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Mechanically Induced Calcium Movements in Astrocytes, Bovine Aortic Endothelial Cells and C6 Glioma Cells

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Abstract. Forces applied to resting primary astrocytes, bovine aortic endothelial cells and C6 glioma cells with collagen-coated magnetite particles produce a fast transient change of intracellular Ca^{2+}. It peaks in the micromolar range as measured by Fura-2. This mechanical response adapts within seconds so that repeated stimulation causes smaller responses requiring >10 min for recovery. When cytoplasmic Ca^{2+} is high after treating with ATP, cyclopiazonic acid and thapsigargin, stimulation causes a transient decrease in Ca^{2+}.

In these three cell types, no influx of ions is required for Ca^{2+} elevation showing the response is not caused by activation of plasmalemmal mechanosensitive channels. Approximately half the cells tested showed similar behavior, while the other half, such as fibroblasts, required extracellular Ca^{2+}. The Ca^{2+} response is not temperature sensitive suggesting the possible involvement of intracellular mechanosensitive channels. We tested a number of second messenger reagents and were only able to block the response in BAECs, but not C6 glioma cells, with Xestospongin C, a blocker of IP_3-activated channels.

Despite the lack of a causal involvement of plasmalemmal mechanosensitive channels, mechanical stimulation immediately activates a persistent Mn^{2+} influx pathway. This Mn^{2+} pathway may be mechanosensitive channels, Ca^{2+}-activated cation channels or depletion-activated Ca^{2+} channels.

Key words: Magnetic — Ion channels — Fluorescence — Integrins — Manganese — Calcium

Introduction

Mechanical stimulation of cells leads to a multitude of responses including elevation of intracellular Ca^{2+} (Furuya & Enomoto, 1990; Sigurdson & Sachs, 1991; Sigurdson, Sachs & Diamond, 1993; Tanaka et al., 1994; Kreimer & Witman, 1994; Duncan & Turner, 1995; Sharma et al., 1995; Xia & Ferrier, 1995; Pommerenke et al., 1996; Glogauer et al., 1997), changes in a variety of second messengers, and the transcription of specific genes. The fundamental transducers for these effects are not known, but possible candidates include mechanosensitive channels (MSCs) and enzymes such as the phospholipases and kinases (Mudo et al., 1993; Roman & Harder, 1993; Noda et al., 1994; Oike, Droogmans & Nilius, 1994; Berk et al., 1995; Wang et al., 1995).

Mechanical stimulation itself is difficult to define and measure precisely. Many different stimuli are used including anisotonic stress (Kawahara, Ogawa & Suzuki, 1991; Izu & Sachs, 1994), flow-induced shear stress (Xiao & Bevan, 1994; Berk et al., 1995), substrate strain (Duncan & Hruska, 1994; Wang et al., 1995; Xu et al., 1996; Maniotis, Chen, Ingber, 1997), membrane tension induced by a pressure gradient (Guharay & Sachs, 1984; Sokabe, Sachs, Jing, 1991; Sigurdson et al., 1997), local stress applied via glass pipettes (Charles et al., 1991; Sigurdson, Ruknudin & Sachs, 1992; Diamond, Sachs & Sigurdson, 1994), and magnetic beads (Glogauer, Ferrier & McCulloch, 1995; Wang & Ingber, 1995; Pommerenke et al., 1996; Bierbaum & Notbohm, 1997; Glogauer et al., 1997; Hafeli et al., 1997; Sachs, 1977; Zorowki, 1997). The stimuli are not generally equivalent. For example, in chick heart cells hypotonic stress opens anionic channels while directly applied stress opens cationic channels (Hu & Sachs, 1996). Flow-induced shear stress can cause changes in the concentration of diffusible factors such as adenosine 5′-triphosphate (ATP) (Grygorczyk & Hanrahan, 1997) as well as pulling on the cell surfaces through adhesion plaques. Stretching the cell cortex in a patch pipette is a general stress that easily stimulates MSCs, but the stress is not applied to specific cellular components. A

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similar lack of specificity arises from poking intact cells, and the technique is not readily extensible to bulk assays. In contrast, magnetic beads coated with specific ligands have been used to remotely apply stress to cells *c.f.,* (Glogauer et al., 1995; Bierbaum & Notbohm, 1997; Simson et al., 1998). The beads can be coated with a wide variety of ligands to stress specific components of the cell surface (Wang et al., 1993; Wang & Ingber, 1994, 1995; Ingber et al., 1995) and the assay can be run in bulk as well as under microscopic examination.

Since Ca^{2+} can have profound effects upon a host of cellular processes, the ability of mechanical stress to acutely alter cell Ca^{2+} represents a key pathway in physiological environments. Cells are normally stressed by the organism's internal and external mobility. While plasmalemmal MSCs are a natural tool to transform mechanical stress into a Ca^{2+} influx (Erxleben, 1993), there are a number of examples in which the effects of mechanical stress can be invoked without extracellular Ca^{2+} (Snowdowne, 1986; Wirtz & Dobbs, 1990; Chen et al., 1997). These latter effects may involve second messengers utilizing phospholipases (Tanaka & North, 1994), autocrine factors such as angiotensin (Sadoshima et al., 1992), endothelin (Hishikawa et al., 1995), and kinases (Berk et al., 1995). In no case other than MSCs has the primary mechanical transducer been identified. In most cases it is extremely difficult to dissociate the initial transduction events from later amplification processes that utilize (multiple) Ca^{2+} stores (Golovina et al., 1996; Golovina & Blaustein, 1997) and enzymatic processes.

The present experiments show that mechanical stimulation of resting cells in culture increases cell Ca^{2+} to micromolar levels. This occurs in all cell types tested (>10) including bovine aortic endothelial cells (BAECs), primary rat astrocytes, C6 glioma cells, osteocyte lines, fibroblasts, HEK-293 (human kidney), opossum kidney cells, and primary chick cardiac myocytes. Most cell types relocate intracellular Ca^{2+} without requiring an influx of ions from the bath and this study focuses on three of these cell types. Other types require extracellular Ca^{2+} for a mechanically stimulated Ca^{2+} elevation. These include AS4.1 (mouse kidney), UMR-106 (rat bone), GH_3 (rat pituitary), and NRK-49F (rat kidney).

We have observed a dual effect of stretch in astrocytes, BAECs, and C6 glioma cells when cytosolic Ca^{2+} is low — a Mn^{2+} permeable pathway in the sarcolemma is opened with no visible delay while intracellular Ca^{2+} is increased. Additionally, when cytosolic Ca^{2+} is elevated in C6 glioma cells, the effect of stretch is an intracellular Ca^{2+} *decrease.*

Ion influx through the plasmalemmal Mn^{2+} pathway is not essential for Ca^{2+} release, but seems to be a correlated rather than causal event. We do not yet know whether the pathway is activated directly by stress, by intracellular Ca^{2+}, or by some other second messenger.

