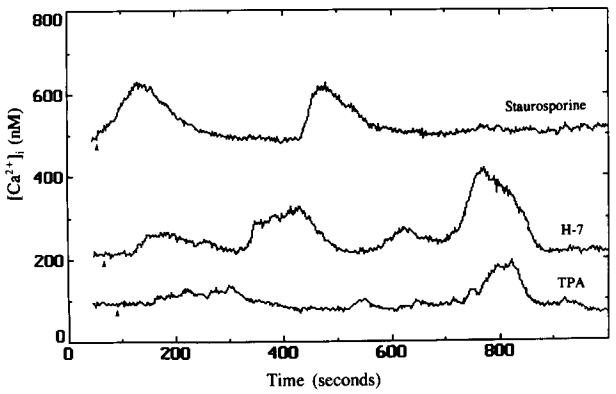

Fig. 15. Top trace demonstrates $[Ca^{2+}]_i$ response to stretch in cell pretreated with PKC inhibitor, staurosporine (100 μM). Middle trace shows response to fibroblast treated with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a more specific inhibitor of PKC than staurosporine. Bottom trace shows response to tetra-phorbol 12-myristate 13-acetate (TPA) at 2 μM to deplete PKC. Transients and oscillations were detected after treatment with all blockers indicating that PKC does not play an important role in the generation of the calcium response to mechanical stretch.

patch clamping study of single channels (e.g., Naruse and Sokabe 1993).

At the two levels of experimental stretches applied, we observed no evidence of cell detachment as detected by confocal microscopy. There was also no loss of membrane integrity as indicated by the insignificant change of fura-2 fluorescence at 356 nm excitation before and after the application of stretch. Further, responding cells were capable of repeated calcium responses after stretches separated over 30 minutes, and there was no sustained increase of $[Ca^{2+}]_i$ after the initial response. Collectively, these findings indicated that the physiological processes regulating cell calcium were intact in stretched cells. However, we have not been able to relate changes in cell area or cell length to the production of a calcium response. Further, the probability of detecting a responsive cell appears low (~12%), a finding consistent with Stockbridge and French (1988), who also found that the probability of cation permeable stretch channels in patches of fibroblasts was low ($P = 0.25$).

Route of calcium influx

We have demonstrated that mechanical stretching of gingival fibroblasts induces increases of intracellular calcium in the form of high amplitude calcium transients and periodic, lower amplitude oscillations. Based on blocking experiments with EGTA, $GdCl_3$, verapamil, and nifedipine, calcium transients and oscillations were probably due to calcium influx through SA channels (Yang and Sachs, 1990) and in part, to L-type Ca^{2+} channels (Davidson et al., 1990). We cannot rule out the contribution of T-type channels, but the methodology used here cannot discriminate accurately between these possibilities. In the presence of $MnCl_2$, the initial calcium influx declined, presumably due to quenching of fura-2 fluorescence. This finding is consistent with the blocking experiments and indicates that the entry of Mn^{2+} was probably through divalent cation permeable SA ion channels (Naruse and Sokabe, 1993). Conceivably, the entry of Ca^{2+} and Na^+ ions through SA channels depolarizes the cell and could induce the opening of L-type voltage Ca^{2+} channels, which in turn further amplifies the calcium influx. The rapidly induced and amplified calcium transient observed after stretching of cells in high external K^+ also suggests that brief depolarization increases the opening of SA Ca^{2+} channels and thus greatly increases the plasma membrane permeability to Ca^{2+} (Harootunian et al., 1988).

A portion of the total calcium influx likely represents activation of the phosphatidyl-inositol pathway induced by membrane perturbations, ultimately leading to calcium release from internal stores (Muallem et al., 1989). This contention is supported by the observation that depletion of calcium from internal stores with thapsigargin decreased the amplitude of the primary and secondary calcium transients. The $[Ca^{2+}]_i$ response in the thapsigargin-treated cells was completely inhibited by 5mM EGTA, suggesting that the observed calcium transients were dependent on flux from both in-

tracellular and extracellular sources. The mechanism for the generation of the secondary transient appears to be functionally linked to the primary transient since all the SA channel blockers and L-type channel blockers strongly inhibited both the primary and secondary transients. Indeed, increased $[Ca^{2+}]_i$ may be essential for IP_3-induced release of calcium from internal stores (Berridge, 1993).

Modulation of intracellular calcium

Our data indicate the virtual complete blockade of calcium transients and oscillations by genistein in stretched cells, suggesting the probable regulation of intracellular calcium by tyrosine kinases. Previously, tyrosine kinases have been shown to regulate $[Ca^{2+}]_i$ responses through PKC (Catarsi and Drapeau, 1993). Evidently, in fibroblasts, the role of PKC is not essential for the stretch-induced calcium response. Protein tyrosine kinases have been immunolocalized in the region of the cell adjacent to focal adhesions, complexes that link the extracellular matrix to the cytoskeleton (Schaller and Parsons, 1993). The complete inhibition of calcium influx by genistein in these substrate-attached cells suggests that regulation of $[Ca^{2+}]_i$ by tyrosine kinases is possibly related to the focal adhesion-associated tyrosine kinase, $pp125^{fak}$ (Schaller and Parsons, 1993). Notably, cells plated 2 hours before stretching exhibited rounded morphologies without long processes and, overall, were more likely to exhibit a calcium response to stretching. As cell shape is dependent on the distribution and densitry of cell-extracellular matrix contacts, the number and force transmitting properties of focal adhesions will likely regulate the conduction of mechanical forces to the cytoskeleton (Wang et al., 1993). Consequently, the large variation in the amplitude of $[Ca^{2+}]_i$ responses between cells to stretch could possibly arise from the enormous variations in the shapes of fibroblasts. In this context, cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ associated with different cell shapes has been observed in cardiac cells (Wier et al., 1987) but has been infrequently noted in fibroblasts.

