Mechanisms of Integrin-mediated Calcium Signaling in MDCK Cells: Regulation of Adhesion by IP$_3$- and Store-independent Calcium Influx

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Peptides containing Arg-Gly-Asp (RGD) immobilized on beads bind to integrins and trigger biphasic, transient increases in intracellular free Ca^{2+} ([Ca^{2+}]$_i$) in Madin–Darby canine kidney epithelial cells. The [Ca^{2+}]$_i$ increase participates in feedback regulation of integrin-mediated adhesion in these cells. We examined influx pathways and inositol 1,4,5-trisphosphate (IP$_3$)-mediated Ca^{2+} store release as possible sources of the [Ca^{2+}]$_i$ rise. The RGD-induced [Ca^{2+}]$_i$ response requires external Ca^{2+} (threshold ≈150 μM), and its magnitude is proportional to extracellular calcium. RGD-induced transients were attenuated by Ca^{2+} channel inhibitors (Ni^{2+} and carboxy-amidotriazole) or by plasma membrane depolarization, indicating that Ca^{2+} influx contributes to the response. Loading cells with heparin reduced the size of RGD-induced [Ca^{2+}]$_i$ transients, indicating that IP$_3$-mediated release of Ca^{2+} from stores may also contribute to the RGD response. Depletion of Ca^{2+} stores with thapsigargin activated Ni^{2+}-sensitive Ca^{2+} influx that might also be expected to occur after IP$_3$-mediated depletion of stored Ca^{2+}. However, RGD elicited a Ni^{2+}-sensitive Ca^{2+} influx even after pretreatment with thapsigargin, indicating that Ca^{2+} influx is controlled by a mechanism independent of IP$_3$-mediated store depletion. We conclude that RGD-induced [Ca^{2+}]$_i$ transients in Madin–Darby canine kidney cells result primarily from the combination of two distinct mechanisms: 1) IP$_3$-mediated release of intracellular stores, and 2) activation of a Ca^{2+} influx pathway regulated independently of IP$_3$ and Ca^{2+} store release. Because Ni^{2+} and carboxy-amidotriazole inhibited adhesion, whereas store depletion with thapsigargin had little effect, we suggest that the Ca^{2+} influx mechanism is most important for feedback regulation of integrin-mediated adhesion by increased [Ca^{2+}]$_i$.

INTRODUCTION

Interaction of cells with extracellular matrix (ECM) is mediated by members of the integrin family of adhesion proteins. Integrin-mediated adhesion regulates cell proliferation, differentiation (Yurochko et al., 1992), gene expression (Streuli et al., 1993), and cell death (Damsky and Werb, 1992; Boudreau et al., 1995) during the development of normal tissue and during the onset and progression of metastatic disease. Integrins also mediate leukocyte and platelet cell–cell in-teractions and are important for initiating the immune response (reviewed in Hynes, 1987, 1992; Clark and Brugge, 1995). The specificity of integrin–ECM binding is determined by recognition sites on ECM[1] molecules and is conferred by the combination of integrin α- and β-subunits (Pierschbacher and Ruoslahti,

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[1] Abbreviations used: AIC, amidoimidazole carboxamide; [Ca^{2+}]$_i$, intracellular calcium; [Ca^{2+}]$_o$, extracellular calcium; CAI, carboxy-amidotriazole; CS, chondroitin sulfate; ECM, extracellular matrix; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; IP$_3$, inositol 1,4,5-trisphosphate; MDCK cells, Madin–Darby canine kidney cells; RGD, Arg-Gly-Asp; TG, thapsigargin.

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1984a,b; Ruoslahti *et al.*, 1994). A widely studied recognition site on the ECM molecules fibronectin and vitronectin is the tripeptide Arg-Gly-Asp (RGD), which is recognized by a subset of common integrins, including $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ (Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1987; Hynes, 1992).

The emerging consensus is that integrins are multipurpose molecules, acting both as receptors ("outside-in" communication) and as effectors ("inside-out" communication), which allows cells to perceive their environment, integrate information, and subsequently take appropriate actions (Ginsberg *et al.*, 1992; O'Toole *et al.*, 1994; Schwartz and Ingber, 1994). Although it is known that integrins regulate cell shape and migration by linking the cellular cytoskeleton to ECM, the mechanisms that transfer information from the extracellular milieu through integrins to the intracellular environment are not fully understood.

Integrin occupancy and clustering activates both cytoskeletal remodeling and intracellular signal transduction pathways (Miyamoto *et al.*, 1995). This outside-in communication leads to formation of focal adhesion complexes containing cytoplasmic cytoskeletal components (talin, vinculin, and α-actinin; Burridge *et al.*, 1988; Clark and Brugge, 1995). Focal adhesions link the actin cytoskeleton to the ECM (Buck and Horwitz, 1987; Hynes, 1987, 1992; Plopper and Ingber, 1993). This cytoskeletal complex also acts as a scaffold for assembly and activation of catalytic signaling proteins (Sastry and Horwitz, 1993; Pavalko and Otey, 1994; Clark and Brugge, 1995; Miyamoto *et al.*, 1995). For example, integrin binding leads to changes in tyrosine phosphorylation of proteins (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Kornberg and Juliano, 1992; Arroyo *et al.*, 1994), including phosphorylation and activation of the focal adhesion kinase (Kornberg *et al.*, 1992; Schaller and Parsons, 1994). Signal transduction can also occur via SH2–SH3 mediated signaling or activation of small-molecular-weight GTPases (Clark and Brugge, 1995).

In addition, integrins can initiate changes in intracellular ion concentration. For example, spreading of endothelial cells on fibronectin and vitronectin triggers increases in intracellular pH via activation of the Na^+/H^+ antiporter and increases in intracellular calcium concentration through other integrin-mediated signaling pathways (Ingber *et al.*, 1990; Juliano and Haskill, 1993; Kanner *et al.*, 1993; Leavesley *et al.*, 1993; Schwartz, 1993; Schwartz and Denninghoff, 1994; Schwartz and Ingber, 1994). Integrin-mediated calcium signaling has also been observed in platelets, macrophages, neutrophils, and osteoclasts (Hendey and Maxfield, 1993; Shankar *et al.*, 1993; Chenu *et al.*, 1994; Zimolo *et al.*, 1994; Ozaki *et al.*, 1995). In some cases phosphorylation events occur in conjunction with calcium signaling and protein kinase C activity

(Pelletier *et al.*, 1992; Juliano and Haskill, 1993; Vuori and Ruoslahti, 1993; Shattil *et al.*, 1994). Recently, it was observed that inhibition of calcium influx in endothelial cells reduced adhesion to collagen, laminin, and fibronectin substrates and inhibited tumor angiogenesis (Kohn *et al.*, 1995). However, mechanisms of integrin-mediated calcium mobilization and their role regulation of adhesion are not fully characterized in endothelial cells (Schwartz, 1993; Schwartz and Denninghoff, 1994).

Although it is known, in many cases, that integrins can initiate signal transduction, the underlying mechanisms by which integrin–ECM interactions initiate intracellular calcium signaling and the immediate consequences of integrin-mediated calcium mobilization remain uncharacterized. In addition, the possibility that distinct roles exist for different calcium mobilization pathways in regulating adhesion and intracellular signaling has not been tested.

We have previously demonstrated integrin-mediated signaling in epithelial cells. Significantly, binding of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins to RGD induces $[Ca^{2+}]_i$ transients in Madin–Darby canine kidney (MDCK) cells, which play a role in feedback regulation of integrin-mediated adhesion to RGD (Sjaastad *et al.*, 1994). Rapid feedback of substrate adhesion may be important during exploratory cell migration in development, wound healing, angiogenesis, and metastasis. In the present study we sought to elucidate the specific mechanisms that underlie the onset of the RGD-induced calcium rise in MDCK cells by examining the role of both inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular calcium stores and activation of plasma membrane calcium channels. In addition, we examined the role of each calcium mobilization pathway in the regulation of adhesion.

MATERIALS AND METHODS

Cell Culture

MDCK epithelial cells were cultured in DMEM with 10% fetal bovine serum (FBS) and antibiotics, as described previously (Nelson and Veshnock, 1986, 1987; Sjaastad *et al.*, 1994). One to two days before experiments, the cells were passaged and replated at single-cell density.

Bead Preparation

RGD peptide (Peptite-2000, Telios Pharmaceuticals, San Diego, CA) was linked to 2.8-μm-tosylactivated polystyrene magnetic beads (Dynal, Lake Success, NY) by incubation of beads in 100 μg/ml peptide overnight in high pH carbonate buffer (pH 9.4), as described previously (Plopper and Ingber, 1993; Sjaastad *et al.*, 1994). Nonspecific sites on the beads were blocked by a 2 h incubation in 1% heat-inactivated bovine serum albumin (BSA). The beads were washed several times to remove uncoupled RGD peptide and stored at 4°C in experimental buffer with antibiotics.

Intracellular Calcium Measurements

Before an experiment, cells were replated at single-cell density on collagen-coated coverslip chambers (Applied Scientific, San Fran-

cisco, CA) for 2–3 h. Cells were loaded with 2 μM fura-2/AM, which was solubilized in dimethyl sulfoxide (DMSO) with 25% pluronic (Molecular Probes, Eugene, OR) for 1 h in the dark at 20°C in DMEM without phenol red and NaHCO$_3$ (NaHCO$_3$ was replaced by 10 mM HEPES, and equivalent osmolarity was maintained by adding NaCl). The experimental buffer was supplemented with 10% FBS, antibiotics, and 250 μM sulfinpyrazone (Sigma, St. Louis, MO) to inhibit dye extrusion (Di Virgilio et al., 1990). Cells were washed free of fura-2 loading solution 30 min before experiments to allow for recovery from the effects of dye loading (Negulescu et al., 1989).

Ratio imaging was conducted at 37°C with a Videoprobe image processor (ETM Systems, Irvine, CA) and an I-CCD camera (Hamamatsu Photonics, Bridgewater, NJ) coupled to a Zeiss Axiovert 35 microscope with a ×40 objective (Achrostigmat, NA 1.30 oil), as described previously (Lewis and Cahalan, 1989). Intracellular calcium was estimated by the use of the equation $[Ca^{2+}]_i = K^*(R - Rmin)/(Rmax - R)$. Rmin, Rmax, and K* were derived from an in vitro calcium solution calibration (Grynkiewicz et al., 1985). Note that this method yields slightly lower $[Ca^{2+}]_i$ values than we obtained previously (Sjaastad et al., 1994) by calibrating with values determined in an situ calibration of Jurkat T cells (Lewis and Cahalan, 1989). For solution changes during experiments, at least five times the volume of the dish was perfused through the chamber. Constant volume of the chamber was maintained by aspiration.