Materials and Methods

Fluorescence Measurements

Performed with an AB2 fluorescence spectrometer (SLM-AMINCO, 820 Linden Avenue, Rochester, NY 14625) with Fura-2 AM (Molecular Probes, Eugene, OR) with excitation at 340 nm and 380 nm and emission at 510 nm unless otherwise specified. Measurements were made in a microchamber with a volume of ~25μL. Cells were grown on 5mm wide coverslips. The chamber was assembled by placing on top a second 5mm coverslip with narrow edge strips of coverglass along its length. Held by a Teflon cap through which solutions could be injected or perfused, this microchamber was then inserted into a quartz cuvette within the fluorimeter. The cuvette was filled with normal saline for index matching to reduce reflection.

Cells included C6 glioma (from Dr. Charles Bowman, VA Hospital, Buffalo & ATCC), bovine aortic endothelial cells (from Dr. Scott Diamond, Department of Chemical Engineering, U. of Penn.), and primary activated astrocytes (from Dr. Thomas Langan, Department of Neurology, SUNY, Buffalo) (Langan et al., 1995). Passage 43–87 C6 glioma cells were maintained in HAMS F-10 Nutrient with 3% FBS, 15% HIHS, and 1% Pen/Strep. Passage 10–19 BAECs and passage 8 astrocytes were maintained in DMEM with 10% FBS and 1% Pen/Strep (HAMS F-10, DMEM, FBS, HIHS, and Pen/Strep were from Gibco BRL, Grand Island, NY). All cells were plated on glass coverslips coated with poly-L-lysine (Sigma, St. Louis, MO) and used within 1–12 days. Loading was done at room temperature for 30 min with 2–4 μM Fura-2 AM and 0.05–0.1% wt/vol Pluronic F-127 (Molecular Probes). An additional 30 min was taken for cleavage of the dye and attachment of coated magnetite beads. Prior to adding the beads, they were sonicated to minimize clumping. The beads were coated by incubating 400 mg of ferric oxide powder, Fe_3O_4, (Aldrich, Milwaukee, WI) with 100 μL of 1.0M NaOH and 1,000 μL of 3.0 mg/mL collagen stock (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) overnight on a rotator at room temperature (Glogauer et al., 1995). The coated beads were then rinsed and resuspended in normal saline (*see below*). Force was applied to the collagen beads either via a round pot electromagnet (Dura Magnetics, Sylvania, OH) using AC current at various voltages or a cylindrical (0.625″ × 0.5″) neodymium boron permanent magnet (Dura Magnetics). We have not attempted to calibrate the absolute forces since the beads are distributed in variable size domains on the cells and the number of bonds per bead is unknown so the absolute magnitude of the relevant forces can't be defined. Typical literature values under these conditions are ~4 pN/bead (Glogauer et al., 1998).

Calcium concentrations were calculated with the following formula: $[Ca^{2+}]_i = K_d\,[(R - R_{min})/(R_{max} - R)]\,(S_{f2}/S_{b2})$ (Grynkiewicz, Poenie & Tsien, 1985), where $K_d = 225$ nM, R = ratio of emission at 340 nm excitation/emission at 380 nm excitation, and S_{f2} and S_{b2} are the emission signals with 380 nm excitation when the dye is free and bound with Ca^{2+}, respectively. Normal saline contained in mM: 135 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 50 mannitol and 10 glucose. R_{max} saline was made by increasing calcium to 5mM and adding 10μM ionomycin. R_{min} saline was made by replacing calcium with 500μM EGTA and adding 10 μM ionomycin. "Sugar water" solution contained in mM: ~350 mM mannitol, 10 HEPES, 10 glucose. High K^+ saline was made by swapping the concentration values for NaCl and KCl as found in the normal saline. Cytochalasin D, colchi-

cine, cyclopiazonic acid (CPA) and thapsigargin were obtained from Sigma and BAPTA AM from Molecular Probes.

Fluorescent Imaging

Astrocytes were prepared by loading with 5 μM Fluo-3 AM (Molecular Probes) in normal saline for 30 min followed by incubation with collagen-coated magnetite beads as described above. Magnetic field stimulation of the bead-decorated cells was made using a home-built electromagnet shaped to focus and localize the field in a restricted area of about 200 μm in diameter. DC and AC (<10 Hz) voltages were used to energize the magnet. Loaded cells were examined with an Axiovert, using a 40× N.A. 1.3 objective (Carl Zeiss, Thornwood, NY). Fluo-3 fluorescent and DIC Images of cells were acquired with an image-intensifier (Video Scope International, Sterling, VA) coupled to a CCD-72 camera (Dage-MTI, Michigan City, IN). Images were stored on S-VHS tape and later digitized using a frame grabber (National Instruments, Austin, TX).

Abbreviations

BAECs, bovine aortic endothelial cells; CPA, cyclopiazonic acid; Gs wv, *Grammostola spatulata* whole venom; MACR, mechanically activated calcium release; MAMI, mechanically activated manganese influx; MSCs, mechanically sensitive ion channels; SAC, stretch-activated ion channel; XC, Xestospongin C.

Results

A typical Ca^{2+} increase in astrocytes to magnetic stimulation is shown in Fig. 1*a* where the fluorescence is in arbitrary units. The upper trace is fluorescence at 510 nm with excitation at 340 nm, the middle is fluorescence at 510 nm with excitation at 380 nm and the lower trace is the ratio (340 nm/380 nm). The beads were collagen coated. (This format is used in all following figures unless otherwise noted). Likewise, Fig. 1*b* and *c* are typical Ca^{2+} increases in BAECs and C6 glioma cells, respectively. The peak Ca^{2+} level in C6 glioma cells is in the range of 1.5–2.0 μM with a mean increase of 0.9 ± 0.2 μM using the standard R_{min}/R_{max} calibration technique described in Materials and Methods (Fig. 1*d*) (Grynkiewicz et al., 1985). In the three cell types, mechanical stimulation caused a transient elevation in Ca^{2+} that inactivated with repeated stimulation (*not shown* for the astrocytes). At the level of single cells, fluorescence microscopy showed a similar response in astrocytes (Fig. 2).

To determine if extracellular calcium was required for the observed intracellular Ca^{2+} increases, we tested C6 glioma cells in what we call sugar water (*see* Materials and Methods). In the absence of extracellular Ca^{2+}, C6 cells still showed an increase in Ca^{2+} with stimulation (Fig. 3). Since this bath solution contained only trace amounts of Na^+, K^+, and Cl^-, the increase in Ca^{2+} does not require the movement of these ions. Obviously, the

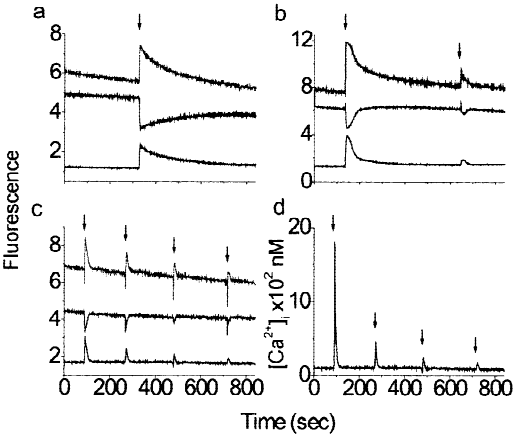


Fig. 1. Intracellular Ca^{2+} increases in astrocytes (*a*) ($n = 12$), BAECs (*b*) ($n = 45$) and C6 glioma (*c*) ($n = 75$) following magnetic stimulation (arrows) in 1 mM Ca^{2+} saline using collagen coated magnetite beads. For clarity, the upper and middle traces in *b* and *c* have been offset 1 and 2 units, respectively. (*d*). Calculated intracellular Ca^{2+} concentration from the data in *c* (*see* Materials and Methods). Peak values were in the range of 1.5–2.0 μM with a mean increase of 0.9 ± 0.2 μM.

