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Cadmium Increases Tritium Inositol Phosphate Production And Releases Stored Calcium(Ii): Evidence For A Novel Calcium (Ii)-Mobilizing Receptor Antagonized By Zinc.

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CADMIUM INCREASES [^{3}H]INOSITOL PHOSPHATE PRODUCTION AND RELEASES STORED Ca^{2+}: EVIDENCE FOR A NOVEL Ca^{2+}-MOBILIZING RECEPTOR ANTAGONIZED BY ZINC

by

SCOTT DOUGLAS DWYER

A DISSERTATION

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Title Cadmium Increases [^{3}H]Inositol Phosphate Production and Releases Stored Ca^{2+}: Evidence for a Novel Ca^{2+} Mobilizing Receptor Antagonized by Zinc.

Cadmium (0.05 - 10 μM) caused a spike in cytoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) similarly to bradykinin in cultured human skin fibroblasts. Cadmium increased $[Ca^{2+}]_i$ in the presence or absence of external Ca^{2+}. After bradykinin produced a spike in $[Ca^{2+}]_i$, the addition of cadmium had no effect on $[Ca^{2+}]_i$ indicating that cadmium and bradykinin release Ca^{2+} from the same intracellular pool. Cadmium triggered a concentration-dependent increase in $^{45}Ca^{2+}$ efflux. Half-maximal stimulation of efflux occurred at 0.1 μM cadmium. Certain other metals also stimulated $^{45}Ca^{2+}$ efflux. The potency order of the metals was $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$. Zn^{2+} competitively inhibited Cd-evoked $^{45}Ca^{2+}$ efflux but had no effect on bradykinin-evoked efflux. Cadmium also decreased total cell Ca^{2+} by 50-60% within 2 min similarly to bradykinin. Decreasing external Na^+ from 120 mM to 3 mM caused a spike in $[Ca^{2+}]_i$, an 8-fold increase in $^{45}Ca^{2+}$ efflux, and rapidly decreased total cell Ca^{2+}

by 40% similarly to cadmium and bradykinin. Decreasing external pH also increased $[Ca^{2+}]_i$, stimulated $^{45}Ca^{2+}$ efflux, and decreased total cell Ca^{2+}. The three unusual stimuli (cadmium, decreasing external Na^+, and decreasing external pH) trigger rapid increases in [^{3}H]inositol 1,4,5-trisphosphate (IP_3), which indicates that IP_3 is the intracellular messenger for release of stored Ca^{2+} by these three unusual stimuli. A single receptor appears to mediate the Ca^{2+} mobilizing response to all three stimuli. First, the response to the three stimuli is cell-type specific; the cell types we have tested respond to all three stimuli or to none of them. Responsive cell types include human skin fibroblasts, coronary endothelial cells, neuroblastoma cells, umbilical artery smooth muscle cells, and WI-38 lung fibroblasts. Second, a 2 hr incubation in 10 μM $ZnSO_4$ desensitizes the cells to the three stimuli but has no effect on Ca^{2+} mobilization evoked by bradykinin or ATP in the fibroblasts and endothelial cells, respectively.

Abstract Approved by: Committee Chairman _____

Program Director _____

Date _____ Dean of Graduate School _____

DEDICATION

To Mary, Steven, and Carrie

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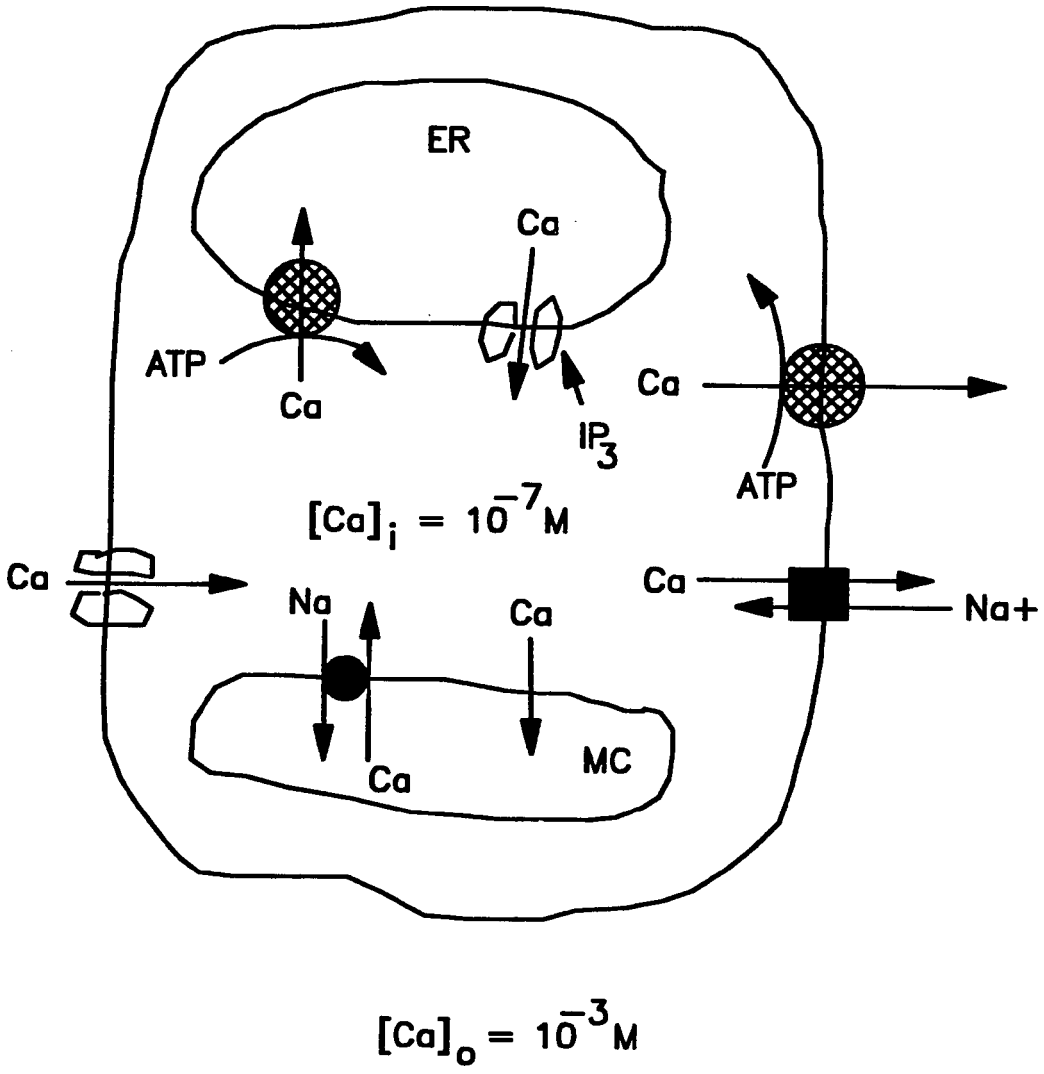
INTRODUCTION

Calcium ions (Ca^{2+}) are a fundamental signal in the regulation of cellular metabolism. Rapid adjustments in the concentration of free Ca^{2+} ions within the cytoplasm ($[Ca^{2+}]_i$) allow a cell to modulate metabolic events quickly and efficiently. In vivo, the extracellular Ca^{2+} concentration is maintained in the millimolar range. Similarly, total cell Ca^{2+} divided by the total cell water space is in the millimolar range, yet, in the unstimulated state, $[Ca^{2+}]_i$ is maintained in the sub-micromolar range (Carafoli, 1987). This 10,000-fold transmembrane gradient of ionized Ca^{2+} suggests a basis for using Ca^{2+} as an intracellular signal; such a large gradient can be utilized to rapidly increase $[Ca^{2+}]_i$ and thus activate many Ca^{2+}-dependent processes. At the same time, a mechanism for rapidly decreasing free Ca^{2+} in the cytoplasm is required. Therefore, cells are endowed with Ca^{2+} transport and storage systems which tightly regulate $[Ca^{2+}]_i$.