Experimental Solutions

Unless otherwise indicated, all experiments were performed in experimental buffer, prepared as described above. Experiments using calcium-free Ringer's or Ringer's with specific concentrations of calcium were prepared from the following protocols: standard Ringer—155 mM NaCl, 4.5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose, and 5 mM HEPES; calcium-free Ringer's—same as standard Ringer's except that 2 mM CaCl$_2$ was replaced with 2 mM MgCl$_2$ (i.e., a total of 3 mM MgCl$_2$); K Ringer—standard Ringer with 155.5 mM KCl instead of 155 mM NaCl (i.e., 160 mM KCl total). To obtain the indicated concentrations of calcium, we added the appropriate volume of a 1.8 M stock solution of CaCl$_2$ to calcium-free Ringer's. The Ringer's solutions did not contain FBS, antibiotics, or sulfinpyrazone. These solutions were placed on the cells immediately before conducting imaging experiments. ATP (Calbiochem, San Diego, CA) was prepared fresh daily as a 1 M stock in ddH$_2$0 and diluted to 10 μM in Ringer's immediately before a solution change. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma) was made as a 0.25 M stock and used at 1 mM final concentration. Thapsigargin (TG), erbstatin analogue, and genistein (LC Laboratories, Woburn, MA) were prepared as 1 mM, 2 mM, and 10 mM stocks, respectively, in DMSO and diluted to the indicated concentrations before addition to cells. Ni^{2+} was used at a concentration of 5 mM from a 1 M stock of NiCl$_2$. Unless otherwise indicated, Ni^{2+} and various external calcium concentration solutions were added 1 min before the addition of RGD beads. The calcium channel inhibitor carboxy-amidotriazole (CAI) and the inactive CAI analogue amidoimidazole carboxamide (AIC) were prepared as a 50 mM stock in DMSO and stored at -80°C. These compounds were a generous gift from Dr. Elise Kohn (National Cancer Institute, Bethesda, MD).

Electroporation

Electroporation was conducted with a Bio-Rad (Richmond, CA) GenePulser Transfection Apparatus. The conditions used for electroporation were optimized to maintain our protocol for cell preparation (Andreason, 1993). Briefly, MDCK cells at 50–75% confluence were trypsinized and re-suspended in standard DMEM growth medium at 10^6 cells/ml. Cells were placed on ice for 10 min and then electroporated in the presence of the indicated amount of 10,000 molecular weight Texas Red-labeled dextran (Molecular Probes, Eugene, OR), low-molecular-weight heparin (H-2149,

Sigma) or chondroitin sulfate (C-8529, Sigma) and then placed back on ice for 10 min. Cells were then plated in coverslip chambers for 2 h and with fura-2/AM for 1 h before loading.

To optimize protein incorporation and cell survival after electroporation, MDCK cells were electroporated at various field strengths by varying only electroporation voltage (150–450 volts at 960 μF) in the presence of 10,000 molecular weight Texas Red-labeled dextran (0.2 mg/ml). Fluorescence of individual cells was measured with the imaging system, and averages were compared with fluorescence of cells electroporated in the absence of dextran and cells that were not electroporated. As shown in Figure 1a, incorporation of dextran increased linearly with increasing field strength. To evaluate cell survival, we estimated plating efficiency by counting the average number of cells in an image field with electroporated and control cells. We chose to perform electroporation at a field strength of 1000 V/cm (i.e., 400 V and 960 μF in a 0.4 cm cuvette) to maximize incorporation of heparin and ensure 60–75% cell-plating efficiency. Cells were plated at 25% greater cell density to maintain a number of cells/field equivalent to that in previous experiments.
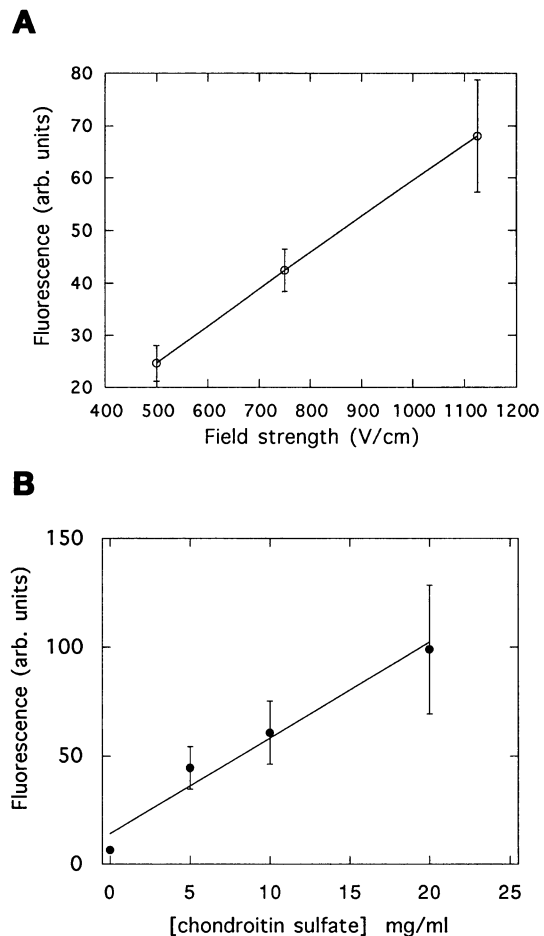

Figure 1. Optimization of electroporation conditions for loading cells with heparin. (a) Texas Red-labeled dextran incorporation into MDCK cells increases with increased electroporation field strength. (b) Incorporation of fluorescent chondroitin sulfate (CS-DTAF) (a glycosaminoglycan of similar structure to heparin labeled with FITC; see MATERIALS AND METHODS) increases with increasing CS-DTAF concentration. Error bars represent SEM; figure is representative of three independent experiments.

Further characterization of electroporation efficiency was conducted with fluorescently labeled chondroitin sulfate (CS), a glycosaminoglycan similar in structure to heparin but that does not inhibit the IP_3 receptor (Hill *et al.*, 1987; Boitano *et al.*, 1992). The incorporation of fluorescent CS (conjugated to D-TAF, see below) increased linearly with increasing external concentration of CS up to 20 mg/ml (Figure 1b). Fluorescent CS was prepared by conjugation to 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF) (Molecular Probes) similar to methods described by De Belder and Granath (1973). Briefly, 0.5 g CS was incubated with D-TAF (50 mg, solubilized in DMSO) at 1:1 molar ratio in 3 ml of carbonate buffer and heated to 50°C for 30 min. Unbound D-TAF was separated from labeled CS by purification on a sephadex G-25 fine column. Fifteen 1 ml fractions were pooled and dried to determine the dry weight of labeled CS. The fluorescent characteristics of D-TAF in cells are not known; therefore, we did not attempt to determine the intracellular concentration of CS by fluorescence.

Adhesion Assays

The effect of various inhibitors on integrin-mediated adhesion was measured by bead binding to MDCK cells and adhesion of MDCK cells to either RGD- or collagen-coated substrates. The bead binding assay has been described previously (Sjaastad, 1994; 46). Briefly, MDCK cells were plated at single-cell density for 3 h on collagen-coated 24-well plates (Falcon). Before an experiment, cells were treated with the indicated compounds for the indicated times. Beads were added to the wells and allowed to settle at $1 \times g$ for 10 min at 37°C in a tissue culture incubator (95% air/5% CO_2). The cells were then washed twice with buffer to remove unbound beads, and bright-field images of the cells with bound beads were captured with the imaging system. The substrate adhesion assay was conducted on 96-well microtiter plates (Corning). Wells were coated with either RGD (10 μg/ml) or collagen (Sjaastad *et al.*, 1994), washed, and filled with buffer containing the indicated experimental conditions. MDCK cells were briefly trypsinized and loaded with 2 μM Calcein-AM (Molecular Probes) in suspension at 37°C for 30 min, 3×10^4 cells were added to each well, and the cells were centrifuged onto the substrates at 24°C for 10 min at $17 \times g$. Each well was then gently washed three times with the appropriate buffer, and the adherent cells were counted with the FITC channel on a Titertek Fluoroskan II Plate Reader (Elfaboy, Finland). Background adhesion to uncoated wells was negligible, and the specificity of adhesion to collagen and RGD was confirmed by inhibition of adhesion with the integrin-specific monoclonal antibodies A2B2 (a gift from Dr. Caroline Damsky, University of California, San Francisco, CA) and LM609 and P3G2 monoclonal antibodies (gifts from Dr. David Cheresh, Research Institute of The Scripps Clinic, La Jolla, CA). Data from adhesion assays are presented either in arbitrary fluorescent units or as percentage of the control.

RESULTS

Attachment of RGD beads to cells induced a characteristic increase in $[Ca^{2+}]_i$ in >80% of cells (Figure 2). The increase was biphasic, with an initial rapid rise in $[Ca^{2+}]_i$ to 1–1.2 μM, followed by a decrease to a variable plateau (0.35–0.45 μM) that lasted from 30 s up to 30 min (our unpublished observations). Figure 2a depicts $[Ca^{2+}]_i$ traces demonstrating the extremes of single-cell $[Ca^{2+}]_i$ responses in a typical experiment. An average trace of all cells from this experiment is shown in Figure 2b. No attempt was made to account for the slight asynchronicity in the onset of the transients resulting from variability in bead settling when averages were calculated. Instead, an average trace and a single-cell trace are presented for comparison in most experiments. In our previous work we found no correlation between the magnitude of the plateau phase and adhesion of RGD beads to MDCK cells. However, inhibition of the initial sharp increase resulted in decreased adhesion to RGD (Sjaastad *et al.*, 1994). Thus, in the present study we focused on mechanisms that elicit the initial sharp increase in $[Ca^{2+}]_i$ (the RGD-induced $[Ca^{2+}]_i$ "transient"). Both bead adhesion and calcium signaling are specific to RGD and are decreased by antibodies that inhibit the function of $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins (Sjaastad *et al.*, 1994). These inhibitory antibodies did not elicit calcium signaling in MDCK cells either in solution or when coupled to beads. However, other (but not all) RGD-containing peptides coupled to beads elicit signaling (our unpublished observations). The beads are not phagocytosed after binding because, even long after their application, beads can be removed from the cell surface by vigorous washes.