Ca^{2+} involved in the transient elevations in C6 cells is predominantly from intracellular sources. In 5 out of 12 experiments the Ca^{2+} increase observed in astrocytes depended on extracellular Ca^{2+}. Although we haven't investigated the source of this variability it appears to be developmentally dependent. Similar to C6 glioma, BAECs do not require the presence of extracellular Ca^{2+}.

What intracellular source of Ca^{2+} is being tapped in C6 glioma cells? In an effort to determine this we mechanically stimulated the cells in either the presence of ATP, bradykinin, thapsigargin, or ryanodine (Fig. 4). Only two of the four drugs, ATP and thapsigargin, suppressed (but didn't eliminate) the mechanically induced increases in intracellular Ca^{2+}. Given that ATP (Creba et al., 1983; Okajima et al., 1987; Phaneuf et al., 1987; Pirotton et al., 1987; Blachier & Malaisse, 1988; Legssyer et al., 1988; van der Merwe et al., 1989; De Young & Scarpa, 1989; Pearce et al., 1989; Arkhammar et al., 1990; Nanoff et al., 1990; Pillai & Bikle, 1992; Sato, Okajima & Kondo, 1992; Idestrup & Salter, 1998) and thapsigargin (Thastrup, Foder & Scharff, 1987; Hanley et al., 1988; Jackson et al., 1988; Takemura et al., 1989; Thastrup et al., 1989; Law et al., 1990; Pepperell & Behrman, 1990; Takemura, Thastrup & Putney, Jr., 1990; Thastrup et al., 1990) have been shown to affect inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} stores, these results strongly suggest that Ca^{2+} from IP_3-sensitive stores is a component of the mechanically activated Ca^{2+} release (MACR) in C6 glioma cells.

If our mechanical stimulus is generating IP_3, then preventing MACR is simply a matter of interrupting the

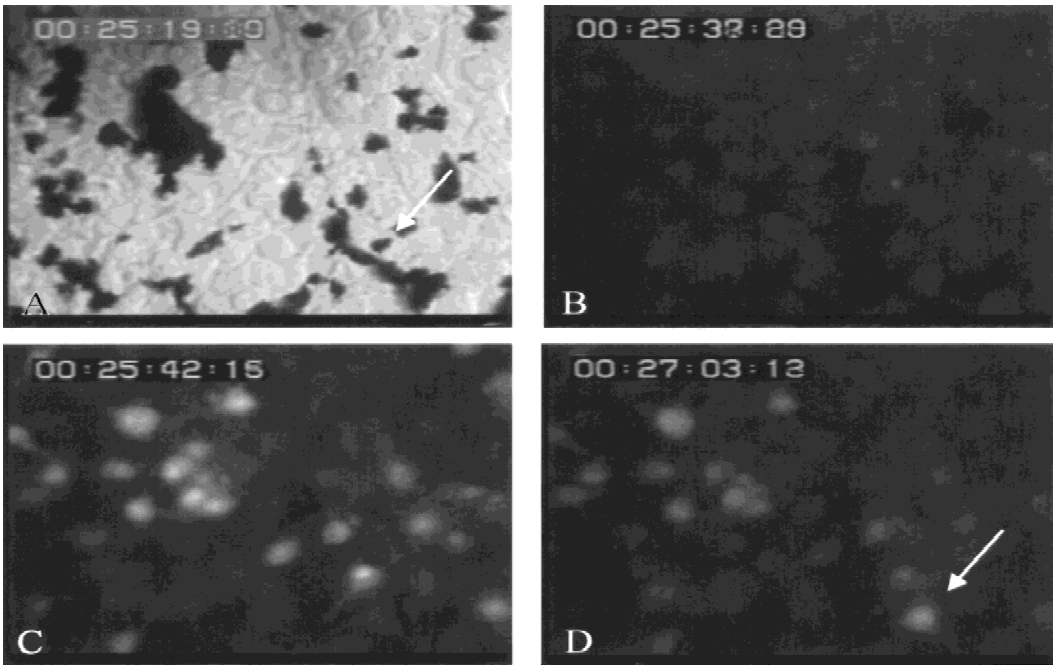


Fig. 2. A time series of images from a monolayer of primary astrocytes loaded with Fluo-3 AM and decorated with collagen-coated magnetite beads. The beads are visible in Panel *A* as clusters of black particles. Panel *B* shows an intensified fluorescent image before stimulation. Panel *C* shows the same field after turning on a nearby electromagnet. Note that regions without beads do not light up. Panel *D* shows the response to a second stimulation imposed on the residual response of the first stimulation. Most of the cells from the first stimulation are still visible, but dimming because of restoration of the Ca^{2+} gradient (not bleaching). The arrow points to a cell that only responded to the second stimulation.

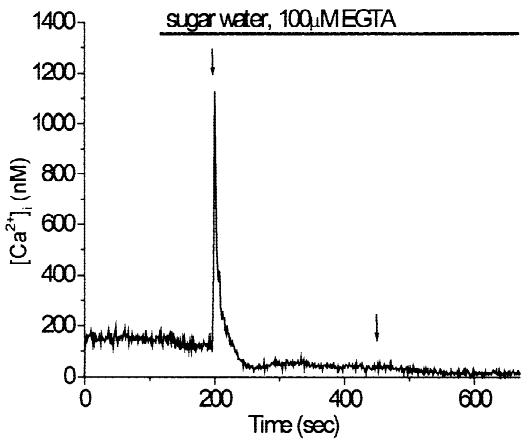


Fig. 3. Sugar water containing 100 μM EGTA does not inhibit the initial intracellular calcium increase in C6 glioma cells following magnetic stimulation (arrows) ($n = 11$). The responses to subsequent magnetic stimuli were inhibited, the most likely cause of which is depletion of intracellular stores.