The illustration on page 3 summarizes the Ca^{2+} transport and storage systems involved in Ca^{2+} regulation in a prototypical cell. These systems are located at three major sites: the plasma membrane, the endo/sarcoplasmic reticulum, and the mitochondria. In the plasma membrane, a ubiquitous ATP-dependent Ca^{2+} pump actively extrudes Ca^{2+} from the

cytoplasm. A Na^+/Ca^{2+} antiport system is present in the plasma membrane of certain cell types - primarily muscle, neural, and some secretory cells - which couples the energy in the Na^+ electrochemical gradient to the extrusion of Ca^{2+}. Some cell types also express voltage-sensitive Ca^{2+} channels in the plasma membrane which, upon depolarization, will open and allow Ca^{2+} to enter the cell down its large electrochemical gradient. An ATP-dependent Ca^{2+} pump is located on the endo/sarcoplasmic reticulum (ER) which is structurally distinct but functionally analogous to the Ca^{2+} pump on the plasma membrane. This pump serves to actively transport Ca^{2+} from the cytoplasm into the ER, a major intracellular Ca^{2+} storage site. The ER contains a high capacity Ca^{2+} binding protein called "calsequestrin" which binds more than 40 moles of Ca^{2+} per mole calsequestrin (MacLennan et al., 1983). Also located on the ER membrane is a Ca^{2+} channel that is opened upon binding of the second messenger inositol 1,4,5-trisphosphate (IP_3) and allows Ca^{2+} to enter the cytoplasm. Recently, a structure distinct from the ER which might contain the IP_3 releaseable Ca^{2+} storage pool was identified (Volpe et al., 1988). This structure, called the "calciosome", was found to co-purify with markers of the IP_3-sensitive pool but not with markers of the ER or other organelles (Volpe, 1988). The mitochondria are of secondary importance in the immediate regulation of cellular Ca^{2+}. The mitochondria store 1-2 nmol Ca^{2+}/mg cell protein depending on the cell type (Carafoli, 1987). A Na^+/Ca^{2+} antiport system appears to mediate Ca^{2+} efflux from the mitochondria while a Ca^{2+} uptake system driven



Major Ca^{2+} Transport and Storage Systems in a Prototypical Cell. ER = endoplasmic reticulum (calciosome); MC = mitochondria.

by the negative mitochondrial membrane potential transports Ca^{2+} into the mitochondria. In concert, these Ca^{2+} transport and storage mechanisms maintain the sub-micromolar $[Ca^{2+}]_i$.

Initially, the goal of the research described here was to characterize the Na^+/Ca^{2+} antiport system in cultured mammalian cells. In vascular smooth muscle cells that have been loaded with Na^+, decreasing the extracellular Na^+ concentration ($[Na^+]_o$) triggers a rapid and transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Smith et al., 1987, 1989c). Decreasing $[Na^+]_o$ inverts the usual Na^+ gradient and drives Ca^{2+} influx via the Na^+/Ca^{2+} antiporter (Smith et al., 1989c). However, decreasing $[Na^+]_o$ has no effect on $[Ca^{2+}]_i$ in smooth muscle cells that have not been loaded with Na^+. This observation can be explained kinetically if the K_m of the antiporter for Na^+ is high, such that a large increase in intracellular Na^+ and a concomitant decrease in $[Na^+]_o$ are required for activation (Smith et al., 1989c). In contrast to the smooth muscle cells, attempts to assess Na^+/Ca^{2+} antiport in fibroblasts cultured from human forearm skin revealed that decreasing $[Na^+]_o$ triggered a rapid increase in $[Ca^{2+}]_i$ whether or not the cells had been loaded with Na^+ (Smith et al., 1989). At least two explanations could account for the increase in $[Ca^{2+}]_i$ caused by decreasing $[Na^+]_o$. First, a kinetically distinct form of the Na^+/Ca^{2+} antiporter mediates Ca^{2+} influx in the fibroblasts; second, release of Ca^{2+} from an intracellular storage pool accounts for the increase in $[Ca^{2+}]_i$. To distinguish between these two possibilities, a comprehensive study of the changes in cell Ca^{2+} regulation

evoked by decreasing $[Na^+]_o$ was undertaken. Thus, changes in $[Ca^{2+}]_i$, Ca^{2+} efflux, Ca^{2+} influx, and total cell Ca^{2+} evoked by decreasing $[Na^+]_o$ were measured. If decreasing $[Na^+]_o$ triggers the release of stored Ca^{2+} in the human skin fibroblasts, then it may do so by a unique mechanism or by a mechanism similar to that of Ca^{2+}-mobilizing hormones.

Ca^{2+}-mobilizing hormones elicit a characteristic sequence of cellular events which trigger the release of stored Ca^{2+} from the ER as illustrated on page 6 (for review see Berridge, 1987). Binding of a hormone or neurotransmitter to a specific cell surface receptor activates a phosphoinositidase, phospholipase C, which in turn catalyzes the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2). A guanine nucleotide binding-protein (G-protein) homologous to the G-protein that links certain receptors to adenylate cyclase appears to couple Ca^{2+}-mobilizing receptors to phospholipase C (Litosch et al., 1985; Baukal et al., 1988). Hydrolysis of PIP_2 concomitantly produces the intracellular messengers IP_3 and diacylglycerol (DAG). Release of IP_3 is unique to the hydrolysis of PIP_2, while hydrolysis of any membrane phospholipid can produce DAG. The immediate effect of IP_3 is the release of stored Ca^{2+} from the ER (Streb et al., 1983). IP_3 appears to bind a specific receptor (Spat et al., 1986) which opens a channel by a ligand-binding mechanism (Smith et al., 1985) to allow the release of Ca^{2+} down its concentration gradient and into the cytoplasm. Hormone-evoked release of stored Ca^{2+} triggers a rapid and transient increase in $[Ca^{2+}]_i$, stimulates Ca^{2+} efflux, decreases total cell Ca^{2+}, and

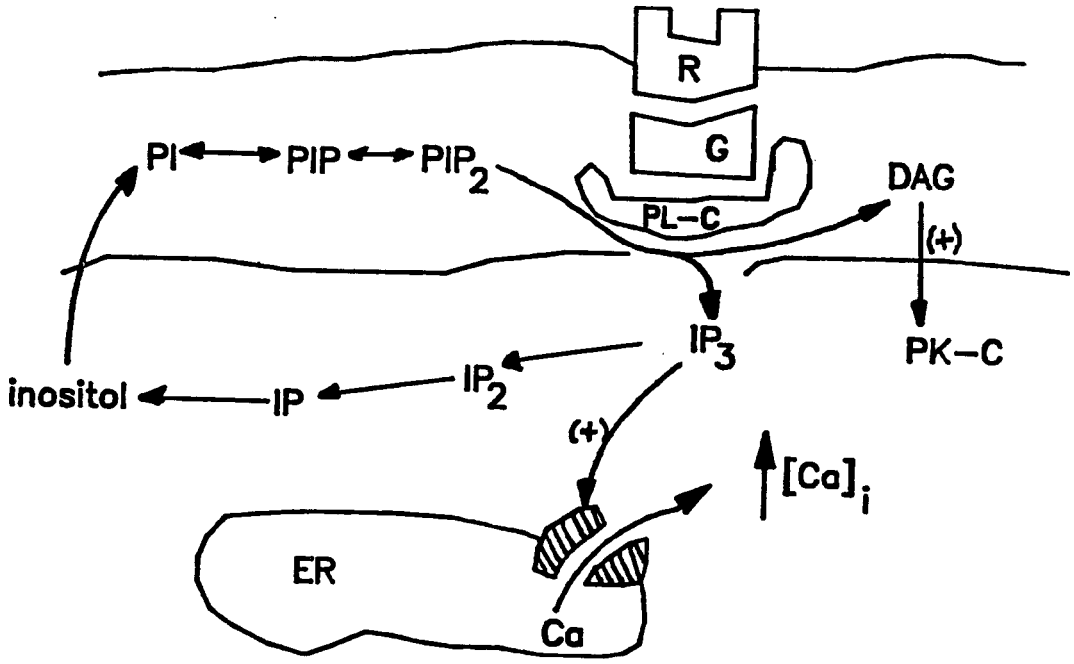


Diagram of the Phosphatidyl Inositol Second Messenger System that is Activated by Ca^{2+}-mobilizing Hormones and Neurotransmitters. R = receptor; G = G-protein; PL-C = phospholipase C; DAG = diacylglycerol; IP_3 = inositol 1,4,5-trisphosphate; IP_2 = inositol 4,5-bisphosphate; IP = inositol monophosphate; PI = phosphatidyl inositol; PIP = phosphatidyl inositol 4-phosphate; PIP_2 = phosphatidyl inositol 4,5-bisphosphate; PK-C = protein kinase C; ER = endoplasmic reticulum.

subsequently increases Ca^{2+} influx (Gill, 1985; Kojima et al., 1987; Smith, 1986; Smith et al., 1987).