Ca^{2+} Influx Contributes to the RGD-induced Calcium Transient

Possible sources of the $[Ca^{2+}]_i$ increase in the response to RGD include intracellular stores and Ca^{2+} influx pathways in the plasma membrane. We characterized
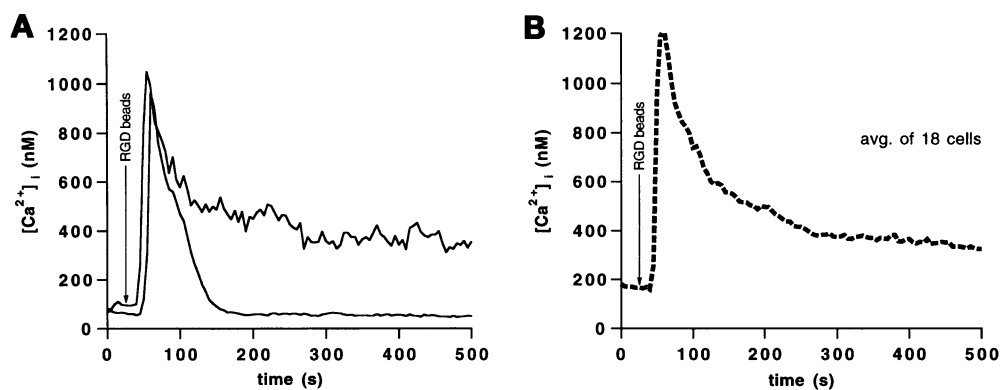

Figure 2. Induction of a transient $[Ca^{2+}]_i$ increase by RGD bead binding to MDCK cells. Extremes of single-cell responses (a) and average trace (b) of $[Ca^{2+}]_i$ are plotted against time after application of RGD beads (arrow). These traces are representative of at least three independent experiments for each condition.

Figure 3. Inhibition of the RGD-induced $[Ca^{2+}]_i$ transients in the presence of 5 mM Ni^{2+} or by membrane depolarization. Single-cell trace (a) and average trace (b) of $[Ca^{2+}]_i$ in cells pretreated with 5 mM Ni^{2+} for 1–2 min. Note that small spikes still occur in the presence of 5 mM Ni^{2+} (see text). (c) Pretreatment of cells with high K^+ Ringer's (complete substitution of K^+ for Na^+) inhibits the RGD-induced transient. (d) Acute addition of high K^+ results in a reversible reduction in the amplitude of the RGD-induced transient. Single-cell traces (solid line) and average traces (dashed line) are shown. The average $[Ca^{2+}]_i$ trace in Figure 3d is slightly lower than that in the single-cell trace because of a higher than normal number of nonresponding cells in this experiment. These traces are representative of at least three independent experiments for each condition.
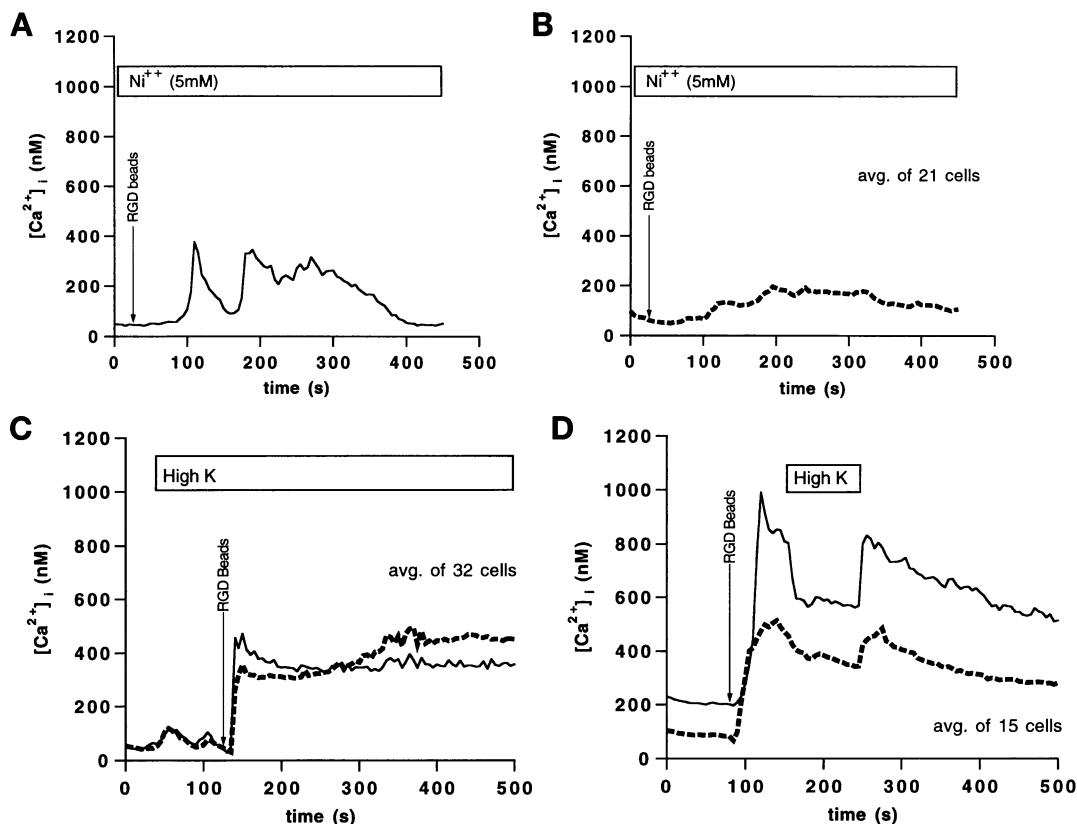
the contribution of Ca^{2+} influx with three independent methods: by blocking influx with Ni^{2+}, by diminishing the driving force for calcium entry by membrane depolarization, or by reducing the concentration of extracellular calcium ($[Ca^{2+}]_o$).

Consistent with our previous work, we show that RGD-induced $[Ca^{2+}]_i$ transients are inhibited by pretreatment with 5 mM Ni^{2+}, a blocker of many types of calcium channels (Hagiwara and Byerly, 1981; Zweifach and Lewis, 1993; Sjaastad et al., 1994) (Figure 3). Inhibition with Ni^{2+} indicates that calcium influx contributes significantly to the RGD-induced response. Note in Figure 3a that small $[Ca^{2+}]_i$ transients still occur in the presence of 5 mM Ni^{2+}, possibly because of the release of intracellular stores (see below). This result shows that Ni^{2+} does not prevent signaling through integrins and that integrins may transduce a small signal even when plasma membrane Ca^{2+} channels are blocked.

Complete substitution of K^+ for Na^+ in the culture medium reduced the driving force for calcium influx by collapsing the plasma membrane potential and simultaneously tested for the presence of voltage-activated calcium channels. We found no evidence for depolarization-activated calcium influx upon switch-

ing from Na^+-Ringer's to K^+-Ringer's before RGD bead addition (Figure 3c). However, K^+-Ringer's inhibited the initial sharp peak of the RGD-induced $[Ca^{2+}]_i$ transient, resulting in only an elevated plateau (Figure 3c). Inhibition of the RGD-induced response indicated that influx contributes significantly to the initial peak of the transient. Application of high K^+ Ringer's to cells immediately after the onset of the RGD response resulted in rapid reduction of the $[Ca^{2+}]_i$ transient, which was reversed when high K^+ was removed (Figure 3d). Application of 5 mM Ni^{2+} (instead of high K^+, as shown in Figure 3d) resulted in a similar reversible inhibition of the $[Ca^{2+}]_i$ transient (our unpublished observation).

We examined the dependence of $[Ca^{2+}]_i$ transients on external calcium. Cells were stimulated with RGD beads in the presence of $[Ca^{2+}]_o$ from 1.0 mM to 100 μM (Figure 4). Because intracellular calcium stores slowly deplete over 10–15 min when MDCK cells are bathed in low $[Ca^{2+}]_o$ (our unpublished observations), cells were maintained at normal $[Ca^{2+}]_o$ (1.8 mM), and $[Ca^{2+}]_o$ was rapidly reduced to the indicated concentration 1 min before the addition of beads by the use of on-stage perfusion. For these experiments, RGD beads were washed and main-