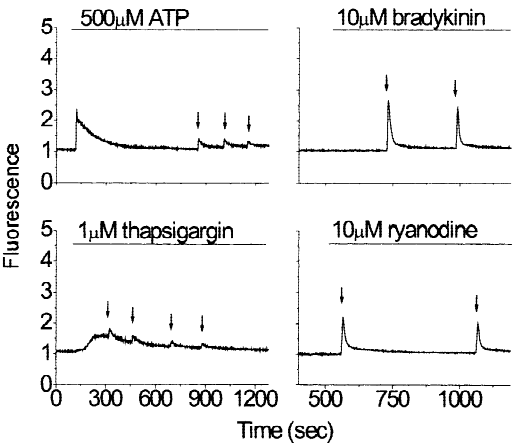


Fig. 4. IP_3 stores appear to be a source for the magnetically induced Ca^{2+} increase in C6 glioma cells. Ca^{2+} increases were suppressed in the presence of ATP ($n = 11$) and thapsigargin ($n = 5$), but not in the presence of bradykinin ($n = 5$) and ryanodine ($n = 5$). All traces shown were taken in buffer containing 1 mM Ca^{2+}. Arrows indicate Ca^{2+} changes due to magnetic stimulation.

IP_3 pathway at or beyond the point affected by the mechanical stimulus. BAECs and C6 glioma cells were mechanically stimulated following a 10 min incubation with 20 μM Xestospongin C (XC), a macrocyclic bis-1-oxaquinolizidine and blocker of the IP_3 receptor isolated from the Australian sponge, Xestospongia (Gafni et al., 1997; Narasimhan, Pessah & Linden, 1998). Following treatment with XC, BAECs, but not C6 glioma cells, showed a significant decrease in MACR (Fig. 5). As a further test we tried the phospholipase C inhibitor, U73122 (Matozaki et al., 1990; Yule & Williams, 1992;

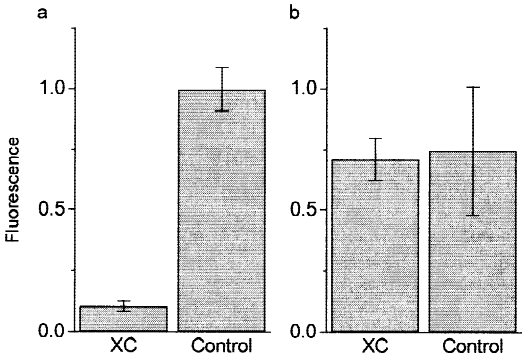


Fig. 5. 20 μM Xestospongin C (XC) blocks MACR in BAECs (*a*) (n = 5), but not in C6 glioma cells (*b*) (n = 3). Both cell types were incubated with XC for 10 min. prior to magnetic stimulation. The peak value of the Ca^{2+} changes was obtained from the ratio emission line and plotted as mean ± sem.

Yule, Essington, Williams, 1993). At a concentration of 20 μM no reduction in MACR was seen in either BAECs or C6 glioma cells (*data not shown*). Since the trimeric GTP-binding protein, G_o, is known to activate phospholipase C-β, we also mechanically stimulated the cells in the presence of 100 ng/mL pertussis toxin, a known blocker of G_i and G_o proteins. In agreement with the U73122 results, we saw no inhibition of MACR (*data not shown*). Without a specific inhibitor of the trimeric G-protein, G_q, we were unable to determine its involvement in MACR. However, given the fact that phospholipase C-β follows G_q in the IP_3-mediated Ca^{2+} release cascade, its involvement in MACR seems highly unlikely since we saw no reduction in MACR in the presence of U73122. The above results suggest that (i) our magnetic stimulus is either generating IP_3 which in turn releases intracellular Ca^{2+} from IP_3-sensitive Ca^{2+} stores without involving the initial IP_3 pathway components, G_q and phospholipase C-β, or (ii) IP_3 channels may themselves be mechanically sensitive and activated by stresses within the cell.

The bead assay is sensitive to the bead coating. We usually coated the beads with collagen following literature references (Glogauer et al., 1995), and this produces a relatively low density of particles on the cells. If we use uncoated magnetite beads, the particles stick in such density as to make the cells virtually black. This observation was true of all three cell types. Stimulating the cells under these conditions produces an increase in intracellular Ca^{2+} that is essentially identical to that seen when using collagen-coated beads (Fig. 6*a*). Statistically there was no difference between the Ca increases seen with plain beads and those seen with collagen-coated beads. Cells coated with plain beads appear less sensitive per bead than cells coated with collagen beads. Still, the response might have saturated if a lower density of beads was used and additional experiments need to be done to definitively answer this question.

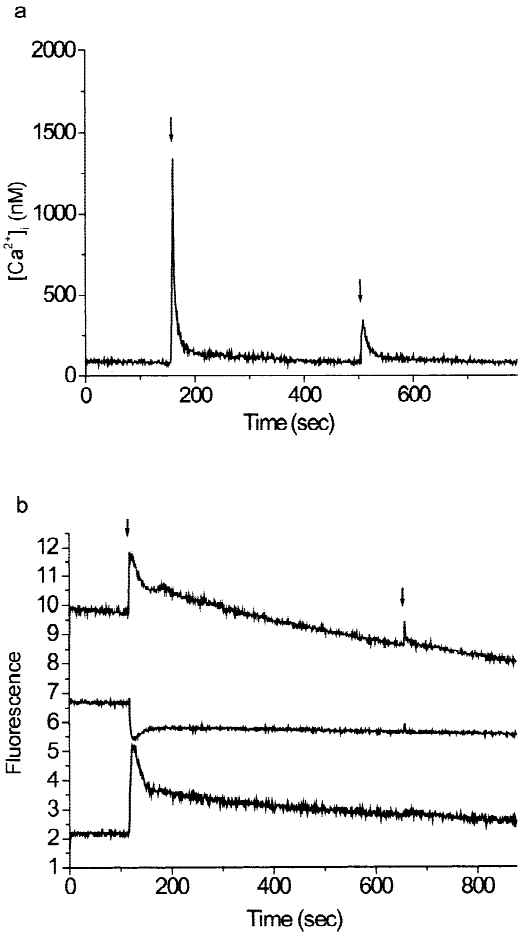


Fig. 6. (*a*) Representative trace of intracellular calcium increases following magnetic stimulation (arrows) in C6 glioma cells covered with uncoated magnetite beads (n = 13). Extracellular saline contained 1 mM calcium. (*b*) Intracellular Ca^{2+} recovery in BAECs is slower with laminin-coated beads than with collagen-coated beads (compare to Fig. 1*b*, collagen beads) (n = 3). The upper and middle emission lines have been offset 4 units for clarity.

Collagen has been proposed to stick to integrins via the RGD sequences (Glogauer et al., 1995, 1997). This, however, is a low affinity site that might not support pulling by the beads, but there are other ligands for collagen (Schlessinger, 1997; Symersky et al., 1997). Interestingly, we found that the collagen-coated beads will not stick to acutely isolated rat heart cells. This is presumably because the enzymes used in isolation have removed the collagen binding sites. The collagen-coated beads do, however, stick to cultured chick heart cells so this lack of adhesion is not a property of heart cells in general. In addition to collagen, we tested the response of BAECs to laminin-coated beads as shown in Fig. 6*b*. Although similar in amplitude, there was a distinct difference in time course between the responses to collagen- and laminin-coated beads. It clearly makes a difference what you pull on.