The present findings indicate that decreasing $[Na^+]_o$ stimulates [^{3}H]inositol phosphate production and evokes the release of stored Ca^{2+} similarly to Ca^{2+}-mobilizing hormones (Smith et al., 1989). In the course of this study, an attempt was made to investigate the Ca^{2+} efflux pathway activated by decreasing $[Na^+]_o$ using divalent heavy metals known to inhibit Ca^{2+} transport systems and to investigate the pH dependence of the effect of decreasing $[Na^+]_o$. Quite unexpectedly, Cd^{2+} and certain other divalent metals as well as decreasing pH_o were found to release stored Ca^{2+} similarly to decreasing $[Na^+]_o$ and Ca^{2+}-mobilizing hormones (Smith et al., 1989a, 1989b). Furthermore, these data suggest that: (1) all three stimuli activate the phosphatidylinositol (PI) second messenger system and that IP_3 causes the release of stored Ca^{2+}; (2) a single receptor apparently mediates the effect of all three stimuli; (3) the receptor, provisionally called the "Cd^{2+} receptor", is present in certain cell types but not in others; and (4) the receptor satisfies several criteria that define a pharmacological receptor (Smith et al., 1989, 1989a, 1989b).

METHODS AND MATERIALS

Cell Culture. Primary cultures of human skin fibroblasts were started from punch biopsies (2 mm dia.) of human forearm skin by L. Smith and E. R. Brown. The tissue was cut into 6 pieces which were placed in a 25 cm^2 flask whose culture surface had been wetted with Dulbecco's Modified Eagle's medium (DME) (GIBCO, Grand Island, NY). DME (1.5 ml) containing 20% (v/v) characterized fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan UT) 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY) was added to the flask. The flask was then gassed with 5% CO_2-95% air, tightly capped, and incubated for 24 hr. Then 2 ml of the same medium was added to the flask which was gassed again and resealed. When outgrowth was observed, the culture medium was exchanged for fresh medium. The flasks usually became confluent in 2 weeks. After passaging with trypsin, the cultures exhibited the usual bipolar spindle shape. Low passage cultures were stored in liquid nitrogen.

Stock cultures (5 to 16 passages) were grown in 80 cm^2 tissue culture flasks (Nunclon-Delta, Kamstrup, Denmark) in DME containing 10% FBS without antibiotics. Stock cultures were passaged 1:2 or 1:3 when they became confluent (2 or 3 times/week). The cultures were passaged by rinsing the

monolayer twice with phosphate buffered saline minus Ca^{2+} and Mg^{2+} and adding 1 ml 0.05% trypsin. The cells rounded up within 1 min and were detached by slapping the flask against the palm of the hand. Nine ml of growth medium was added, and the cells were counted and diluted to the desired plating density. One flask yielded about 2 x 10^6 cells.

Tissue culture dishes (Falcon, 35-mm diameter, Becton Dickinson and Company, Oxnard, CA) were seeded with 2 to 3 x 10^5 cells in 2 ml DME containing 10% FBS. The dishes were usually used 5 to 7 days later when they contained a confluent monolayer with no visible mitotic cells. Each experiment was done with dishes plated at the same time only. Total cell protein was measured by the method of Lowry et al. (1951) on duplicate cultures from each plating on the day they were used.

Smooth muscle cells were cultured from explants of umbilical arteries as described for the skin biopsies, and from the medial portion of human and rat aortae after enzymic dispersion (Smith and Brock, 1983; Smith, 1984). The identity of the cells as smooth muscle was confirmed by positive staining with a monoclonal antibody (CGA7) to the alpha isoform of actin (Gown et al., 1985). Endothelial cells were grown from scrapings of dog coronary arteries. The identity of the cells as endothelial cells was confirmed by greater than 98% positive staining with 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Biomedical Technologies Inc., Cambridge, MA) which is accumulated only by endothelial cells and macrophages (Voyta, et al., 1984).

The smooth muscle and endothelial cells were grown in Medium 199 containing 10% FBS. Rat embryo fibroblasts were grown in DME containing 10% FBS. Human neuroblastoma cells (SK-N-SH) and epidermoid carcinoma (A431) cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DME containing 10% FBS. Both the A431 and neuroblastoma cells were subcultured (1:2) 2-3 times per week as described for the fibroblasts (Smith et al., 1989). A confluent 100 mm (dia) culture dish was used to make 16 dishes (35 mm dia) (Falcon, Becton Dickinson and Company, Oxnard, CA).

Cytosolic Free Ca^{2+}. The cells were grown on cover glasses (no. 2 thickness, 18 x 18 mm, Fisher Scientific, Pittsburgh, PA) that were cut to 12 x 18 mm with a diamond pointed pencil. The coverslips were soaked in dichromate sulfuric acid cleaning solution (Chromerge, Manostat, New York, NY), rinsed with water, and dried. They were put in 95% ethanol, flamed, and transferred to 12-well tissue culture plates which were seeded with 2 ml DME containing 10% FBS and 1 x 10^5 cells. The cover glasses were used 2 to 4 days later after the cells formed a confluent monolayer. The cells were loaded with fura-2 by placing the cover glass in a 35 mm diameter petri dish and adding 1 ml DME containing 2 μl 5 mM fura-2 acetoxymethyl (AM) ester (Molecular Probes, Inc., Eugene, OR) which was dissolved in dimethyl sulfoxide. After a 30 min incubation in a humidified atmosphere of 5% CO_2-95% air, the cover glass was rinsed with a physiological salts solution (PSS) which contained (in mM): 120 NaCl, 20 HEPES-Tris (HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic

acid; Tris, Tris[hydroxymethyl]aminomethane) adjusted to pH 7.4, 5 KCl, 1 $MgCl_2$, and 1 $CaCl_2$. After 30 min in PSS containing 10 mM glucose, the cover glass was rinsed again with PSS and placed on the diagonal face of a triangular cuvette containing 1 ml PSS.

Fluorescence was recorded at two excitation wavelengths with a Deltascan 1 fluorometer (Photon Technology International Inc., Princeton, NJ) equipped with a temperature jacketed, magnetically stirred cuvette compartment. The excitation and emission specifications were the same as previously described (Smith and Smith, 1987). The fluorescence of a cover glass with attached cells was subtracted in real time while recording the 345/380 fluorescence ratio. Autofluorescence was about 10% of the fluorescence of dye-loaded cells at both excitation wavelengths. The treatments that affected dye fluorescence as reported here had no effect on autofluorescence.

$^{45}Ca^{2+}$ Uptake. Cultures were incubated overnight in DME containing 2% FBS and then for 1 hr in PSS containing 10 mM glucose. Uptake was started by removing the PSS and adding PSS or choline-PSS containing 4 μCi/ml $^{45}Ca^{2+}$ (20 to 30 Ci/g in water, Dupont-NEN, Boston, MA). Uptake was stopped at the indicated times by removing the uptake solution and rinsing the monolayer 7 times with ice cold MLB. $^{45}Ca^{2+}$ taken up by the cells was extracted and analyzed as previously described (Smith and Smith, 1987).

$^{45}Ca^{2+}$ Efflux. Cultures were incubated overnight with $^{45}Ca^{2+}$ (10 μCi/ml) in DME containing 2% FBS. This medium was

removed and replaced with 1 ml of PSS containing $^{45}Ca^{2+}$ at the same specific radioactivity as the overnight labeling medium. One hr later the cultures were rinsed 8 times (<20 sec total) with the efflux buffer. For efflux assayed in Ca^{2+}/Mg^{2+}-free PSS, cultures were rinsed 5 times with PSS and 8 times with Ca^{2+}/Mg^{2+}-free PSS. One ml of efflux solution was added immediately after aspirating the last rinse. The efflux solution was removed for counting and replaced with fresh solution at 10-sec intervals. At the end of the efflux assay, the cultures were extracted twice with 1 ml of 0.1 N HNO_3 for 20 minutes at room temperature to measure the amount of $^{45}Ca^{2+}$ remaining. Efflux data are presented as semi-log plots of the percentage $^{45}Ca^{2+}$ that remained intracellular at the end of each 10-sec interval (Smith et al., 1985).