A Individual traces — 1000 μM [Ca++]o

B Average traces — 1000 μM [Ca++]o — avg. of 33 cells

C 500 μM [Ca++]o

D 500 μM [Ca++]o — avg. of 34 cells

E 150/200 μM [Ca++]o

F 150/200 μM [Ca++]o — avg. of 43 cells

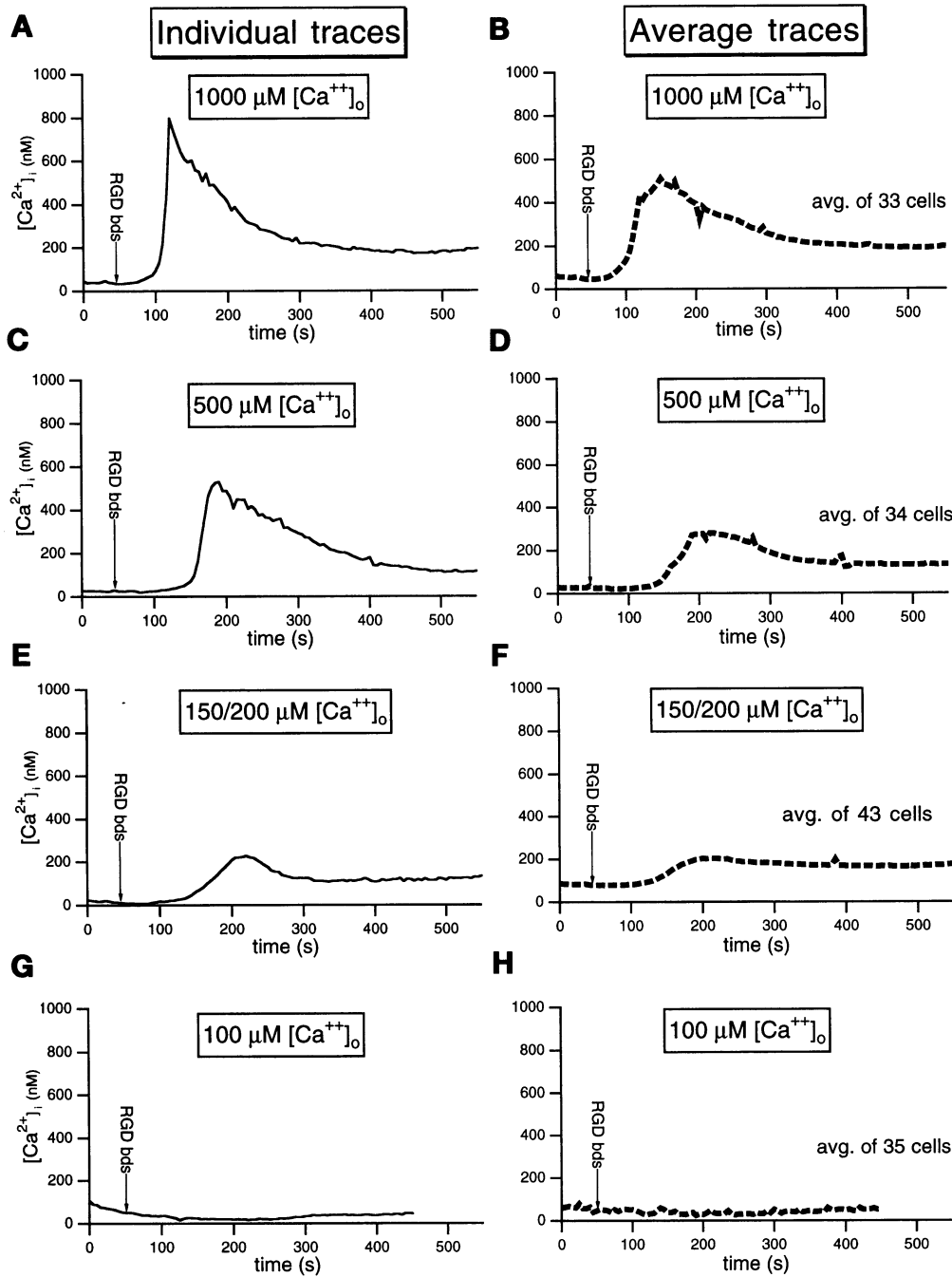**G** 100 μM [Ca++]o

H 100 μM [Ca++]o — avg. of 35 cells

Figure 4. RGD-induced $[Ca^{2+}]_i$ transients require external calcium and decrease in magnitude with decreasing extracellular calcium concentration. (a, c, e, and g) Representative single-cell traces of $[Ca^{2+}]_i$ are shown in experiments in which beads were added to cells that had been switched to Ringer's solutions containing the indicated $[Ca^{2+}]_o$ concentrations 1–2 min before bead addition. (b, d, f, and h) Average traces from the same experiments. There were no RGD-induced changes in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was below ≈150 μM (g and h). The traces are representative of at least three independent experiments.

tained in calcium-free Ringer's. Single-cell traces are shown in the left column, and average traces are shown in the right column. As a general trend, the amplitude of RGD-induced $[Ca^{2+}]_i$ transients decreased with decreasing $[Ca^{2+}]_o$. We also observed a threshold requirement of 150 μM $[Ca^{2+}]_o$ for induction of $[Ca^{2+}]_i$ transients by RGD beads. No responses were observed at a $[Ca^{2+}]_o$ of 100 μM or below. There are two possible interpretations of

these results. First, the decrease in the magnitude of the $[Ca^{2+}]_i$ transient as $[Ca^{2+}]_o$ was lowered from 1 mM to 200 μM is consistent with the conclusion that $[Ca^{2+}]_i$ transients result in part from influx of external calcium. However, the complete lack of response in very low concentrations of $[Ca^{2+}]_o$ (<200 μM) indicate that, second, there may also be a threshold $[Ca^{2+}]_o$ required for the RGD–integrin binding event, which elicits proximal signal transduction.

To examine effects of low $[Ca^{2+}]_o$ on integrin–ECM binding, we conducted adhesion assays with either collagen or RGD substrates. We previously observed that MDCK cells remain spread and bound to collagen-coated coverslips even in nominally calcium-free media (≈ 5 μM calcium). In substrate adhesion assays, cells adhere readily to collagen over a broad range of calcium concentrations (in a representative experiment, 85–90% of cells bound at 5 μM or 100 μM $[Ca^{2+}]_o$ compared with binding at 2 mM $[Ca^{2+}]_o$; our unpublished observations). However, there was little binding (14% of control) in the complete absence of calcium (calcium-free Ringer's containing 1 mM EGTA). By the use of the same assay, MDCK cells bound to RGD substrates in low $[Ca^{2+}]_o$ concentrations (48 and 64% of control at 5 and 100 μM, respectively, vs. 6% in 1 mM EGTA; our unpublished observations). We conclude that a variety of integrins on MDCK cells can bind their ligands at concentrations of Ca^{2+}_o below those required to elicit $[Ca^{2+}]_i$ transients. Thus, the absence of $[Ca^{2+}]_i$ transients below 150–200 μM $[Ca^{2+}]_o$ may result from a failure of bound integrins to signal properly. Taken together, the combination of decreased driving force and loss of integrin signaling function would lower the extent of calcium influx as $[Ca^{2+}]_o$ was decreased.

MDCK Cells Have IP_3-sensitive Calcium Stores that Can Be Released by ATP

Although calcium influx seems to contribute to the RGD-induced $[Ca^{2+}]_i$ transient, the occurrence of small transients in the presence of 5 mM Ni^{2+} (Figure 3a) indicates that intracellular Ca^{2+} release may also play a role. However, because of the requirement of $[Ca^{2+}]_o$ for RGD-induced signaling, removal of external calcium could not be used to distinguish between the contributions of intracellular stores and calcium influx. Instead, we used ATP to examine the role of intracellular Ca^{2+} release. ATP (10–20 μM) stimulates maximal IP_3 production and release of Ca^{2+} from intracellular stores in MDCK cells (Paulmichl et al., 1990). Ca^{2+} release occurs because of activation of phospholipase C and generation of IP_3 via a phorbol ester and pertussis toxin-sensitive mechanism (Paulmichl et al., 1990). We observed that treatment of MDCK cells with 10 μM ATP resulted in a large $[Ca^{2+}]_i$ transient (Figure 5a). Dose–response experiments confirmed maximal responses at 10 μM ATP (our unpublished observations). When ATP was removed and subsequently reapplied 150 s later, a second transient occurred with a magnitude similar to that of the first (Figure 5a). Addition of 10 μM ATP to cells in the absence of extracellular Ca^{2+} (Ca^{2+}-free buffer with 1 mM EGTA) elicited a $[Ca^{2+}]_i$ transient of a magnitude similar to that in the presence of calcium (Figure 5b). This suggests that the Ca^{2+} stores contain

significant signaling capacity in the absence of Ca^{2+} influx and that Ca^{2+} influx does not make a large contribution to the observed ATP response. However, sustained Ca^{2+} influx has been observed in response to ATP treatment in other clones of MDCK cells (Paulmichl et al., 1990; Dietl and Volkl, 1994). Note that, in the absence of external calcium, subsequent removal of ATP followed by a second application of ATP stimulated little or no response, indicating that, once calcium stores are depleted, they require extracellular calcium to refill.

Inhibition of IP_3-mediated Ca^{2+} Release Diminishes the RGD-induced Ca^{2+} Transient

Integrin binding to ECM has been linked to changes in inositol phosphate turnover (McNamee et al., 1993), tyrosine phosphorylation-dependent activation of phospholipase C (Kanner et al., 1993; Blake et al., 1994; Somogyi et al., 1994), and production of IP_3 (Sultan et al., 1991; Somogyi et al., 1994). To address the involvement of IP_3-mediated Ca^{2+} release in the RGD response, we examined the effect of heparin on $[Ca^{2+}]_i$ transients. Heparin competes with IP_3 for the IP_3-receptor and inhibits the release of calcium from stores in many cell types (Hill et al., 1987; Ghosh et al., 1988; Harootunian et al., 1991). MDCK cells were preloaded by electroporation in the presence of low-molecular-weight heparin or unlabeled CS as a control (Boitano et al., 1992) before plating and stimulation with RGD beads (see MATERIALS AND METHODS). To control for the efficiency of electroporation and inhibitory activity of heparin on IP_3-mediated store release, we compared ATP responses in cells preloaded with heparin and CS (Figure 6). Concentrations of 20–40 mg/ml heparin reduced the average amplitude of ATP-stimulated $[Ca^{2+}]_i$ transients by 30–50% (Figure 6a). However, similar concentrations of CS had little, if any, effect on the response to ATP (Figure 6b).

In a similar manner, increasing concentrations of heparin decreased the average amplitude of $[Ca^{2+}]_i$ transients induced by RGD beads (Figure 6c), whereas identical concentrations of CS had little or no effect (Figure 6d). The maximum degree of inhibition of the RGD-induced $[Ca^{2+}]_i$ transients by heparin is similar to that seen with ATP stimulation. Heparin loading also seemed to cause a dose-dependent effect on resting intracellular calcium levels, perhaps because of an inhibitory effect on IP_3-mediated regulation of basal $[Ca^{2+}]_i$. Even when peak values of the transient were measured relative to initial baseline $[Ca^{2+}]_i$ levels, heparin reduced the RGD-induced $[Ca^{2+}]_i$ response. These results indicate that IP_3-mediated release of Ca^{2+} from intracellular stores contributes to RGD-induced $[Ca^{2+}]_i$ transients.
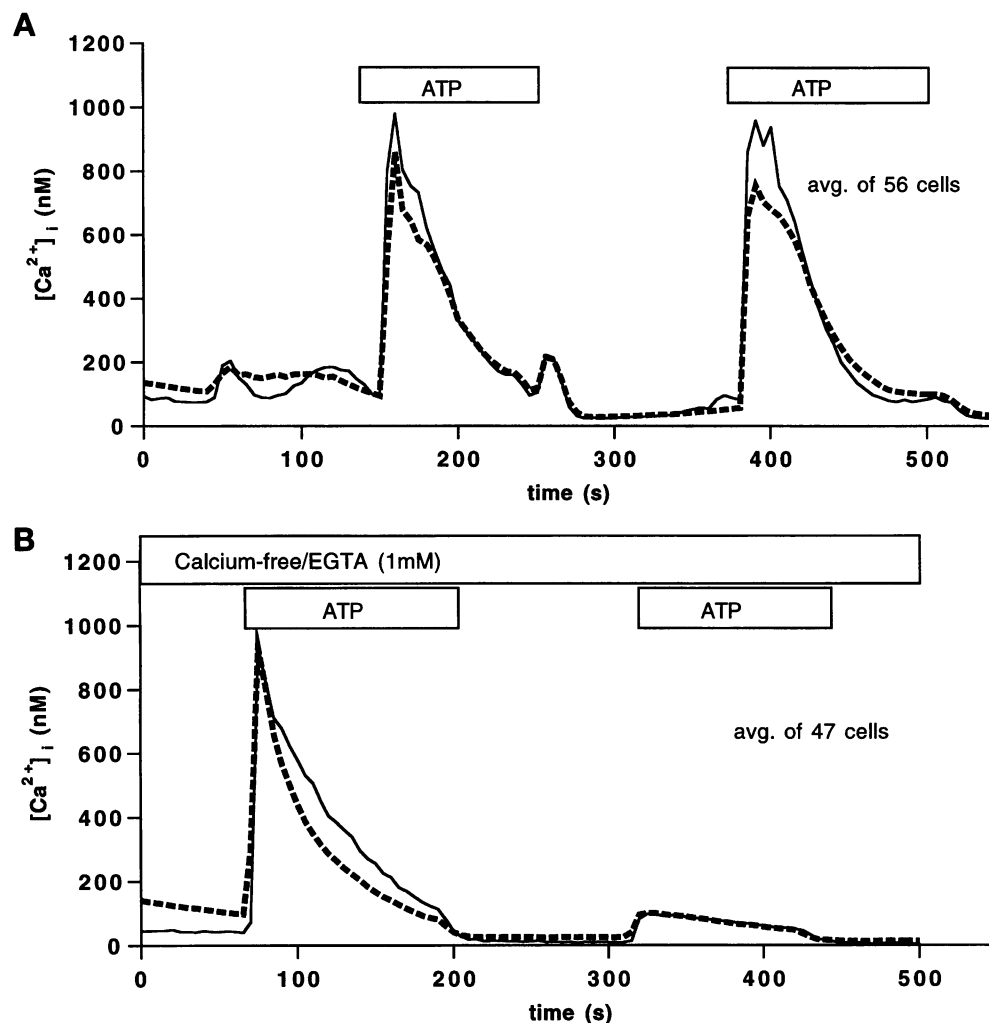
A