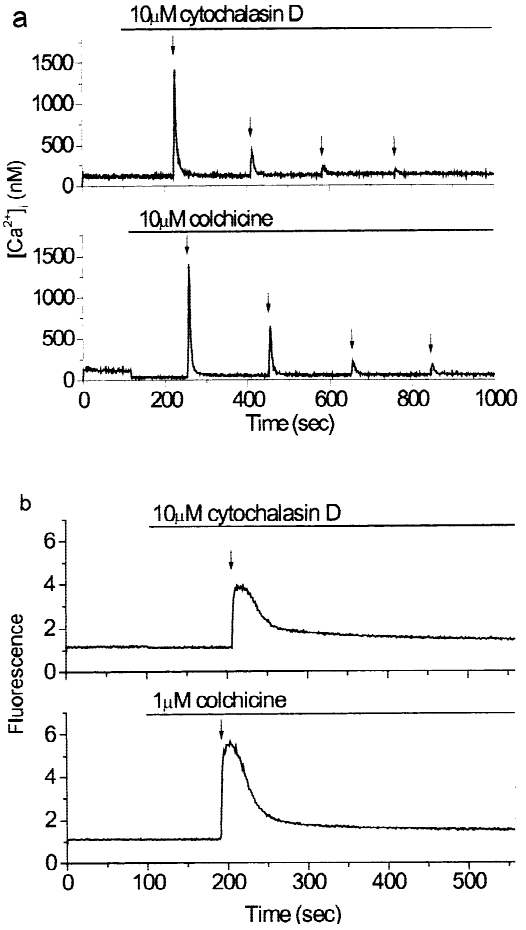


Fig. 7. (*a*) 10 μM Cytochalasin D ($n = 3$) (upper trace) and 10 μM colchicine ($n = 4$) (lower trace) do not affect the magnetically induced calcium increases seen in C6 glioma cells. Both traces were done in 1 mM calcium saline. The experiment in each trace was paused for 20 min. (@ 105 sec in upper and @ 116 sec in lower) following the addition of the drug. (*b*) Pretreatment of BAECs with 10 μM cytochalasin D ($n = 5$) (upper trace) or 1 μM colchicine ($n = 3$) (lower trace) did not block the magnetically induced Ca^{2+} increases in 1 mM Ca^{2+} saline. The experiment in each trace was paused for 15 min (at 100 sec) following the addition of the drug. Each trace is of the ratio (340 nm/380 nm) and not of calibrated Ca^{2+} concentrations as in Fig. 7*a*, thus care should be taken in interpreting an effect of the drug when comparing the traces in *a* and *b*. Arrows indicate Ca^{2+} changes due to magnetic stimulation.

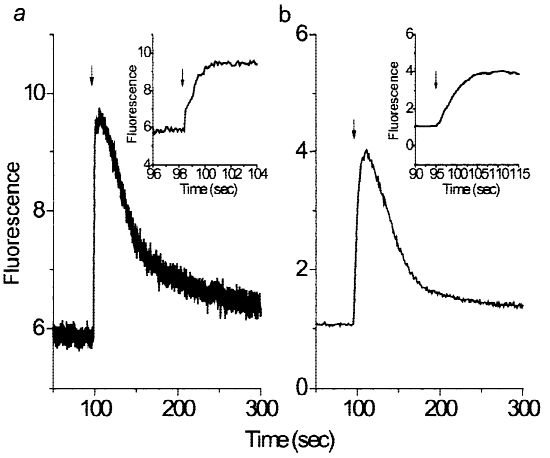


Fig. 8. Rapid elevation of Ca^{2+} in BAECs. (excitation at 340 nm only to speed up the recording rate to 100 msec/pt). Inset: Magnification of the *x*-axis between 96 and 104 sec. Time from baseline to peak Ca^{2+} levels is within 2 sec. *b.* Cooling BAECs to ~6°C does not slow down the magnetically induced Ca^{2+} response ($n = 3$). The rise time from baseline to peak Ca^{2+} levels is <10 sec. Controls at room temperature show similar rise times. Unlike figure *a*, these data were taken at a slower rate of 1 sec/pt. Ratio only shown (340 nm/380 nm). Arrows indicate Ca^{2+} changes due to magnetic stimulation.

Given the mechanical nature of this response, do cytoskeletal components play a role in the activation mechanism? Neither actin nor tubulin reagents block the response in C6 glioma (Fig. 7*a*) or BAECs (Fig. 7*b*) or significantly affect the response in any way. Thus, actin filaments and microtubules do not appear to participate in the activation mechanism responsible for the release of intracellular Ca^{2+}. These reagents have not been tested on astrocytes.

The magnetic response to stimulation is fairly rapid. To speed up the fluorimeter output, we monitored a single wavelength so that data were obtained at 100 msec/pt. The fastest population response we observed peaked within 2 sec (Fig. 8*a*). Other experiments show response times to peak Ca^{2+} levels as slow as approximately 10 sec. Although more precise experiments are necessary to accurately define the limits, a time scale of 2–10 seconds leaves plenty of room for biochemistry.

If the magnetic response invokes elaborate chemistry, then we would expect the response to be slower in a cooled environment. BAECs cooled to ~6°C still show a robust Ca^{2+} increase following magnetic stimulation, peaking in approximately 10 sec, similar to the 2–10 sec range for control Ca^{2+} increases (Fig. 8*b*).

Does membrane potential play a role in the Ca^{2+} response? Since MACR is essentially the same in high K^+ saline as it is in normal saline, membrane potential isn't important (Fig. 9). In fact, if all Na^+ is replaced with K^+, baseline Ca^{2+} levels rapidly drop as previously reported (Manor, Moran & Segal, 1994) and the mechanical response is maintained (*data not shown*). In most cases replacing Na^+ with K^+ will suppress or even reverse the Na^+/Ca^{2+} exchanger resulting in an increase of intracellular Ca^{2+}. If anything, depolarization would have also activated voltage-dependent Ca channels tending to increase Ca^{2+} levels. The drop in Ca^{2+} we see when replacing Na^+ with K^+ in C6 glioma cells is likely due to a decrease in the Ca^{2+} electrochemical gradient for passive Ca^{2+} entry-coupled with extremely efficient clearing of intracellular Ca^{2+}, swamping any effect the reversal of the Na^+/Ca^{2+} exchanger might have.

While there is adaptation to the magnetic stimula-

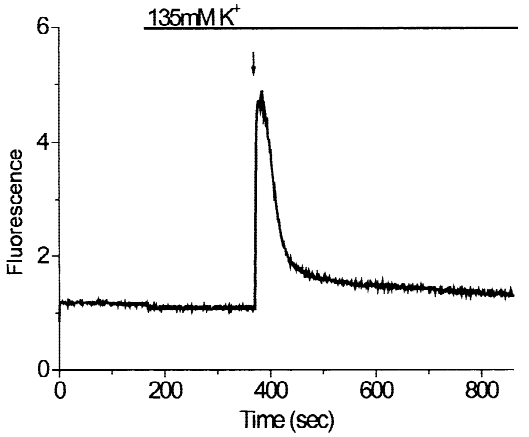


Fig. 9. Intracellular Ca^{2+} increase in BAECs following magnetic stimulation (arrow) in the presence of 135 mM KCl ($n = 5$). Data shown is the ratio of the 340 nm emission line to the 380 nm emission line in arbitrary fluorescence units.