Total Cell Ca^{2+}, Na^+, and Water Space. The culture medium was aspirated and replaced with fresh DME containing 2% FBS and 10 uCi/ml $^{45}Ca^{2+}$. The cultures were incubated overnight (15 to 24 h) to equilibrate intra- and extracellular Ca^{2+}. One hr before starting the assay, the medium was replaced with PSS containing $^{45}Ca^{2+}$ at the same specific radioactivity as the overnight labeling medium. Then the medium was replaced again with PSS containing $^{45}Ca^{2+}$ or with Na^+-free PSS containing $^{45}Ca^{2+}$ and 120 mM of either choline chloride, LiCl (lithium chloride), KCl (potassium chloride), NMG (N-methyl-D-glucamine chloride), or 240 mM sucrose. After incubating the cultures for the indicated times, the assay was stopped by rinsing the cultures 4 times with ice-cold magnesium-lanthanum buffer (MLB). MLB contained (in mM): 100 $MgCl_2$, 10 $LaCl_3$, and 10

HEPES adjusted to pH 7.4 with Tris. The cultures were incubated on ice in MLB for 10 min to displace slowly exchangeable extracellular $^{45}Ca^{2+}$ before rinsing 4 more times with MLB. MLB prevents $^{45}Ca^{2+}$ efflux (Smith and Smith, 1987). The cultures were extracted with 1 ml of 0.1 N HNO_3 for 20 min at room temperature. Radioactivity in a 0.9 ml sample was measured by liquid scintillation spectrometry.

The following data indicate that the 20 hr incubation with $^{45}Ca^{2+}$ was sufficient to equilibrate intracellular and extracellular $^{45}Ca^{2+}$. First, cultures that were incubated for 20, 48, or 72 hr had the same $^{45}Ca^{2+}$ content: 47,890 ± 838, 46410 ± 387, and 48,247 ± 1462 cpm/mg, respectively. The data (mean ± S.E.) are from two experiments (n = 8). The cultures were incubated with 1 ml DME containing 2% (v/v) FBS and 5 μCi $^{45}Ca^{2+}$ for the indicated time, rinsed with a solution containing 10 mM $LaCl_3$ (lanthanum chloride), and extracted as described (Smith et al., 1989). Second, the specific radioactivity of intracellular $^{45}Ca^{2+}$ was not significantly different from that of the labeling medium. Ten cultures (100 mm dia) were incubated for 20 hr in the medium described above with 1 μCi/ml $^{45}Ca^{2+}$. The cultures were rinsed as described (Smith et al., 1989) with an ice-cold solution containing 120 mM NaCl, 5 KCl, 1 mM $LaCl_3$, and 20 mM HEPES-Tris, pH 7.4. Before $LaCl_3$ was added to the solution, it was passed through a Chelex 100 column and the absence of Ca^{2+} verified using fura-2. Rinsing cultures with the solution containing 1 mM $LaCl_3$ gave the same intracellular $^{45}Ca^{2+}$ content/mg protein as rinsing with solution containing 10 mM $LaCl_3$ (Smith et al.,

1989). The cultures were extracted with 10 mM HNO_3 for 1 hr. The extract was neutralized with Tris and applied to a 0.3 ml Chelex 100 column. Ca^{2+} was eluted with 1 ml 2 N HCl. The yield of $^{45}Ca^{2+}$ was about 70%. Ca^{2+} was measured by atomic absorption spectroscopy. The specific radioactivity of intracellular $^{45}Ca^{2+}$ was 94 ± 5% that of the labeling medium (mean ± SE, 4 experiments).

Cell Na^+ and water space were measured as previously described (Smith, 1984; Smith et al., 1989c). Cell water space was 6.6 ± 0.7 μl/mg protein (n = 3 experiments in duplicate). Intracellular Na^+ concentration was calculated by dividing total Na^+ by the water space.

[^{3}H]Inositol Phosphates. The cells (1 x 10^7) were seeded in 100-mm diameter culture dishes (Falcon, Becton Dickinson Company, Oxnard, CA) in 10 ml DME containing 10% FBS. Two days later they were rinsed twice with Medium 199 and refed with 10 ml Medium 199 containing 2% dialyzed FBS and 10 uCi [^{3}H]inositol ([2-^{3}H(N)]myo-inositol, 15 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO). The serum was dialyzed against three changes of saline containing 1 mM HEPES-Tris, pH 7.5 at 4°C to remove inositol. The [^{3}H]inositol was treated with anion exchange resin (AG 1-X8, 100-200 mesh, Bio-Rad Laboratories, Richmond, CA) to remove impurities. [^{3}H]inositol (3.9 ml 100 uCi/ml water) was incubated with 0.1 ml resin for 30 min and sterilized by filtration before adding it to the cultures. The cultures were incubated in a humidified atmosphere containing 5% CO_2-95% air for 48 hr. Then they were rinsed 5 times with PSS and incubated for 30

min with 10 ml PSS containing 10 mM glucose. To start the reaction, the medium was replaced with fresh PSS (control cultures) or choline-PSS. After the indicated time interval (Figs. 41, 42, and 43), the medium was removed and 3 ml ice-cold 10% trichloroacetic acid was added to stop the reaction and extract inositol phosphates. After 20 min at 4°C, the extract was removed and the culture was rinsed 3 times with 2 ml 5 mM myo-inositol. The inositol rinses and acid extract were combined and washed 4 times with 3 ml of ethyl ether to remove the acid. The pH of the extract was adjusted to 7.0 to 7.5 by adding 40 μl of 1 M Tris. The extracts were stored at -20°C for 1 to 3 days.

[^{3}H]Inositol phosphates, contained in the extracts, were separated on 0.8 ml AG 1-X8 columns as described (Smith et al., 1989). The sample (9 ml) was applied, and the column was rinsed with 20 ml water to elute [^{3}H]inositol. [^{3}H]Inositol phosphates were eluted sequentially with 20 ml each of the following: 5 mM sodium borate/60 mM sodium formate; 0.2 M ammonium formate/0.1 M formic acid; 0.4 M ammonium formate/0.1 M formic acid; and 0.9 M ammonium formate/0.1 M formic acid. Four ml fractions were collected and analyzed by liquid scintillation counting with 10 ml aqueous counting fluid (Budget-Solve, Research Products International Corporation, Mount Prospect, IL). The count yield of [^{3}H]inositol was the same in all eluent solutions. [^{3}H]inositol phosphate standards (Dupont-NEN, Boston, MA) were used to verify the separation of glycerophosphoinositol (GPI),

inositol monophosphate (IP), inositol-1,4,-bisphosphate (IP_2), IP_3, inositol-1,3,4,5-tetrakisphosphate (IP_4).

Cell pH. Fibroblasts were grown on cover glasses (12 x 18 mm) as described (Smith et al., 1989) and incubated with 3 μM BCECF acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) in 1 ml DME for 30 min at 37°C. Then the cover glass was rinsed and incubated with 2 ml PSS with 10 mM glucose for 30 min before placing the cover glass on the diagonal face of a triangular cuvette. Fluorescence emission at 527 nm minus the autofluorescence of a cover glass that was not loaded with BCECF was recorded at two excitation wavelengths, 500 and 440 nm, with a Deltascan dual wavelength fluorometer (Photon Technology International Inc., Princeton, NJ). Autofluorescence was not greater than 5% dye fluorescence. Calibration of cytoplasmic dye fluorescence was done with a high K^+ PSS (KCl substituted for NaCl) pH 8.0 to 6.0 containing 10 μM nigericin as described (Moolenaar et al., 1984). PIPES-PSS contained 20 mM PIPES (Piperazine-N,N'-bis[2-ethanesulfonic acid]) instead of HEPES. The pH of PIPES-PSS was adjusted with Tris or HCl to the desired value. For the neuroblastoma cells, PSS was buffered with Tris/maleic acid instead of HEPES: for pH values 5.7-6.7, 20 mM maleic acid was used and adjusted to the indicated pH with Tris; for pH values greater than 6.7, 20 mM Tris was used and adjusted to the indicated pH with maleic acid. All incubations were at 37°C unless otherwise indicated.