B

Figure 5. Release of intracellular calcium stores after addition of ATP. (a) Application of ATP (10 μM) results in a large $[Ca^{2+}]_i$ transient. Removal of ATP and subsequent re-addition of ATP results in a similar transient. (b) After 1–2 min preincubation of cells in nominally calcium-free Ringer containing 1 mM EGTA, application of ATP (10 μM) results in a large $[Ca^{2+}]_i$ transient, indicating that ATP mobilizes Ca^{2+} from internal stores. Removal and subsequent re-addition of ATP results in a very small $[Ca^{2+}]_i$ transient, indicating that the stores require extracellular Ca^{2+} to refill. The solid traces indicate a representative single cell, and the dashed trace is the average of all cells in a single experiment. Results are representative of at least four independent experiments.

Depletion of Intracellular Ca^{2+} Stores Activates Ca^{2+} Influx in MDCK Cells

In many cell types, depletion of intracellular stores by IP_3 activates "capacitative" calcium entry (Hoth and Penner, 1992; McDonald *et al.*, 1993; Putney and Bird, 1993; Zweifach and Lewis, 1993). Activation of these influx pathways may contribute to the amplitude and duration of the calcium signal as well as to the refilling of intracellular calcium stores. Recently, a highly selective calcium current activated by intracellular calcium release and a nonselective cation current have been identified in MDCK cells (Dietl and Volkl, 1994; Delles *et al.*, 1995). These conductance mechanisms could, in principle, contribute to Ca^{2+} influx triggered by RGD in MDCK cells.

We applied TG to determine whether depletion of intracellular stores activates calcium influx in MDCK cells. TG specifically inhibits the sarco-endoplasmic reticular calcium ATPase (SERCA) family of Ca^{2+}-ATPases and irreversibly depletes intracellular cal-

cium stores (Thastrup *et al.*, 1990). Addition of 1 μM TG results in a large, transient increase in $[Ca^{2+}]_i$, followed by a slightly elevated $[Ca^{2+}]_i$ plateau (Figure 7). The plateau was reversibly decreased by the addition of 5 mM Ni^{2+}, suggesting that depletion of intracellular Ca^{2+} stores activates calcium influx. In the absence of external calcium, 1 μM TG consistently elicited smaller $[Ca^{2+}]_i$ transients (<500 nM peaks; our unpublished observations), suggesting that Ca^{2+} influx largely determines the magnitude of TG-induced $[Ca^{2+}]_i$ transients. Interestingly, this component of the transient is not inhibited by 5 mM Ni^{2+} (compare Figure 7 with Figure 8b). Thus, TG-induced $[Ca^{2+}]_i$ transients result from the mobilization of Ca^{2+} from intracellular stores and activation of capacitative Ca^{2+} influx, possibly through more than a single type of store-operated plasma membrane Ca^{2+} channel. Although the existence of Ni^{2+}-insensitive Ca^{2+} entry pathways activated by TG is not well documented, it should be noted that multiple types of store-operated
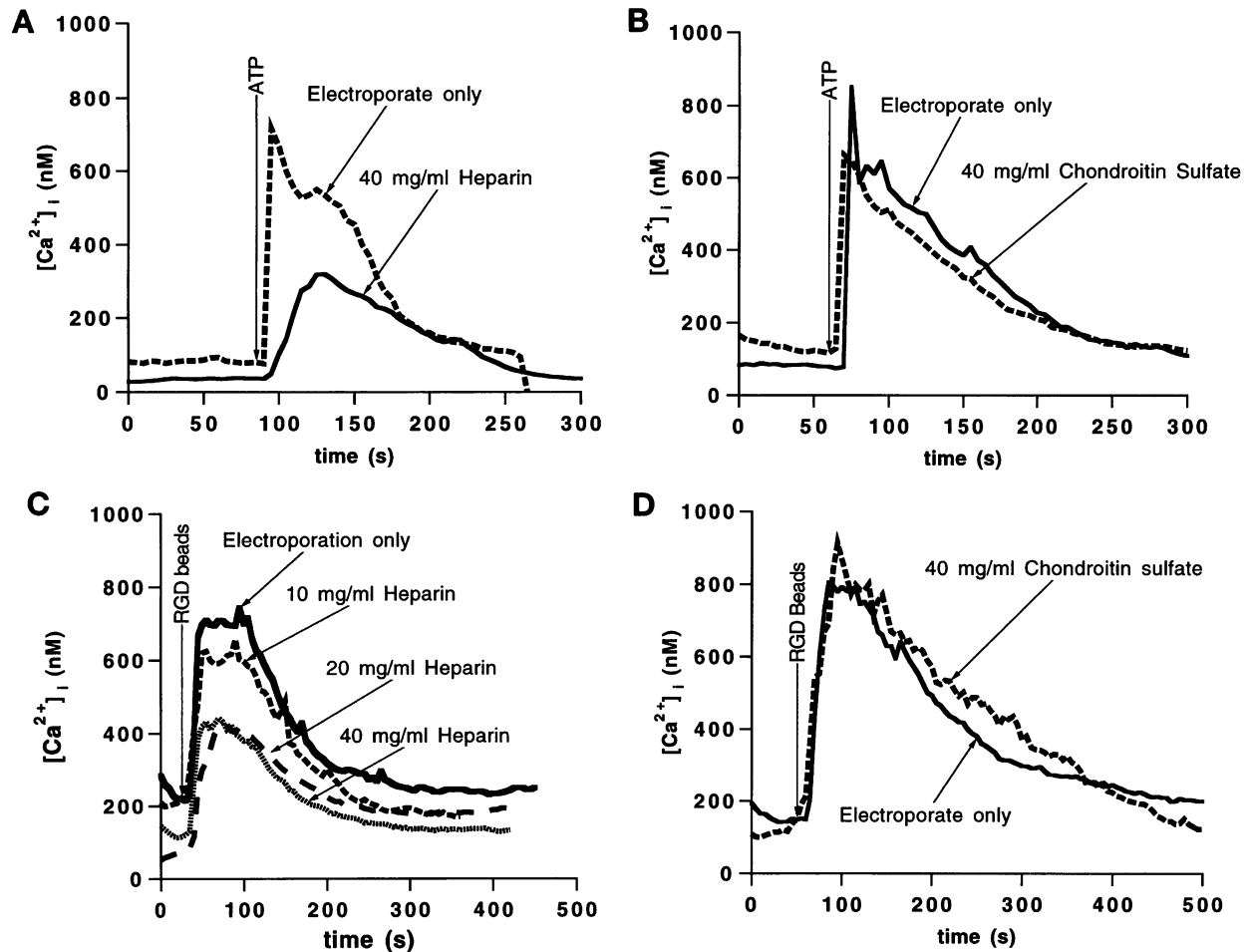
Figure 6. Incorporation of heparin, but not chondroitin sulfate (CS), inhibits ATP- and RGD-induced $[Ca^{2+}]_i$ transients. Results shown are average traces from experimental runs conducted from a single experiment of heparin or CS electroporations. (a) Heparin (40 mg/ml) inhibits ATP-induced (10 μM), IP_3-mediated release of calcium from intracellular stores. (b) However, the same concentration of CS had no effect. (c) Increasing concentrations of heparin decrease the magnitude of the average $[Ca^{2+}]_i$ transient induced by RGD beads. (d) However, even at maximal concentration, (40 mg/ml) CS did not significantly inhibit the Ca^{2+} response. The traces are averages and representative of at least three independent experiments.

Ca^{2+} channels have been described (Fasolato *et al.*, 1994) and that MDCK cells contain at least two known Ca^{2+} entry pathways (Dietl and Volkl, 1994; Delles *et al.*, 1995).

RGD-activated Ca^{2+} Influx Is Independent of IP_3-mediated Pathways

We used TG to address whether capacitative Ca^{2+} influx was responsible for the Ca^{2+} influx occurring in response to RGD. First we tested whether TG completely depleted IP_3-sensitive stores in MDCK cells. ATP was applied to cells after treatment with TG. The TG treatment completely inhibited $[Ca^{2+}]_i$ transients normally triggered by addition of 10 μM ATP (Figure 8a). We conclude that, in MDCK cells, TG effectively depletes the IP_3-sensitive $[Ca^{2+}]_i$ stores. In addition,

these data indicate that, after depletion of Ca^{2+} stores, subsequent IP_3 production does not stimulate Ca^{2+} influx via direct activation of plasma membrane channels. However, subsequent addition of RGD beads to these TG-treated cells elicited a significant $[Ca^{2+}]_i$ transient (Figure 8a). On average, this response is slightly blunted, which may be due to the inhibition of IP_3-sensitive store release by TG pretreatment.