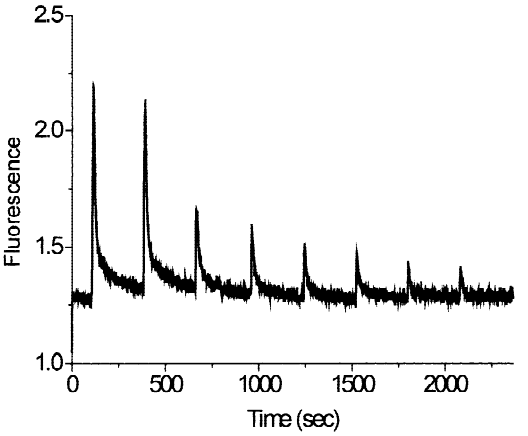


Fig. 10. Repeatable MACR in C6 glioma cells using collagen beads in 1 mM calcium saline ($n = 12$). Data shown are the ratio of the 340 nm emission line to the 380 nm emission line in arbitrary fluorescence units.

tion, it is possible to obtain repeated responses (Fig. 10). However, even after 10 min we have not observed complete recovery. Is this true adaptation? Although some sort of intrinsic adaptation is expected, we suspect that complete recovery won't be seen due to a repositioning of the beads following the initial stimulus as seen in the microscope. In other words, the beads do not return to their "starting" positions and thus the cells do not experience the same force with subsequent stimuli. As can be seen in Fig. 10 there is a significant difference between the first two responses and those following. The first two responses were elicited with separate neodymium boron permanent magnets (*see* Materials and Methods) situated 180 degrees apart. A second response with the same magnet is always much smaller than the first response. For example, the first and third responses were produced with one magnet while the second and fourth responses were produced with the other magnet. Subsequent stimulation with both magnets showed a more gradual adaptation.

In addition to MACR, we observed a plasmalemmal, Mn^{2+} permeable pathway activated with magnetic stimulation in all three cell types. Figure 11 shows this mechanically activated Mn^{2+} influx (MAMI) in Ca^{2+}-free saline. There is a resting permeability to Mn^{+2} as shown by the slight negative slope following the addition of Mn^{2+}. When the magnetic stimulus is applied, there is a short spike of increased intensity due to movement of the beads causing changes in light scattering, followed by a higher rate of quenching.

MAMI is inhibited by high concentrations of La^{3+} (Fig. 12) and Gd^{+3} (Fig. 13*a*). At higher concentrations of Gd^{+3} there is further suppression (Fig. 13*b*). Gd^{3+} causes an elevation in the ratio but in solution experiments we have found that adding Gd^{3+} to Fura in the presence of Ca^{2+} causes an increase in the ratio (*data not shown*). Thus, this slow rise in the ratio is probably due to Gd^{3+} entering the cell and interacting with Fura. Note in Fig. 12*a* that the Mn^{2+} influx pathway remains open for a relatively long period of time following magnetic stimulation. An increased quenching rate is seen approximately 8 min after stimulation when Mn^{2+} is added.

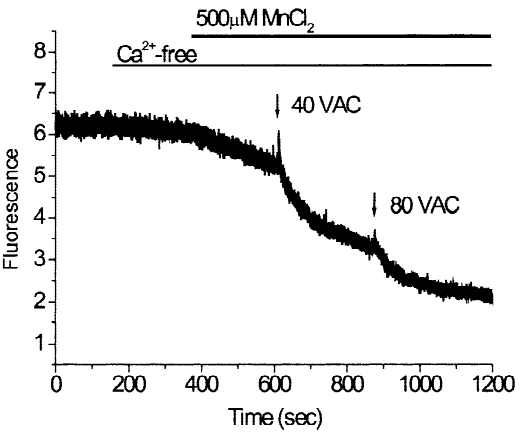


Fig. 11. Fura-2 quench by Mn^{2+} in C6 glioma cells following magnetic stimulation ($n = 45$) (collagen beads in Ca^{2+}-free saline containing 10 mM $MnCl_2$). Data were collected at a 100 msec/pt resolution at 356 nm only (isosbestic point of Fura-2). AC magnetic fields were applied at the indicated voltages (arrows).

In addition to the MACR and MAMI effects of stimulation we observed a unique decrease in Ca^{2+} following magnetic stimulation when intracellular Ca^{2+} levels were elevated (Fig. 14). The decrease in Ca^{2+} following the first stimulation amounts to ~180 nM. Intracellular Ca^{2+} was elevated by treating C6 glioma cells with a cocktail containing 1mM ATP, 10 μM CPA and 1 μM thapsigargin in 1mM Ca^{2+} normal saline. Upon ex-

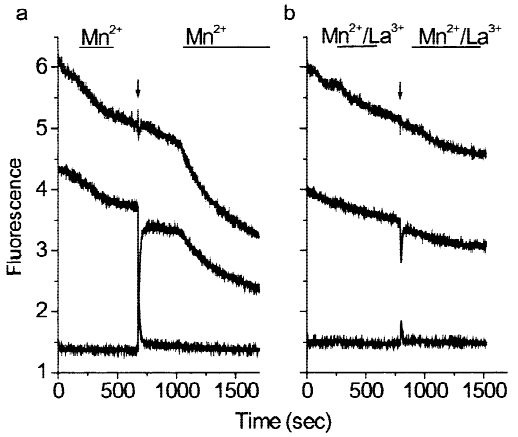
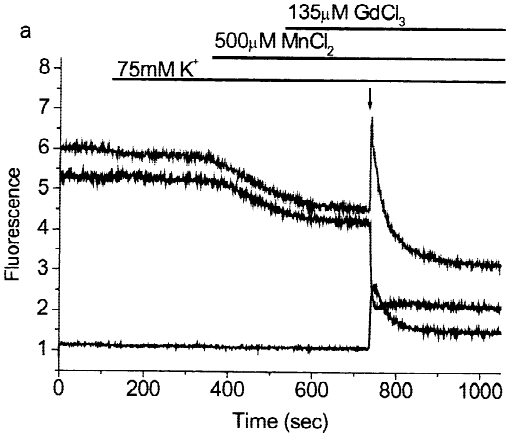


Fig. 12. (*a*) Representative trace of the Mn^{2+} influx pathway before and after magnetic stimulation in C6 glioma cells in normal saline containing 1 mM Ca^{2+} and 50 μM Mn^{2+} ($n = 6$). Note that although Ca^{2+} levels have returned to near normal following magnetic stimulation, the MAMI pathway remains activated as seen by the change in slope upon adding Mn^{2+} to the bath. (*b*) Addition of 1 mM La^{3+} under the same conditions as in Panel a blocks the Mn^{2+} influx following magnetic stimulation ($n = 3$). In both traces the upper data line shows 510 emission due to excitation at the isosbestic point of Fura-2, the middle data line 510 emission due to excitation at 380 nm and the lower data line the ratio of the upper to the middle.