Ca^{2+}/Mg^{2+} Free Solutions. Ca^{2+}/Mg^{2+}-free PSS containing 120 or 3 mM Na^+ (choline replacement) were prepared by passing

the solution without added Ca^{2+} or Mg^{2+} through a 3 x 9 cm column containing Chelex 100 (BioRad, Richmond, CA) in the Na^{+} or Tris form, respectively. $[Ca^{2+}]$ in the chelex solutions was <10 nM as measured by passing a 1 μM $CaCl_2$ solution containing tracer $^{45}Ca^{2+}$ (specific radioactivity, 17286 cpm/nmol) through the chelex column. To produce the Tris form of Chelex 100, the resin was rinsed sequentially with 2 bed volumes of 1 N HCl, 5 volumes of Milli Q water, 2 volumes of 1 M Tris-free base, 5 volumes of Milli Q water, 2 volumes of 0.5 M HEPES, and, finally, several volumes of Milli Q water.

RESULTS

HYPOTHESIS I

Decreasing $[Na^+]_o$, adding Cd^{2+} or certain other divalent metals, or decreasing pH_o causes the release of stored Ca^{2+} in human skin fibroblasts, canine endothelial cells, and neuroblastoma cells, similarly to Ca^{2+}-mobilizing hormones and neurotransmitters.

Effect of Decreasing $[Na^+]_o$ or Adding Bradykinin on $[Ca^{2+}]_i$ in Human Skin Fibroblasts. Decreasing $[Na^+]_o$ from 120 mM to 0 mM triggered a rapid and transient increase in $[Ca^{2+}]_i$ from the basal level, 154 ± 4 nM (n = 158), to 1133 ± 48 nM (n = 58) (Fig. 1). The basal to peak rise time was 15 ± 1 sec (n = 24). For these experiments, equimolar choline chloride was substituted for NaCl in the physiological salt solution (PSS). Substituting K^+ or NMG for extracellular Na^+ induced $[Ca^{2+}]_i$ increases which were similar to those evoked with choline chloride. Replacing Na^+_o with Li^+ produced a much smaller increase in $[Ca^{2+}]_i$ compared to the other Na^+ replacements. $[Ca^{2+}]_i$ increased to 336 ± 30 nM (n = 18) when Na^+ in PSS was replaced with Li^+. Decreasing $[Na^+]_o$ from 120 to 14 mM evoked the half-maximal increase in $[Ca^{2+}]_i$ (Fig. 2). Bradykinin increased $[Ca^{2+}]_i$ similarly to decreasing $[Na^+]_o$ (Fig. 3). Bradykinin (100 nM) rapidly increased $[Ca^{2+}]_i$ to

1295 ± 177 nM (n = 11). The basal to peak rise time was 12 ± 1 sec (n = 18). Bradykinin and decreasing $[Na^+]_o$ may release Ca^{2+} from the same intracellular pool because prior stimulation with bradykinin strongly inhibited the response to a subsequent decrease in $[Na^+]_o$ and vice versa (Fig. 3). The calcium ionophore, A23187, releases stored Ca^{2+} from mammalian cells (Chen, 1979; Artalejo and Garcia-Sancho, 1988). A23187 produced a rapid and transient increase in $[Ca^{2+}]_i$ similarly to bradykinin and decreasing $[Na^+]_o$ (Fig. 3); subsequent addition of bradykinin or decreasing $[Na^+]_o$ did not produce an increase in $[Ca^{2+}]_i$. This result suggests that the release of stored Ca^{2+} produces the increase in $[Ca^{2+}]_i$ by bradykinin or decreasing $[Na^+]_o$.

Effect of Decreasing $[Na^+]_o$ or Addition of ATP on $[Ca^{2+}]_i$ in Coronary Artery Endothelial Cells. Decreasing $[Na^+]_o$ from 120 to 4 mM transiently increased $[Ca^{2+}]_i$ about 5-fold, from 273 ± 9 nM (n = 105) to 1436 ± 96 nM (n = 30) (Fig. 4). Lowering $[Na^+]_o$ to 27 mM half-maximally increased $[Ca^{2+}]_i$ (Fig. 5). For these experiments, equimolar choline chloride was substituted for NaCl in PSS. Substitution of LiCl for NaCl produced a very small increase in $[Ca^{2+}]_i$ from 194 ± 12 to 309 ± 38 nM (n = 9). Li^+ may partially mimic the effect of Na^+ because the ions have similar physicochemical properties.

ATP (100 μM) increased $[Ca^{2+}]_i$ from 256 ± 23 nM (n = 13) to 1203 ± 189 nM (n = 13) (Fig. 4). ATP increases inositol polyphosphates and releases stored Ca^{2+} in endothelial cells (Pirotton et al., 1987). Prior stimulation with ATP

completely prevented the effect of decreasing $[Na^+]_o$ on $[Ca^{2+}]_i$ (Fig. 4).

Effect of Cd^{2+} and Certain Other Divalent Metals on $[Ca^{2+}]_i$ in Human Skin Fibroblasts. Cd^{2+} (0.05-10 μM) and Fe^{2+} (1-100 μM) evoked large spikes in $[Ca^{2+}]_i$ in the fibroblasts (Fig. 6A, B). Fura-2 binds Fe^{2+} about 3-10 times more tightly than Ca^{2+} and quenches its fluorescence (Grynkiewicz et al., 1985), whereas Cd^{2+} shifts the excitation spectrum of fura-2 similarly to Ca^{2+} (Fig. 6C). The fluorescence yield of Cd^{2+}-fura-2 is somewhat greater than that of Ca^{2+}-fura-2 (Fig. 6C). Because Fe^{2+} and Cd^{2+} evoked similar $[Ca^{2+}]_i$ spikes, it is likely that the low permeability of the cells to the metals prevented them from binding fura-2. After evoking a $[Ca^{2+}]_i$ spike with 1 μM Cd^{2+}, increasing the Cd^{2+} concentration to 1 mM caused a slow increase in the fluorescence ratio (Fig. 6A). Cd^{2+}, not Ca^{2+}, influx probably causes the slow increase in the fluorescence ratio at 1 mM Cd^{2+} because it was unaffected by removing external Ca^{2+}.

Cd^{2+} evoked similar increases in $[Ca^{2+}]_i$ in the presence or absence of external Ca^{2+}. For this experiment, PSS with no added Ca^{2+} was passed through a Chelex-100 column to remove contaminating Ca^{2+}. Ca^{2+} was not detectable (<10 nM) by $^{45}Ca^{2+}$ tracer after one passage through the Chelex-100 column. Replacing PSS with Ca^{2+}-free PSS decreased basal $[Ca^{2+}]_i$ from 206 ± 9 nM (n = 12) to 163 ± 11 nM (n = 6, p = 0.015). The Ca^{2+}-free PSS was removed after about 15 sec and replaced with Ca^{2+}-free PSS containing 5 μM Cd^{2+}. Cd^{2+} increased $[Ca^{2+}]_i$ to

756 ± 46 nM (n = 6) in the absence of Ca^{2+}, compared to 847 ± 85 nM (n = 6) in the presence of external Ca^{2+}. The difference in peak $[Ca^{2+}]_i$ was not statistically significant by Student's t test for unpaired samples (p = 0.372). The addition of bradykinin produced a similar increase in $[Ca^{2+}]_i$. After removal of bradykinin, a subsequent addition of Cd^{2+} did not increase $[Ca^{2+}]_i$, indicating that bradykinin and Cd^{2+} release stored Ca^{2+} from the same intracellular pool.

Effect of Cd^{2+} on $[Ca^{2+}]_i$ in Endothelial Cells. One μM Cd^{2+} produced a rapid increase in $[Ca^{2+}]_i$ from 197 ± 29 nM to 562 ± 120 nM (n = 5). $[Ca^{2+}]_i$ decreased from the peak and returned almost to the basal level within 2 min. Cd^{2+} at 1 mM had a much different effect on $[Ca^{2+}]_i$; after the rapid rise in $[Ca^{2+}]_i$, the 340/380 ratio of fura-2 fluorescence increased slowly for several minutes. Cd^{2+} influx may cause the slow increase in the fluorescence ratio because Cd^{2+} shifts the excitation spectrum of fura-2 similarly to Ca^{2+} (see above, Fig. 9C).