We tested whether this component of the $[Ca^{2+}]_i$ transient resulted from release of a TG-insensitive intracellular Ca^{2+} pool or from activation of a noncapacitative, IP_3-independent Ca^{2+} influx. Cells were treated sequentially with 5 mM Ni^{2+} and TG to simultaneously block calcium influx and deplete the IP_3-sensitive stores. This treatment completely inhibited the RGD-induced $[Ca^{2+}]_i$ transients (Figure 8b; com-
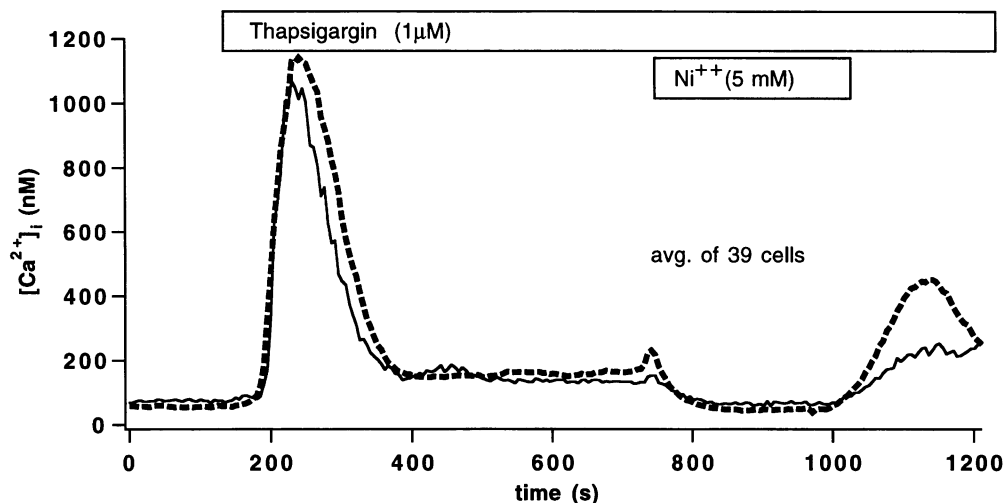
Figure 7. Treatment of cells in buffer containing 1 μM thapsigargin results in a $[Ca^{2+}]_i$ transient followed by slightly elevated baseline $[Ca^{2+}]_i$. Application of 5 mM Ni^{2+} reversibly returns baseline $[Ca^{2+}]_i$ to normal levels. The solid line represents a single cell and the dashed line an average from the same experiment. Results are representative of three independent experiments.

pare with small transients occurring in the presence of Ni^{2+} in Figure 3a). Similar results were obtained with an organic Ca^{2+} channel inhibitor CAI (see below) rather than Ni^{2+} to block Ca^{2+} influx, suggesting that the inhibitory effect of Ni^{2+} is due to inhibition of Ca^{2+} entry rather than nonspecific interference of RGD–integrin binding. Thus, these data indicate that part of the RGD-induced $[Ca^{2+}]_i$ transient is due to Ni^{2+}-sensitive Ca^{2+} influx rather than the release of calcium from a TG-insensitive intracellular Ca^{2+} pool. Furthermore, the RGD-induced Ca^{2+} influx pathway seems to be functionally distinct from the capacitative calcium entry pathway.

Role of Ca^{2+} Store Release and Influx in Regulation of Integrin-mediated Adhesion

We have previously demonstrated a role for RGD-mediated Ca^{2+} signaling in rapid feedback regulation of integrin-mediated substrate adhesion (Sjaastad *et al.*, 1994). To distinguish between roles of separate Ca^{2+} mobilization pathways in feedback regulation of adhesion, we independently inhibited the Ca^{2+} store release and Ca^{2+} influx and determined the effects of each on RGD bead binding.

Depletion of intracellular Ca^{2+} stores with TG for either 10 or 30 min before addition of RGD beads only slightly inhibited adhesion of RGD beads (Figure 9). The two different periods of pretreatment with TG were examined to chronologically separate the onset of the $[Ca^{2+}]_i$ transient induced by TG treatment from the subsequent binding of RGD beads. In contrast, inhibition of Ca^{2+} influx with 5 mM Ni^{2+} resulted in ≈80% reduction of bead binding. The adhesion that remained in the presence of Ni^{2+} was not further decreased by pretreatment with TG, indicating that it was not dependent on intracellular store release. Together, these data suggest that Ca^{2+} influx is a more

potent regulator of integrin-mediated adhesion to RGD than is Ca^{2+} store release.

Tyrosine phosphorylation has been implicated in the regulation of integrin-mediated adhesion in platelets and other cell systems (Klemke *et al.*, 1994; Clark and Brugge, 1995). Thus, it is possible that tyrosine phosphorylation event(s) could be required for calcium signaling or that a downstream phosphorylation event (Pelletier *et al.*, 1992) acts to regulate integrin adhesion to RGD. We observed no inhibition of RGD-induced $[Ca^{2+}]_i$ transients after treatment of MDCK cells with the tyrosine kinase inhibitors genistein (50–100 μM) or erbstatin analogue (1–5 μM) (our unpublished observations). However, low concentrations of erbstatin analogue significantly inhibited adhesion of RGD beads (Figure 9), suggesting that tyrosine phosphorylation events may be important for stabilizing RGD-mediated adhesion. Therefore, we conclude that these phosphorylation events are not required for RGD to induce $[Ca^{2+}]_i$ transients and must lie downstream of the onset of the $[Ca^{2+}]_i$ transient.

Inhibition of RGD-induced Signaling and Cell Adhesion with CAI

CAI is a potent Ca^{2+} channel inhibitor that recently has been shown to inhibit adhesion and migration of endothelial cells (Felder *et al.*, 1991; Kohn *et al.*, 1994, 1995). We tested whether CAI similarly inhibited Ca^{2+} channels and adhesion in MDCK cells. As an independent Ca^{2+} channel inhibitor, CAI provided an important control for possible nonspecific effects of 5 mM Ni^{2+} in our previous studies of integrin-mediated Ca^{2+} signaling and adhesion. CAI inhibited the average peak of RGD-induced $[Ca^{2+}]_i$ transients compared with untreated cells or cells treated with equivalent concentrations of the inactive CAI analogue AIC (Figure 10, a and b). CAI (20 μM) completely inhibited the
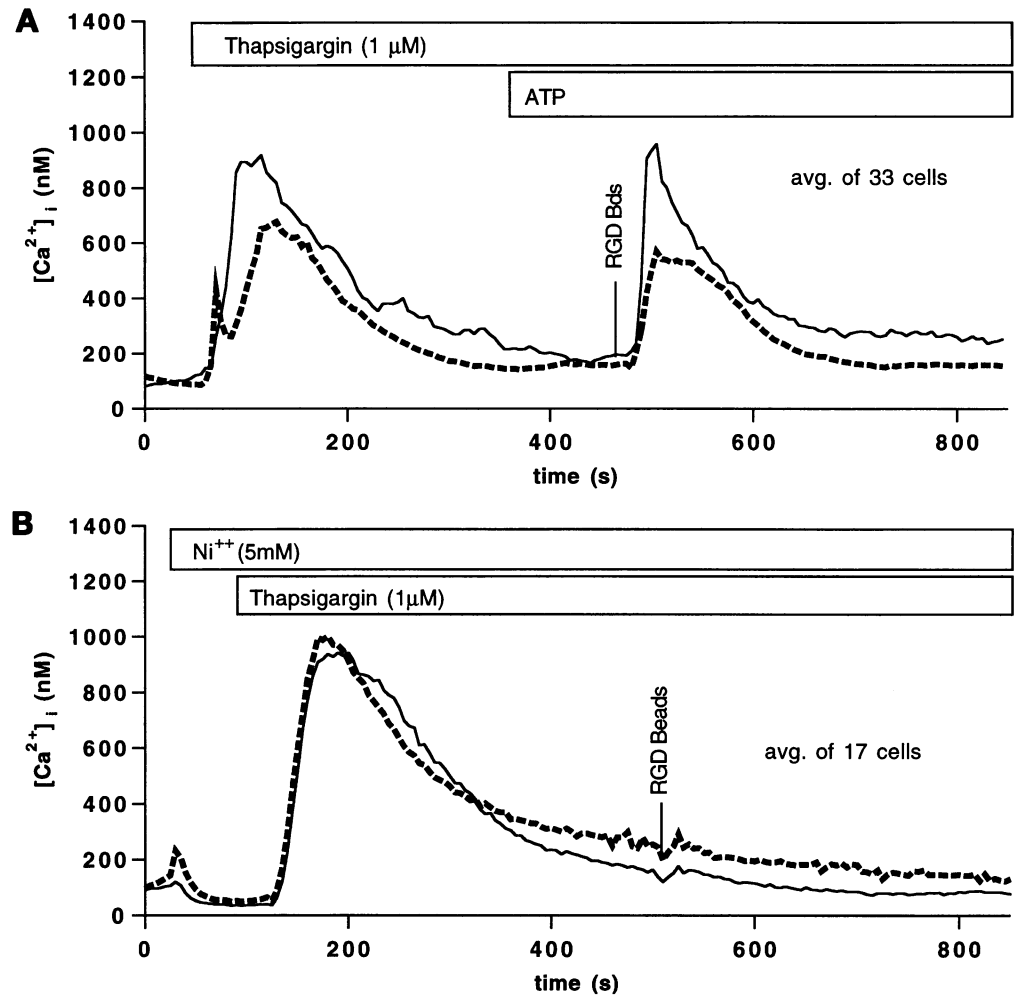
Figure 8. IP_3-independent activation of Ca^{2+} influx. After depletion of the stores with thapsigargin, ATP does not induce a Ca^{2+} transient. (a) However, subsequent addition of RGD beads still elicits a large response. (b) Addition of 5 mM Ni^{2+} to block calcium influx channels and subsequent depletion of stores completely inhibit the RGD-induced $[Ca^{2+}]_i$ transient. The solid lines represent single cells, and the dashed lines are averages. Results are representative of three independent experiments.

RGD transient, except for small spikes, similar to pretreatment with 5 mM Ni^{2+} (Figure 10a; compare with Figure 3a). In addition, pretreatment of cells with 20 μM CAI followed by depletion of stores with TG (similar to the protocol used for Ni^{2+} in Figure 8b) also completely eliminated the RGD-induced calcium transient (our unpublished results). The similarity of the inhibition of $[Ca^{2+}]_i$ transients by CAI and Ni^{2+} and the similar presence of small spikes in the presence of CAI (possibly because of the release of Ca^{2+} stores) is consistent with the assumption that their mechanism of inhibition occurs via inhibition of calcium channels and not inhibition of the RGD–integrin interactions necessary for signaling.