Treatment with cytochalasin D inhibited calcium responses to stretch, indicating the existence of membrane-bound gating systems that are dependent on the transmission of extracellular force through intact actin filaments. Indeed, disruption of microfilaments has been shown to influence the activity of stretch-activated ion channels in embryonic skeletal muscle cell (Guharay and Sachs, 1987). Our results indicate that the transmission of force from the substrate to the mechanosensors that regulate $[Ca^{2+}]_i$ are absolutely dependent on filamentous actin. This finding is also consistent with the view that tyrosine kinases localized in actin-rich, focal adhesion contacts play a major regulatory role in mechanotransduction.

In this study, stimulation of the $G_{s\alpha}$ subunits of G-proteins by cholera toxin did not inhibit $[Ca^{2+}]_i$ transients or oscillations, indicating no apparent role of $G_{s\alpha}$ subunits in regulating SA calcium permeable channels in gingival fibroblasts. In contrast, pertussis toxin completely inhibited all calcium responses to stretch, indicating that $G_{i\alpha}$ and $G_{o\alpha}$ subunits of G-proteins exert an important regulatory role in the stretch-induced calcium responses. These findings support the existence of mechanosensitive, membrane-bound gating systems (Lansman et al., 1987; Sachs, 1987; Davidson et al., 1990) that are regulated by GTP binding proteins. These data are also consistent with G-protein dependent regulation of actin assembly in response to mechanical stretching of cells (Pender and McCulloch, 1991) and intracellular calcium regulation after hypoosmotic buffer treatment (Suzuki et al., 1990).

Calcium oscillations

Agonist-evoked Ca^{2+} oscillations have been observed in several cell types (Orchard et al., 1983; Schlegel et al., 1987; Ambler et al., 1988; Berridge et al., 1988; Jacob et al., 1988; Woods et al., 1990; Hartoonian et al. 1991; Bird et al. 1993; Uneyama et al., 1993). The amplitude and the period of these oscillatory patterns vary from one cell type to another and also within one cell type depending on the agonist used (Woods et al., 1987; Byron and Villereal, 1989; Rooney et al., 1989). Several different models have been proposed to explain calcium oscillations in cells (Jacob, 1988; Meyer and Stryer, 1988; Berridge, 1990; Rink and Merritt, 1990; Tsien and Tsien, 1990; Dupont et al., 1991; Harootunian et al., 1991), but there is some agreement that the generation of Ca^{2+} oscillations may involve the synthesis of IP_3 and the subsequent release of Ca^{2+} (Bird et al., 1993) from IP_3-sensitive (Berridge, 1990) or IP_3-insensitive Ca^{2+} stores (Berridge and Irvine, 1989). Indeed, the existence of these two releasable pools and their potential interactions with one another are the basis for the two pool calcium oscillation model (Berridge, 1990). To our knowledge, this is the first report in which calcium oscillations have been reported in response to low level mechanical stretch of a single, nonexcitable cell without previous agonist or depolarization treatment. There have been previous electrophysiological studies of oscillations of calcium ion concentration in fibroblasts after high amplitude mechanical perturbation of cell membranes, but the physiological significance of these observations is uncertain (Henkart and Nelson, 1979). In the present study, long period, variable amplitude Ca^{2+} oscillations varied widely between cells, in spite of the uniformity of applied stretch. Notably, very long period oscillations have also been observed in rat parotid acinar cells after caffeine or thapsigargin treatment (Foskett and Wong, 1991).

Incubation of cells with thapsigargin, caffeine, H-7, or staurosporine failed to inhibit calcium oscillations, suggesting that release from IP_3-sensitive and IP_3-insensitive Ca^{2+} stores and PKC were not absolutely essential for the generation of stretch-induced calcium oscillations (Harootunian et al., 1991). Although thapsigargin and caffeine are capable of inducing calcium oscillations in rat acinar cells (Foskett and Wong, 1991), we found no generation of Ca^{2+} oscillations after these treatments in unstretched fibroblasts. Thus the persistence of oscillations in stretched cells after thapsigargin or caffeine treatment implies that when either one of the IP_3-sensitive or IP_3-insensitive pools is blocked, then the other, unblocked pool is capable of releasing Ca^{2+}. Such a backup system would permit a single Ca^{2+} pool at any given time to generate oscillations (e.g., when one pool is exhausted). Blockade of L-type calcium channels by nifedipine or verapamil largely inhibited oscillations. Replacement with a high

K^+ buffer induced rapid and enhanced calcium transients and oscillations, suggesting that depolarization increases the plasma membrane permeability to Ca^{2+} (Lipsombe et al., 1988). As EGTA incubation of thapsigargin-treated cells completely abolished oscillations, it is evident that there is an absolute requirement for extracellular Ca^{2+} in the generation of oscillations. This observation leads us to believe that the entry of Ca^{2+} from the bathing medium is essential for refilling intracellular stores and subsequently for the generation of oscillations and the control of the calcium feedback mechanism.

In the context of existing models that attempt to explain stimulus-induced calcium oscillations in nonexcitable cells (Berridge, 1990; Dupont et al., 1991; Harootunian et al., 1991), the important determinants in the various models appear to be the presence or absence of IP_3 oscillations, Ca^{2+} feedback, PKC regulation, and Ca^{2+} stimulation of phospholipase C. Taken together, our data are most consistent with a model that invokes a calcium feedback mechanism, is PKC independent, and can function without Ca^{2+}-ATPase-dependent calcium stores. These data do not permit the inclusion or exclusion of IP_3 oscillations or phospholipase C in the generation of the calcium oscillations.

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