Effect of Decreasing pH_o on $[Ca^{2+}]_i$ in Human Skin Fibroblasts. Decreasing pH_o from 7.4 to 6.0 evoked a large spike in $[Ca^{2+}]_i$ similarly to bradykinin in the fibroblasts (Fig. 7A, B). $[Ca^{2+}]_i$ increased rapidly in response to both stimuli to a peak of about 1 μM, and then fell to nearly the basal level. Smaller decreases in pH_o evoked smaller increases in $[Ca^{2+}]_i$. Changing pH_o to 6.9 increased $[Ca^{2+}]_i$ to about twice the basal level (Fig. 7A). Decreasing pH_o increased $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. Changing pH_o from 7.4 to 6.0 increased $[Ca^{2+}]_i$ from 134 ± 9 nM (n = 9)

to 978 ± 52 nM (n = 5) in the presence of Ca^{2+} or to 952 ± 70 nM (n = 4) in PIPES-PSS pH 6.0 containing 0.1 mM EGTA and no added Ca^{2+}. The free [Ca^{2+}] present in the cuvette at this pH and [EGTA] was calculated to be 17 μM. These data indicate that the release of stored Ca^{2+} is largely responsible for the increase in $[Ca^{2+}]_i$ produced by lowering pH_o. Prior stimulation of the cells with bradykinin prevented the increase in $[Ca^{2+}]_i$ of a subsequent decrease in pH_o. This result indicates that the release of stored Ca^{2+} is largely responsible for the increase in $[Ca^{2+}]_i$ produced by lowering external pH.

Changing pH_o from 7.4 to 6.0 transiently increased $[Ca^{2+}]_i$ in endothelial cells (Fig. 8). $[Ca^{2+}]_i$ increased rapidly from 187 ± 11 nM (n = 12) to 527 ± 48 nM (n = 7) and returned to the basal level about 2 min after lowering pH_o. Removing external Ca^{2+} from the low pH buffer and adding 0.1 mM EGTA only slightly altered the $[Ca^{2+}]_i$ response. $[Ca^{2+}]_i$ increased to 489 ± 93 nM (n = 5) in response to the pH 6.0 buffer containing EGTA and no added Ca^{2+} (Fig. 8).

Effect of Decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ Efflux in Human Skin Fibroblasts. Removal of Na^+_o by replacement with choline increased $^{45}Ca^{2+}$ efflux about 8-fold (Table 1). The half-time of efflux was 349 ± 17 sec in PSS compared to 40 ± 2 sec in the absence of Na^+_o. Decreasing $[Na^+]_o$ from 120 to 12 mM caused the half-maximal stimulation of efflux (Fig. 9). This value agrees well with the concentration of extracellular Na^+ that produced the half-maximal peak increase in $[Ca^{2+}]_i$ (Fig. 2). For these experiments, choline chloride was substituted

Table 1. Effect of replacing external Na^+ on the rate of $^{45}Ca^{2+}$ efflux in human skin fibroblasts.

Extracellular cation	K (x 10^{-3} sec^{-1})	n
Na^+	2.0 ± 0.1	8
Choline	17.2 ± 0.9	8
K^+	14.5 ± 1.9	5
Sucrose	18.3 ± 1.9	4
Li^+	6.8 ± 2.1	5

Values are means ± SE for n independent experiments. Rate constants were obtained by exponential curve fitting to the initial portion of the efflux curve which was linear on a semi-log plot.

for NaCl in PSS. Replacing Na^+ with K^+ or sucrose stimulated efflux by almost the same degree as replacement with choline chloride, but replacement with LiCl caused a smaller increase (Table 1). Removing extracellular Ca^{2+} had no effect on the rate or extent of $^{45}Ca^{2+}$ efflux evoked by Na^+_o removal (Fig. 10). Efflux was started in PSS or PSS containing 1 mM EGTA and no added Ca^{2+} and switched at 60 sec to choline PSS or choline PSS containing 1 mM EGTA and no added Ca^{2+}. Because the stimulation of efflux was independent of extracellular Ca^{2+}, it is not a secondary effect of changing Ca^{2+} influx, nor is it due to Ca^{2+}/Ca^{2+} exchange.

Comparison of $^{45}Ca^{2+}$ Efflux Evoked by Bradykinin, Histamine, or Decreasing $[Na^+]_o$ in Human Skin Fibroblasts. The initial rate and extent of $^{45}Ca^{2+}$ efflux evoked by saturating concentrations of bradykinin or histamine were similar to those evoked by decreasing $[Na^+]_o$ (Figs. 11, 12). The onset of the increase in efflux evoked by decreasing $[Na^+]_o$ was 10 to 20 sec slower than that produced by bradykinin or histamine (Figs. 11, 12). Slower and smaller production of inositol phosphates or slower activation of the Ca^{2+} efflux pathway in response to decreasing $[Na^+]_o$ may account for the lag in the onset of efflux compared to bradykinin (Figs. 8, 40) or histamine (Fig. 9).

Effect of Decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ Efflux in Endothelial Cells. The rate of $^{45}Ca^{2+}$ efflux in PSS was very slow; the half-time was 481 ± 27 sec (n = 6). Replacing Na^+_o with choline decreased the half-time of efflux more than 4-fold to 111 ± 2 sec (n = 4) (Fig. 13A). Other Na^+ replacements,

Table 2. Effect of removing external Na^+ on $^{45}Ca^{2+}$ efflux from endothelial cells in the presence and absence of external Ca^{2+}.

	$^{45}Ca^{2+}$ efflux (x 10^{-3} sec^{-1})		
External Ca^{2+}	Plus Na^+_o	Minus Na^+_o	fold increase
No	1.1 ± 0.1 (4)	5.8 ± 0.1 (8)	5.3
Yes	1.5 ± 0.1 (4)	5.7 ± 0.1 (6)	3.8

First-order rate constants were estimated from the initial, linear portion of a semi-log plot of the data using an HP-11C calculator programmed for exponential curve fitting by linear regression. Values are mean ± SE (n). To start the assay, the cultures were rinsed 8 times with the solution in which efflux was started. In the absence of external Ca^{2+}, efflux was assayed in PSS containing 0.1 mM EGTA and no added Ca^{2+} and switched at 60 sec to choline PSS containing 0.1 mM EGTA and no added Ca^{2+}. In the presence of external Ca^{2+}, efflux was assayed in PSS from 0 to 60 sec and switched to choline PSS from 60 to 180 sec.

sucrose, or NMG increased $^{45}Ca^{2+}$ efflux similarly to choline (Fig. 13B).

Removing Na^{+}_{o} stimulated $^{45}Ca^{2+}$ efflux to the same extent in the presence or absence of extracellular Ca^{2+} (Table 2). For this experiment, efflux was started in PSS containing EGTA (1 mM) and no added Ca^{2+} and then switched to choline PSS containing EGTA (1 mM) and no added Ca^{2+} at 60 sec. These data indicate that the stimulation of efflux does not depend on Ca^{2+}_{o}.

Effect of Decreasing $[Na^{+}]_{o}$ on $^{45}Ca^{2+}$ Efflux in Neuroblastoma Cells. Decreasing $[Na^{+}]_{o}$ by equimolar replacement with choline evoked a 12-fold increase in the rate of $^{45}Ca^{2+}$ efflux; the first-order rate constant of efflux increased from $1.4 \pm 0.1 \times 10^{-3}$ sec^{-1} (n = 5) to $17.2 \pm 1.1 \times 10^{-3}$ sec^{-1} (n = 4) (Fig. 14). Acetylcholine (10 μM) similarly increased the rate of efflux; the first-order rate constant was $19.2 \pm 0.8 \times 10^{-3}$ sec^{-1} (n = 6). Atropine (20 μM) completely blocked acetylcholine-stimulated $^{45}Ca^{2+}$ efflux and inhibited efflux stimulated by choline replacement by about 50% (Fig. 14); the first-order rate constant in choline-PSS containing atropine was reduced to $8.1 \pm 1.4 \times 10^{-3}$ sec^{-1} (n = 2). Replacement of Na^{+}_{o} with NMG or sucrose also stimulated $^{45}Ca^{2+}$ efflux; the first-order rate constants were $9.0 \pm 0.5 \times 10^{-3}$ sec^{-1} (n = 8), and $7.5 \pm 0.9 \times 10^{-3}$ sec^{-1} (n = 2), respectively. Atropine had no effect on $^{45}Ca^{2+}$ efflux stimulated by replacement of Na^{+}_{o} with NMG or sucrose. Removal of Ca^{2+}_{o} had no effect on efflux stimulated by acetylcholine or decreasing $[Na^{+}]_{o}$. Loading the cells with Na^{+}, by incubation

with 0.1 mM ouabain for 30 min, did not inhibit $^{45}Ca^{2+}$ efflux stimulated by decreasing $[Na^+]_o$, suggesting that decreasing extracellular, not intracellular, Na^+ triggered the stimulation of $^{45}Ca^{2+}$ efflux.