We tested the ability of CAI to inhibit RGD-mediated cell adhesion. Figure 11 (inset) depicts the dose–response relation for CAI-dependent inhibition of adhesion in a substrate adhesion assay. Nearly complete inhibition of cell adhesion was obtained with 20 μM CAI, whereas equivalent concentrations of AIC had no effect. These results and those shown in Figure 9 sug-

gest that Ca^{2+} influx plays the dominant role in regulating RGD bead adhesion. We next asked whether adhesion could be rescued by restoring the $[Ca^{2+}]_i$ transient in cells treated with channel inhibitors. MDCK cells were treated with either 5 mM Ni^{2+} or 20 μM CAI before the addition of RGD beads, and then 10 μM ATP was added to trigger substantial $[Ca^{2+}]_i$ transients by releasing Ca^{2+} stores at the time the beads began contacting the cells. As shown in Figure 10c, the $[Ca^{2+}]_i$ transients elicited by 10 μM ATP were not inhibited by pretreatment with 20 μM CAI. Similarly, pretreating cells with 5 mM Ni^{2+} did not significantly inhibit ATP-induced $[Ca^{2+}]_i$ transients (our unpublished results). Using ATP to restore the $[Ca^{2+}]_i$ transient in Ni^{2+}- or CAI-treated cells resulted in little, if any, rescue of RGD bead adhesion. Similar results were obtained when ATP was used to restore the $[Ca^{2+}]_i$ transients in CAI-treated cells in the substrate adhesion assay (our unpublished observations). Because the global rises in $[Ca^{2+}]_i$ elicited by ATP and RGD beads are similar in magnitude, these results
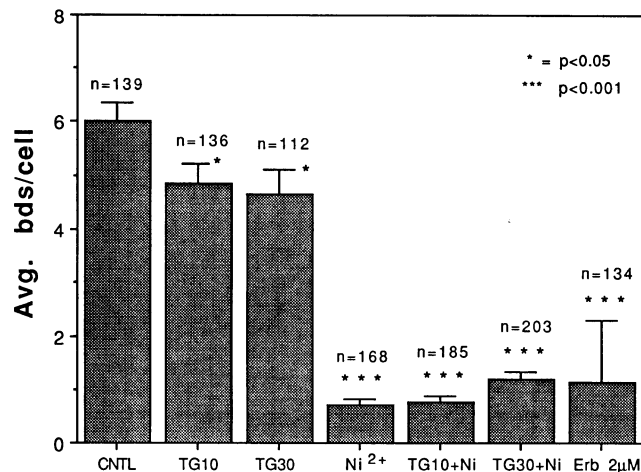
Figure 9. Adhesion of RGD beads is inhibited by blocking Ca^{2+} influx and inhibition of tyrosine phosphorylation. Ni^{2+} (5 mM) and erbstatin analogue (2 μM) or DMSO as a vehicle control was added immediately before the addition of RGD beads. Cells were gently washed twice to remove unbound beads; the average number of beads bound to single cells was determined by counting bright-field images captured with the imaging system. Data are presented as mean $\pm$ SEM. At least five fields of cells were analyzed for each condition. The number of cells counted for each condition and the statistical significance (Student's t test) from control are indicated in the figure. This experiment is representative of at least three independent experiments.

further support the notion that RGD-stimulated Ca^{2+} influx is more important for modulating integrin-mediated adhesion to RGD than equivalent global cell calcium changes occurring by release of intracellular Ca^{2+} stores. These findings indicate that Ca^{2+} influx is the decisive factor in the RGD-induced feedback regulation of adhesion in MDCK cells.

DISCUSSION

Rapid and dynamic regulation of integrin-mediated cell–ECM adhesion is required for exploratory cell movements during early development, wound healing, angiogenesis, inflammatory and immune responses, and the progression of metastatic disease (Hynes, 1992; Clark and Brugge, 1995). Furthermore, integrin-mediated signaling may directly regulate gene expression and cell death (Jones *et al.*, 1993; Boudreau *et al.*, 1995). Our previous study demonstrated the importance of integrin-mediated calcium signaling in rapid (<10 min) feedback regulation of cell–substrate adhesion in MDCK cells (Sjaastad *et al.*, 1994). In this system and many others, the mechanisms that underlie calcium signaling and their role in regulating adhesion are uncharacterized. Here, we report that Ca^{2+} release and Ca^{2+} influx occur simultaneously to generate a $[Ca^{2+}]_i$ transient in response to RGD–integrin binding in MDCK cells and that Ca^{2+} influx is the

more potent of the two mechanisms for rapid feedback regulation of integrin-mediated adhesion.

RGD triggers release of IP_3-sensitive Ca^{2+} stores. Intracellular heparin, an inhibitor of IP_3 binding to its receptor (Hill *et al.*, 1987; Ghosh *et al.*, 1988), decreased the RGD-induced $[Ca^{2+}]_i$ transients. Moreover, we observed that small $[Ca^{2+}]_i$ transients still occurred in the presence of Ni^{2+} but not in the presence of Ni^{2+} after Ca^{2+} store depletion with TG pretreatment (Figure 8b). Finally, both ATP and RGD beads increased the rate of inositol phosphate turnover in MDCK cells, but beads coated with BSA did not (M.D. Sjaastad, R.S. Lewis, W.J. Nelson, unpublished results). These results are consistent with recent findings in pancreatic acinar cells that cell adhesion to the RGD sequence in collagen and RGD peptides stimulates IP_3 production via activation of phospholipase C (Somogyi *et al.*, 1994).

Several lines of evidence suggest that calcium influx is also a key component of the RGD-mediated $[Ca^{2+}]_i$ transient. The RGD response was significantly inhibited by Ca^{2+} channel inhibitors (5 mM Ni^{2+} or CAI) and reduction of the driving force for Ca^{2+} entry by either membrane depolarization or reduced $[Ca^{2+}]_o$. To control for the possibility that the inhibitory effects of Ni^{2+} on $[Ca^{2+}]_i$ transients and adhesion were due to disruption of RGD–integrin binding, we also tested the effects of a second Ca^{2+} channel inhibitor (CAI) and its control compound (AIC) on Ca^{2+} influx and adhesion. We observed identical inhibition of RGD-induced $[Ca^{2+}]_i$ transients and adhesion with Ni^{2+} and CAI and little effect with AIC, suggesting that Ni^{2+} and CAI both act via inhibition of Ca^{2+} influx and not by hindering initial RGD–integrin binding. In the case of membrane depolarization, we observed inhibition of the initial $[Ca^{2+}]_i$ peak followed by a substantial plateau (Figure 3c). Mechanisms regulating the plateau amplitude in response to RGD are not known; however, the complete substitution of K^+ for Na^+ in this experiment may result in elevated $[Ca^{2+}]_i$ by inhibiting Ca^{2+} efflux mechanisms. Inhibition of the initial $[Ca^{2+}]_i$ peak by depolarization suggests that Ca^{2+} influx contributes to the early phase of the RGD response. Finally, when $[Ca^{2+}]_o$ is decreased from millimolar levels to concentrations above 150 μM, reduction of the RGD response is likely due to reduced driving force; however, a loss of integrin signaling function may explain the lack of RGD-mediated Ca^{2+} signaling below 150 μM $[Ca^{2+}]_o$. Note that cells adhere to RGD and collagen substrates at $[Ca^{2+}]_o$ concentrations well below 150 μM (Leavesley *et al.*, 1993), suggesting that integrin function in adhesion and signal transduction may have different sensitivities to low $[Ca^{2+}]_o$.

In many cell types, depletion of Ca^{2+} stores elicits capacitative Ca^{2+} entry (Putney and Bird, 1993; Fasolato *et al.*, 1994). Recently, a calcium current activated
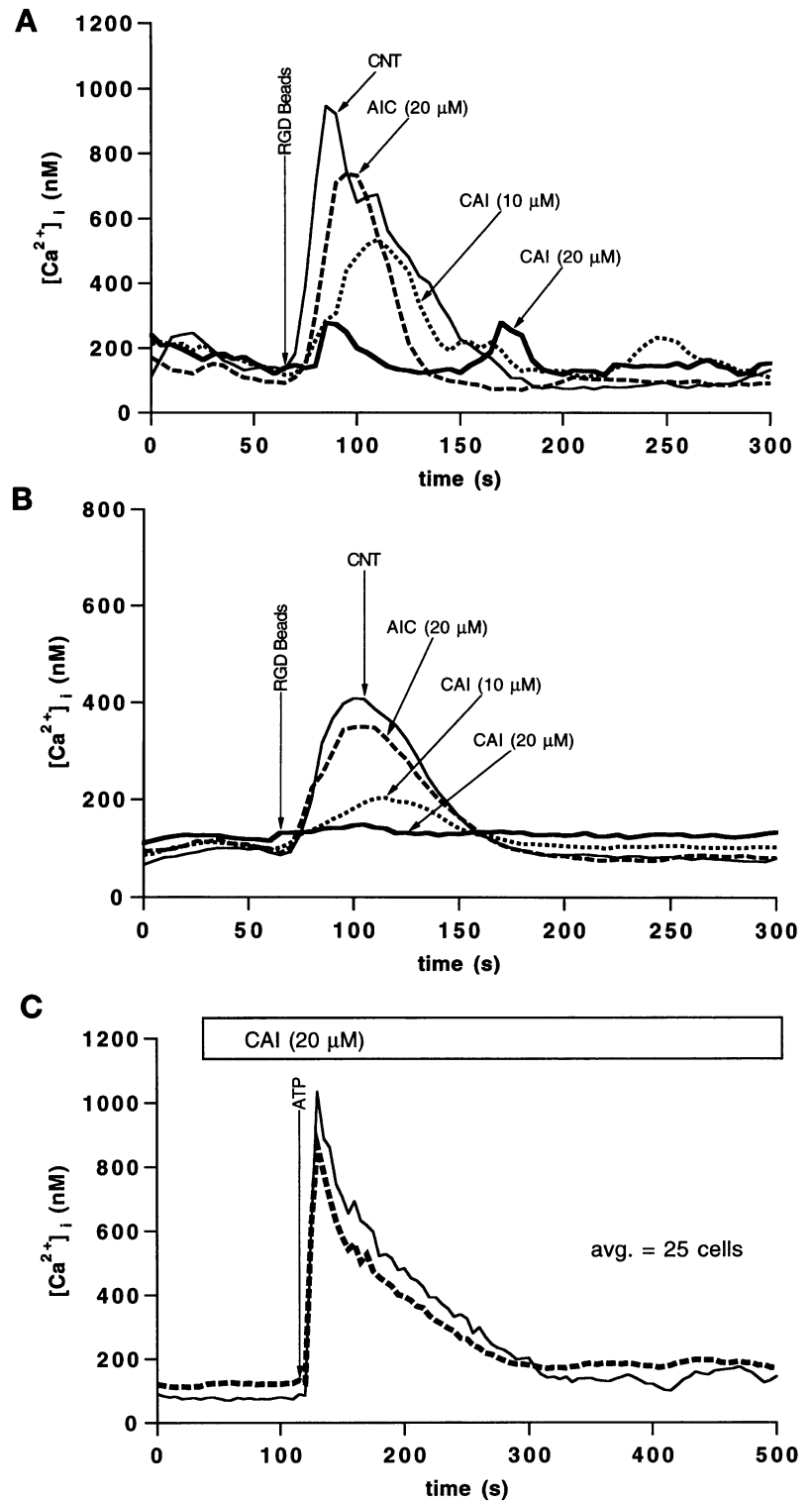
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Figure 10. CAI inhibits RGD-induced $[Ca^{2+}]_i$ transients but does not inhibit transients triggered by ATP. (a) Single-cell traces of $[Ca^{2+}]_i$ after application of RGD beads in control cells (solid line) or in the presence of AIC (20 μM, dashed line) or CAI (10 μM, dotted line; 20 μM, heavy solid line). Note that small spikes of $[Ca^{2+}]_i$ increase are still present in the presence of 20 μM CAI. (b) Average traces from the same experiments depicted in a. (c) In the presence of 20 μM CAI, the transients triggered by ATP are similar to those in the absence of CAI (compare with Figure 5, a and b). Results are representative of at least three independent experiments.