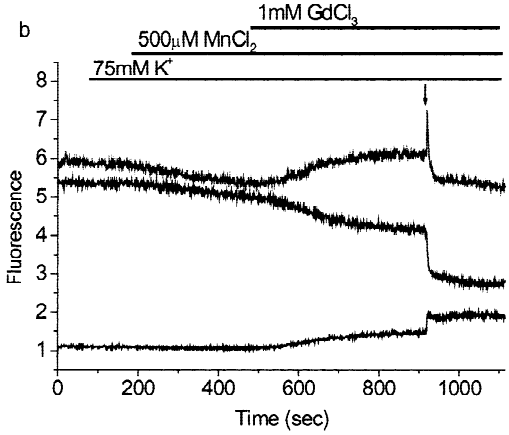


Fig. 13. (*a*) 135 μM Gd^{+3} only slightly blocks the magnetically induced Fura-2 quenching by Mn^{2+} (BAECs in high K^+ saline, 500 μM $MnCl_2$, 1 mM Ca^{2+}) ($n = 3$). The magnetic field was applied manually for approximately 2 sec with an electromagnet. There is a resting permeability to Mn^{+2} that is increased by magnetic stimulation. Stimulation also produces a transient spike in intracellular Ca^{2+}. (*b*) At 1 mM, Gd^{+3} strongly inhibits MAMI in BAECs; same conditions as in panel (*a*) ($n = 3$). There may be some permeation by Gd^{+3} that is a low affinity, low yield mimic of Ca^{2+} with Fura.

posure to the cocktail, the cells showed a transient spike in Ca^{2+} (probably release from intracellular IP_3-sensitive stores) followed immediately by a slower rise to approximately the same amplitude as the initial transient (presumably this represents capacitative calcium entry and prevention of uptake by CPA and thapsigargin). This second elevation then exhibited a long, slow decrease back to baseline (clearance of Ca^{2+} to the extracellular environment and into intracellular stores). If the cells were stimulated during this return to baseline, then Ca^{2+} transiently decreased, returning to values expected from the normal recovery time course in approximately 80 sec. This effect has not been tested in the other two cell types.

Because of the unexpected nature of the response, we tried a variety of controls searching for artifacts. We applied magnetic stimulation to cells without beads or dye, without beads and with dye, and with beads and without dye. We saw no change in the ratio (*data not shown*).

Discussion

In astrocytes, BAECs and C6 glioma cells the mechanical stimulus has two effects: increases in cell Ca^{2+} and increases in Mn^{2+} influx when cytoplasmic Ca^{2+} is low. Additionally, C6 cells show a decrease in cell Ca^{2+} when cytoplasmic Ca^{2+} is high. Currently, it is unknown whether astrocytes and BAECs exhibit the latter behavior.

Considering the results of both the sugar water experiment (Fig. 3) and the intracellular Ca^{2+} store reagent experiments (Fig. 4), it is clear that mechanical stimulation is releasing stored Ca^{2+}. Furthermore, IP_3-sensitive Ca^{2+} stores seem to be a major source of this Ca^{2+}. Are known second messenger pathways responsible for the release of this intracellular Ca^{2+}? The XC (Fig. 5), U73122, and pertussis toxin results suggest that in BAECs mechanical stimulation generates IP_3 without involving the initial components of the IP_3 pathway, namely G_q and phospholipase C-β. Since XC had no

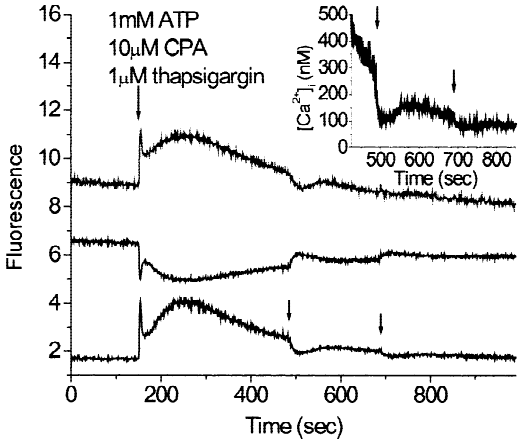


Fig. 14. Magnetic stimulation (arrow) in C6 glioma cells produces a decrease in calcium when intracellular calcium is already elevated ($n = 20$). The initial drop in Ca^{2+} is ~180 nM. Intracellular calcium was elevated by treating the cells with 1 mM ATP, 10 μM CPA and 1 μM thapsigargin in 1 mM Ca^{2+} saline at 150 sec. Inset: Intracellular Ca^{2+} concentration values for the raw data from 425 to 850 sec.

effect on C6 glioma cells, MACR in this case must be due to other mechanosensitive enzymes or direct activation of a mechanically sensitive channel located in the stores, possibly the IP_3 channel itself. All we can say for sure at this point is that in C6 glioma cells IP_3-mediated Ca^{2+} release is not involved. IP_3-sensitive Ca^{2+} stores are being emptied, but by a pathway or mechanism other than IP_3.

In exploring possible artifacts we looked for a magnetically activated autofluorescent response that might mimic Fura, but there was none (*data not shown*). In those cells not requiring extracellular Ca^{2+}, the increase in Ca^{2+} may arise from direct mechanical activation of a stretch-activated ion channel (SAC) in a Ca^{2+} storing organelle or perhaps from other second messenger pathways activated by mechanosensitive enzymes. The low temperature experiments didn't show a slowing of the Ca^{2+} response, but that is unfortunately at best a result suggestive that complicated second messenger pathways aren't involved. In the cells tested that required extracellular Ca^{2+}, the increase in Ca^{2+} may arise from direct mechanical activation of SACs in the plasma membrane.

One possible explanation for the Ca^{2+} increase is that we are ripping holes in the membrane allowing an influx of extracellular Ca^{2+}. The fact that stimulating the cells in Ca^{2+}-free saline still generates an increase in intracellular Ca^{2+} strongly opposes this explanation. Secondly, in some experiments the response to a second stimulation was larger than the first. And finally, *decreases* in intracellular Ca^{2+} following magnetic stimulation (Fig. 14) in 1 mM Ca^{2+} saline clearly demonstrate that we are not making holes.