Effect of Decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ Efflux in Other Cell Types. Decreasing $[Na^+]_o$ from 120 mM to 0 mM stimulated $^{45}Ca^{2+}$ in two other cell types: smooth muscle cells cultured from human umbilical artery (HUA) (Fig. 15) and WI-38 lung fibroblasts. Decreasing $[Na^+]_o$ had no effect on $^{45}Ca^{2+}$ efflux in rat embryo fibroblasts or rat aortic smooth muscle cells. In contrast, decreasing $[Na^+]_o$ markedly stimulated $^{45}Ca^{2+}$ efflux from A431 epidermoid carcinoma cells. The mechanism by which decreasing $[Na^+]_o$ stimulates $^{45}Ca^{2+}$ efflux in the A431 cells is clearly different from the other cell types tested because incubation with ouabain prevented the stimulation of efflux in A431 cells but had no effect on the stimulation of efflux in the other cell types.

Effect of Cd^{2+} and Certain Other Divalent Metals on $^{45}Ca^{2+}$ Efflux in Human Skin Fibroblasts. Cd^{2+} stimulated $^{45}Ca^{2+}$ efflux similarly to bradykinin (Figs. 16A, B). Half-maximal stimulation of efflux occurred at about 0.1 μM Cd^{2+}. Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} also stimulated $^{45}Ca^{2+}$ efflux (Figs. 16B, 17). The maximal stimulation of $^{45}Ca^{2+}$ efflux by the five Ca^{2+}-mobilizing metals was similar (Fig. 17 and data not shown). The potency order was $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$ (Fig. 16B). Other divalent and trivalent metals at concentrations up to 100 μM (Zn^{2+}, Cu^{2+}, Pb^{2+}, Pd^{2+}, Sr^{2+}, Ba^{2+}, Be^{2+}, La^{3+}, Gd^{3+}, Sm^{3+}, Tb^{3+}, Al^{3+}, Fe^{3+}) had little or no effect on basal $^{45}Ca^{2+}$ efflux

(Figs. 17-20). The lanthanides (La^{3+}, Sm^{3+}, Gd^{3+}, Tb^{3+}) were potent inhibitors of $^{45}Ca^{2+}$ efflux stimulated by decreasing $[Na^+]_o$ (Fig. 18). La^{3+} caused a concentration-dependent inhibition of $^{45}Ca^{2+}$ efflux stimulated by decreasing $[Na^+]_o$ (Fig. 18). La^{3+} does not readily enter cells (Wendt-Gallitelli and Isenberg, 1985; Gylfe et al., 1986; Negulescu and Machen, 1988), which implies that La^{3+} inhibits the effect of decreasing $[Na^+]_o$ by binding at an external receptor. The other lanthanides were more potent than La^{3+} as inhibitors of $^{45}Ca^{2+}$ efflux; at 1 μM, these metals completely blocked the stimulation of efflux by decreasing $[Na^+]_o$ (Fig. 18). Two mechanisms, acting separately or in concert, could account for lanthanide inhibition of $^{45}Ca^{2+}$ efflux. First, the lanthanides may bind at an external receptor to prevent the release of stored Ca^{2+} triggered by decreasing $[Na^+]_o$. Second, the lanthanides block transport of Ca^{2+} across the plasma membrane (Dos Remedios, 1981). The results described under Hypothesis VI, page 56, suggest that the lanthanides inhibit $^{45}Ca^{2+}$ efflux triggered by decreasing $[Na^+]_o$ by both mechanisms.

Zn^{2+} (100 μM) prevented 2 μM Cd^{2+} or 10 μM Fe^{2+} from stimulating $^{45}Ca^{2+}$ efflux (Figs. 17, 21). Zn^{2+} inhibition was rapidly reversible; removing Zn^{2+} after incubating the cultures with 100 μM Zn^{2+} during the last 5 min of the labeling with $^{45}Ca^{2+}$ had no effect on Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux. Cu^{2+} and Pb^{2+} also inhibited Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux (Fig. 20). Zn^{2+} had no effect on the stimulation of efflux by bradykinin (Fig. 21).

Effect of Cd^{2+} and Certain Other Divalent Metals on $^{45}Ca^{2+}$ in Endothelial Cells. Cd^{2+} caused a concentration-dependent

increase in $^{45}Ca^{2+}$ efflux (Fig. 22). About 0.1 μM Cd^{2+} half-maximally stimulated efflux. Cd^{2+} at 10 μM decreased the half-time of efflux about 5-fold, from 487 ± 27 sec (n = 6) to 105 ± 5 sec (n = 3). Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} also stimulated efflux in the endothelial cells (Fig. 23). The potency order for stimulation of $^{45}Ca^{2+}$ efflux was the same as that in the fibroblasts: $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$.

Zn^{2+} and Cu^{2+} markedly inhibited Cd^{2+}-stimulated efflux in the endothelial cells (Fig. 24). ATP-stimulated $^{45}Ca^{2+}$ efflux was not affected by Zn^{2+} or Cu^{2+} (Fig. 24). Therefore, Zn^{2+} and Cu^{2+} probably do not inhibit either the phosphoinositidase that produces IP_3 or the Ca^{2+} efflux pathway, which presumably involves an intracellular release channel and the plasma membrane Ca^{2+} ATPase (Smith, 1986; Smith et al., 1989). Zn^{2+} and Cu^{2+} apparently inhibit the binding of Cd^{2+} to a cell surface receptor, the Cd^{2+} receptor, because Zn^{2+} competitively antagonizes the effect of Cd^{2+} on Ca^{2+}-mobilization (Smith et al., 1989a).

Effect of a 2 Hr Incubation with $ZnSO_4$ on the Stimulation of $^{45}Ca^{2+}$ Efflux by Cd^{2+}, Decreasing $[Na^+]_o$, Decreasing pH_o, or Adding ATP. Because Zn^{2+} is a potent inhibitor of $^{45}Ca^{2+}$ efflux evoked by Cd^{2+}, we wanted to find out if prolonged incubation with Zn^{2+} would desensitize the endothelial cells to the Ca^{2+} mobilizing effects of Cd^{2+}, or decreasing $[Na^+]_o$ and decreasing pH_o. For these experiments, cultures were incubated with 10 μM $ZnSO_4$ during the 2 hr $^{45}Ca^{2+}$ labeling. Incubation with $ZnSO_4$ decreased $^{45}Ca^{2+}$ labeling about 10% (data not shown). The cultures were rinsed 8 times to remove $ZnSO_4$, and $^{45}Ca^{2+}$ efflux

Table 3. Effect of Cd^{2+}, decreasing $[Na^+]_o$, decreasing pH_o, or addition of ATP on $^{45}Ca^{2+}$ efflux in endothelial cells after a 2 hr incubation with $ZnSO_4$.