by store depletion has been described in MDCK cells (Delles *et al.*, 1995), which may account for the Ni^{2+}-sensitive Ca^{2+} plateau we observed after TG treatment (Figure 7). However, a second component of store-

operated Ca^{2+} entry is indicated by the transient $[Ca^{2+}]_i$ rise induced by TG in the presence of Ni^{2+} (Figure 8b). The pathway responsible for this influx has not been identified in MDCK cells but may be
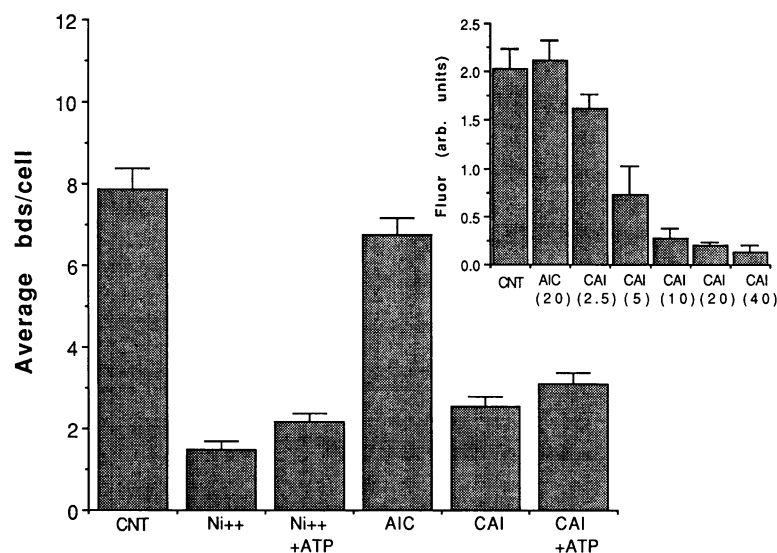
Figure 11. CAI inhibits cell adhesion to RGD beads and RGD-coated substrate. Inset: In a substrate adhesion assay (see MATERIALS AND METHODS), CAI inhibits adhesion of cells to RGD-coated wells in a dose-dependent manner; nearly maximal inhibition occurred at 20 μM CAI. Equivalent concentration of the inactive analogue (AIC) had no effect. Data are shown as the mean $\pm$ SEM; n = 4 wells for each condition. Bead adhesion (see MATERIALS AND METHODS) was significantly inhibited by 5 mM Ni^{2+} and 20 μM CAI. Addition of 10 μM ATP to induce a calcium rise in cells pretreated with 5 mM Ni^{2+} and 20 μM CAI resulted in only a modest increase in adhesion. Data are shown as mean $\pm$ SEM; n = 5 fields analyzed (at least 150 cells) for each condition. $p = 0.021$ for Ni^{2+}+ATP compared with Ni^{2+} alone, and $p = 0.136$ for CAI + ATP compared with CAI alone; Student's t test. Results are representative of three independent experiments.

related to rapidly inactivating store-operated Ca^{2+} channels studied in A431 epithelial cells (Lückhoff and Clapham, 1994).

Despite their presence, activation of store-operated channels does not seem to contribute significantly to RGD-induced Ca^{2+} influx. Instead, RGD triggers a store- and IP_3-independent Ca^{2+} influx pathway, as shown by the activation of additional Ca^{2+} influx after TG pretreatment (Figure 8a). Because exposure to TG is expected to fully activate store-operated channels in the cells, the additional influx triggered by RGD is likely to be regulated through an independent mechanism. Two additional lines of evidence indicate that store-operated channels make, at most, a minor contribution to the RGD-induced influx. First, we found that the magnitude of $[Ca^{2+}]_i$ transients resulting from ATP stimulation is similar in the presence or absence of external calcium, suggesting that, although it is substantial in other clones (Paulmichl *et al.*, 1990), stimulation of store release does not trigger a large store-operated influx component in the clone of cells we have used. Second, we can rule out the possibility that RGD beads induce a rapidly inactivating store-operated Ca^{2+} influx mechanism like that triggered by TG, because that influx was Ni^{2+}-insensitive, unlike the RGD bead response, which is Ni^{2+}-sensitive (Figure 8b). Thus, Ca^{2+} influx resulting from IP_3-mediated store depletion is likely to make only a minor contribution to the RGD-induced transient.

In the experiments shown in Figure 6, heparin blocks the responses to both ATP and RGD by ~50% at maximum, suggesting that IP_3-induced Ca^{2+} release contributes equivalently to the two responses. It is therefore surprising that store depletion by TG eliminates the ATP response but not the RGD response and that the latter seems to include an additional

contribution from Ca^{2+} influx (Figure 8). We do not know the basis for this discrepancy, but one possibility is that heparin has nonspecific effects in addition to inhibiting the IP_3 receptor, perhaps blocking Ca^{2+} influx as well. In most cells, the relative contributions of Ca^{2+} release and influx to a response are most easily assessed by comparing responses obtained in the presence and absence of extracellular Ca^{2+}. This test could not be applied in the present study because of the requirement of extracellular Ca^{2+} for proper signaling through RGD-binding integrins (Figure 4; Hynes, 1992). Thus, we cannot determine quantitatively the relative contributions of Ca^{2+} release and influx to the RGD response, although the important qualitative conclusions of this study remain valid.

Taken together, our results indicate that two independent mechanisms are activated by RGD–integrin binding in MDCK cells: an IP_3-mediated release of Ca^{2+} stores and an IP_3- and store-independent activation of Ca^{2+} influx that is inhibited by Ni^{2+}, CAI, and membrane depolarization. This set of characteristics may aid in the identification of RGD-regulated channels by the use of the patch-clamp methodology. On the basis of findings in adhesion studies, we propose a model for RGD-induced Ca^{2+} signaling and feedback regulation of adhesion in MDCK cells. RGD binding stimulates elevated $[Ca^{2+}]_i$ levels by a combination of activation of an IP_3-dependent pathway that releases calcium stores and activation of IP_3-independent calcium influx that regulates adhesion. Three lines of evidence show that Ca^{2+} influx, rather than store release, is the dominant regulatory component of feedback regulation of adhesion. First, blocking Ca^{2+} influx dramatically inhibits adhesion. Second, blocking release of IP_3-sensitive stores with TG pretreatment has little affect on adhesion. Finally, restoration of

Ca^{2+} transients by releasing Ca^{2+} stores with ATP in the presence of Ni^{2+} or CAI does not enhance adhesion. The target of the calcium signal is currently unknown. However, a tyrosine phosphorylation event(s)is important for adhesion and occurs downstream of the Ca^{2+} response (Figure 9). The importance of Ca^{2+} influx in regulation of adhesion suggests that a close coupling of integrins to Ca^{2+} channel activation may exist. Presently, there is no evidence that integrins and Ca^{2+} channels are directly coupled, but an intriguing possibility is that specific spatial information may be encoded by direct coupling of Ca^{2+} influx proximal to regions of integrin activity.

Although Ca^{2+} influx regulates integrin-mediated adhesion in MDCK cells, it may be less critical in other cell types. For example, spreading of endothelial cells on collagen activates $\alpha2\beta1$ integrins, resulting in an elevation of pH_i, whereas attachment to vitronectin activates $\alpha v\beta3$ integrins, resulting in increased pH_i and $[Ca^{2+}]_i$ through different signaling pathways (Leavesley et al., 1993). In addition, adhesion and signaling assays conducted on endothelial cells spreading on fibronectin indicate that activation of $\alpha5\beta1$ integrin elevates pH_i and participates significantly in adhesion, whereas αv integrins mobilize intracellular calcium but play only a minor role in adhesion (Schwartz and Denninghoff, 1994). Note, however, that tumor angiogenesis requires both acute expression of $\alpha v\beta3$ (Brooks et al., 1994a,b) and Ca^{2+} influx in endothelial cells (Kohn et al., 1995). Unlike endothelial cells, MDCK cells do not express detectable levels of $\alpha5\beta1$ (Ojakian and Schwimmer, 1994; Schoenenberger et al., 1994), nor do RGD beads induce an increase in pH_i (M.D. Sjaastad, R.S. Lewis, W.J. Nelson, unpublished observations). However, MDCK cells express $\alpha v\beta3$ and $\alpha v\beta5$, both of which contribute to RGD-specific bead binding, $[Ca^{2+}]_i$ signaling, and rapid regulation substrate adhesion, likely via calcium channel activation. Taken together, these studies indicate that the signaling function of specific integrins may vary with cell type, ligand presentation, and changes in cell activity.

ACKNOWLEDGMENTS

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