Although much work still needs to be done in characterizing MACR, we believe it to be a legitimate, physiological response of cells to physical changes within their immediate environment. Increases in intracellular Ca^{2+} due to mechanical deformation initiate responses to these physical changes. Due to their location, vascular endothelial cells are continuously subjected to normal and shear stresses imposed by blood flow. Increases in shear stress have been shown to cause significant alterations in endothelial cell structure and function. These changes include elongation and orientation of the cells major axis and actin fibers with flow (Shirinsky et al., 1989; Nerem & Girard, 1990), protein synthesis, cell proliferation (Dartsch & Betz, 1989), activation of extracellular-regulated kinases and tyrosine phosphorylation of focal adhesion kinase (Takahashi et al., 1997), increases in IP_3, Ca^{2+} (Nerem & Girard, 1990), prostacyclin (Frangos et al., 1985; Grabowski, Jaffe & Weksler, 1985), nitric oxide (Taylor et al., 1991; Takahashi et al., 1997), and tissue-plasminogen activator secretion (Diamond, Eskin & McIntire, 1989; Diamond et al., 1990), as well as decreases in preproendothelin (endothelin-1) mRNA expression and endothelin-1 peptide release (Yanagisawa et al., 1988; Sharefkin et al., 1991).

Injury or pathology in glial cells has been shown to cause astrocytes to transform into "active astrocytes" and microglia into phagocytic macrophages (Landis, 1994; Gehrmann, Matsumoto & Kreutzberg, 1995). Some evidence suggests that Ca^{2+} is the trigger for this change in function (Haun et al., 1992; Wood et al., 1993; Bader et al., 1994; Codazzi et al., 1995; Codazzi et al., 1996; Duffy & MacVicar, 1996; Nolte et al., 1996; Moller et al., 1997). Increases in Ca^{2+} in glia have also been shown to affect numerous other responses such as modulation of differentiation, proliferation, DNA synthesis (Meador-Woodruff, Lewis & Devries, 1984; Saunders & Devries, 1988; Hart et al., 1989; Supattapone, Simpson & Ashley, 1989; Puro & Mano, 1991), mRNA transcription and stabilization (Vogt & Bos, 1989; Benveniste et al., 1990; Gabellini et al., 1991; Trejo & Brown, 1991; Kerr, Inoue & Verma, 1992), protein synthesis (Brostrom et al., 1990; Brostrom et al., 1991), metabolic functions like histamine-induced glycogenolysis (Arbones, Picatoste & Garcia, 1990), glutamine synthesis (Benjamin, 1987), and phagocytosis (Mano & Puro, 1990), reorganization of cytoskeleton (Schlaepfer & Zimmerman, 1981; Blaurock, Yale & Roots, 1986; Yang, Kong & Babitch, 1988; Noetzel, 1990; Ropte, Scheidt & Friede, 1990; Harrison & Mobley, 1992), and the regulation of the uptake and release of ions and water (Latzkovits et al., 1982; Olson et al., 1990).

Is the increase in Mn^{2+} permeability caused by direct activation of MSCs or activation of other Mn^{2+} permeant channels by second messengers generated by intracellular Ca^{2+} release or other mechanosensitive enzymes? Blockage of the Mn^{2+} influx pathway with La^{3+} and Gd^{3+} supports a role for MSCs. However, failure to see

blockage in more discriminating experiments using *Grammostola spatulata* spider venom, a blocker of cationic MSCs in astrocytes (T.M. Suchyna et al., *unpublished results*), suggests MSCs are not involved.

As with MACR, membrane potential doesn't seem to play a role in MAMI since the response is similar in normal saline and high K^+ saline. Also, clamping cell Ca^{2+} with BAPTA AM uncouples MACR from MAMI, yet the Mn^{2+} influx is still activated (*data not shown*).

Several pieces of evidence lead us to believe that the best explanation for MAMI is that it is a Ca^{2+} store depletion current. First, our data suggests that IP_3 stores are involved in the MACR response. Second, depletion of IP_3 stores in many cell types has been shown to activate a plasmalemmal, Ca^{2+} permeable pathway (Parekh & Penner, 1995; Krause et al., 1996; Centinaio, Bossi & Peres, 1997; Parekh, Fleig & Penner, 1997; Somasundaram, Mason & Mahaut-Smith, 1997; Wu et al., 1997; Fasolato & Nilius, 1998; Hofer, Fasolato & Pozzan, 1998). Third, the rate of Mn^{2+} influx following exposure to Ca^{2+}-free saline or ATP mimics the influx rate following magnetic stimulation (*data not shown*). Incubation in Ca^{2+}-free saline and ATP depletes IP_3-sensitive Ca^{2+} stores. Fourth, treatment with thapsigargin alone produces Mn^{2+} influx at rates comparable to those following magnetic stimulation (*data not shown*). One can conclude from this that depletion of intracellular Ca^{2+} is all that is necessary to elicit Mn^{2+} influx. In future experiments we plan to test the effectiveness of XC in blocking MAMI.

The picture that emerges is that stretching the cells with magnetite beads causes a release of intracellular Ca^{2+} from IP_3-sensitive stores through multiple mechanisms depending on which cell one looks at. Elevation of Ca^{2+} in C6 glioma cells appears to occur through direct mechanical activation of SACs in the IP_3 store or from a second messenger pathway activated by mechanosensitive enzymes. Generation of Ca^{2+} in BAECs appears to occur by the generation of IP_3 through an unknown mechanism, possibly SACs, which in turn causes the release of intracellular Ca^{2+}. Depletion of IP_3-sensitive Ca^{2+} stores results in the opening of a capacitative Ca^{2+} influx pathway in the plasma membrane in both cell types. It has been shown in C6 glioma cells that the amplitude of this store depletion current is highly dependent on the $[Ca^{2+}]$ within IP_3-sensitive stores (Wu et al., 1997).

The transient Ca^{2+} *decrease* observed when $[Ca^{2+}]_i$ is elevated may be a result of deformation exposing Ca^{2+}-binding sites within the cytoplasm. To account for the 180 nM drop in Ca^{2+} following stimulation, we need to expose an equivalent concentration of binding sites. For a 10 μm diameter cell where the cytoskeleton comprises 50% of the cell volume, we need approximately 30,000 binding sites/cell. The affinity of these putative sites must be <100 nM since they are not visible at normal cytoplasmic levels.

The previous explanation for the Ca^{2+} decrease assumes negligible sequestration of the loaded dye. However, if we assume the opposite, that a significant concentration of the dye has been sequestered, mechanical stimulation may result in the movement of Ca^{2+} between organelles. Considering our experimental conditions, we would also have to assume that C6 glioma cells contain Ca^{2+} stores not depleted by mechanical stimulation, ATP, thapsigargin, or CPA. C6 glioma cells do possess a lysophosphatidic acid sensitive Ca^{2+} store that is independent of their IP_3 store (Hildebrandt & Hildebrandt, 1997). In addition, gastric epithelia have also demonstrated a pool of intracellular Ca^{2+} resistant to IP_3 and thapsigargin (Hofer & Machen, 1994). Testing such an explanation will require careful imaging experiments with dextran labeled Fura-2 in place of the acetoxymethyl ester form to keep the dye contained within the cytoplasm. Imaging of cells, which have been permeabilized following dye loading, could also provide information on the extent of dye sequestration.

Regardless of the mechanisms involved, deformation of cells is a potent stimulus for Ca^{2+} movements that can drive a host of secondary responses. This appears to be a universal means by which cells sense and react to physical changes within their surroundings.

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