	Stimulated $^{45}Ca^{2+}$ efflux ($K \times 10^{-3}$ sec^{-1})		
Stimulus	Control	Zn^{2+} treated	%inhibition
Cd^{2+}	3.74 ± 0.42	0.69 ± 0.16	82
Low $[Na^+]_o$	3.33 ± 0.38	1.36 ± 0.31	59
pH 6.0	4.68 ± 0.42	1.05 ± 0.25	78
ATP	4.17 ± 0.25	4.43 ± 0.37	0

Zn^{2+} treated cultures received 10 μM $ZnSO_4$ during the 2 hr labeling with $^{45}Ca^{2+}$. Zn^{2+} was not present during the efflux assay. The basal rate of efflux (20-60 sec) of each culture was subtracted from the rate obtained after adding the stimulus (80-110 sec). $ZnSO_4$ had no effect on basal efflux. The first-order rate constants of efflux in the absence and presence of Zn^{2+} were 1.35 ± 0.05 and 1.30 ± 0.08 x 10^{-3} sec^{-1} (n = 8), respectively. Values are mean ± SE (n = 8). This series of experiments was conducted by Yingxin Zhuang.

was assayed in the absence of $ZnSO_4$. The 2 hr incubation with $ZnSO_4$ markedly inhibited $^{45}Ca^{2+}$ efflux evoked by Cd^{2+}, decreasing $[Na^+]_o$ and decreasing pH_o but had no effect on ATP-evoked $^{45}Ca^{2+}$ efflux (Table 3). These results suggest that prolonged exposure to $ZnSO_4$ selectively desensitizes endothelial cells to the three unusual stimuli and suggest that a single receptor, the Cd^{2+} receptor, mediates the Ca^{2+} mobilization response of endothelial cells to the three stimuli, as proposed in Smith et al. (1989b).

Effect of Cd^{2+} and Other Divalent Metals on $^{45}Ca^{2+}$ Efflux in Neuroblastoma Cells. Cd^{2+} and certain other divalent metals markedly stimulated $^{45}Ca^{2+}$ efflux (Fig. 25). Cd^{2+} increased the first-order rate constant to $8.8 \pm x\ 10^{-3}$ sec^{-1} (n = 12). The potency order of the Ca^{2+}-mobilizing metals was the same as that in the fibroblasts and endothelial cells: $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$ (Fig. 25). Zn^{2+} (100 μM) completely prevented 10 μM Cd^{2+} from stimulating $^{45}Ca^{2+}$ efflux. Neither atropine nor removal of Ca^{2+}_o inhibited Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux.

Effect of Cd^{2+} on $^{45}Ca^{2+}$ Efflux in Other Cell Types. Cd^{2+} stimulated $^{45}Ca^{2+}$ efflux in WI-38 lung fibroblasts and HUA cells. Although Fe^{2+} was not tested, the potency order of the Ca^{2+}-mobilizing metals in the HUA cells was similar to that in the fibroblasts, endothelial and neuroblastoma cells: $Cd^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+}$ (Fig. 26). Cd^{2+} did not stimulate $^{45}Ca^{2+}$ efflux in three other cell types: rat embryo fibroblasts, rat aortic smooth muscle cells, and A431 epidermoid carcinoma cells.

Effect of Decreasing pH_o on $^{45}Ca^{2+}$ Efflux in Human Skin Fibroblasts. Decreasing pH_o strikingly stimulated $^{45}Ca^{2+}$ efflux

(Fig. 27A). The maximal rate of efflux evoked by decreasing pH_o was similar to that evoked by bradykinin. Changing pH_o from 7.4 to 6.4 caused the half-maximal increase in the first-order rate constant of efflux (Fig. 27B). The effect of pH_o was independent of the buffer used: changing pH_o from 7.4 to 6.0 produced similar increases in $^{45}Ca^{2+}$ efflux when phosphate or imidazole was used to buffer PSS instead of PIPES.

Effect of Decreasing pH_o on $^{45}Ca^{2+}$ Efflux in Other Cell Types. Lowering pH_o strongly stimulated $^{45}Ca^{2+}$ efflux in endothelial and neuroblastoma cells (Figs. 28A, B). Lowering pH_o from 7.4 to 6.4 or 6.3 half-maximally stimulated $^{45}Ca^{2+}$ efflux in the endothelial and neuroblastoma cells. Therefore, the pH_o dependence of Ca^{2+} mobilization in fibroblasts, endothelial and neuroblastoma cells was similar. Decreasing pH_o did not stimulate $^{45}Ca^{2+}$ efflux in three other cell types: rat embryo fibroblasts, rat aortic smooth muscle cells, or A431 epidermoid carcinoma cells (data not shown).

Effect of Bradykinin or Decreasing $[Na^+]_o$ on Total Cell Ca^{2+} in Human Skin Fibroblasts. Removing Na^+_o rapidly decreased total cell Ca^{2+} (Fig. 29). The maximal decrease (37 ± 1.1%; n = 11) occurred within 5 min of Na^+_o replacement. Replacing Na^+_o with choline, K^+, or sucrose for 5 min caused similar decreases in total Ca^{2+} (data not shown). The drop in total Ca^{2+} lasted for more than 60 min in the presence of choline PSS. Returning the cultures to PSS 5 min after removing Na^+_o caused the cells to recover the lost Ca^{2+} (Fig. 29). The recovery was about half complete within 15 min and complete recovery occurred in about 60 min (Fig. 29).

Bradykinin rapidly decreased total cell Ca^{2+} (Fig. 30). The effect of bradykinin on total Ca^{2+} depended on the hormone concentration (Figs. 30A, 31) and time (Fig. 30B). The concentration of bradykinin that half-maximally decreased total Ca^{2+}, measured after a 2 min incubation, was 0.6 nM (Fig. 30A). The maximal decrease (50-60%) occurred within 1 min of adding 100 nM bradykinin (Fig. 30B, inset). In the presence of bradykinin, the cells slowly regained the lost Ca^{2+} (Fig. 30B). Total Ca^{2+} returned almost to the basal unstimulated level 60 min after bradykinin addition (Fig. 30B).

Effect of Ca^{2+}-mobilizing Divalent Metals on Total Cell Ca^{2+} in Human Skin Fibroblasts. Cd^{2+} decreased total cell Ca^{2+} by about 60% in 2 min, similarly to bradykinin (Fig. 32A). A 2 min treatment with 10 μM Co^{2+}, Ni^{2+}, Fe^{2+}, or Mn^{2+} decreased total Ca^{2+} by 64 ± 2, 38 ± 10, 38 ± 6, and 14 ± 7%, respectively (mean ± SE, n = 4-9). Removing Cd^{2+} (Fig. 32A) or Co^{2+} (Fig. 33) accelerated the recovery of the lost Ca^{2+}. After cell Ca^{2+} had nearly returned to the basal level, the addition of bradykinin or a second addition of Cd^{2+} decreased total Ca^{2+} similarly to the first addition of metal, indicating that Ca^{2+} had been restored to the hormone-sensitive pool. Zn^{2+} competitively inhibited the effect of Cd^{2+} on total cell Ca^{2+} (Fig. 32B), presumably by binding to the same site as Cd^{2+}. The $K_{1/2}$ and K_i values of Cd^{2+} and Zn^{2+} were 0.09 ± 0.02 and 0.37 ± 0.06 μM, respectively (mean ± SE). Cu^{2+} and Pb^{2+} also inhibited Cd^{2+}-induced Ca^{2+}-mobilization; the potency order of the inhibitors was $Zn^{2+} > Cu^{2+} > Pb^{2+}$ (Fig. 20). At 100 μM, Zn^{2+}, Cu^{2+}, and Pb^{2+} did not inhibit Ca^{2+} transport under

Table 4. Responsiveness of various cell types to three stimuli that release stored Ca^{2+}.

Cell type	Stimulus of cell Ca^{2+} mobilization		
	Decreasing $[Na^+]_o$	Cd^{2+}	Decreasing pH_o
HSF	+	+	+
WI-38	+	+	+
HUA	+	+	+
CEC	+	+	+
SK-N-SH	+	+	+
RASMC	-	-	-
REF	-	-	-
A-431	+	-	-

Ouabain abolishes the cell Ca^{2+} mobilization produced by decreasing $[Na^+]_o$ in A-431 cells in contrast to all the other cell types. Therefore, the mechanism of Ca^{2+} mobilization evoked by decreasing $[Na^+]_o$ is different in the A-431 cells compared to the other cell types. HSF, human skin fibroblasts; WI-38, human lung fibroblasts; HUA, human umbilical artery smooth muscle; CEC, canine coronary artery endothelial cells; SK-N-SH, human neuroblastoma cells; RASMC, rat aorta smooth muscle; REF, rat embryo fibroblasts; A-431, human epidermoid carcinoma cells.

these conditions because they had no effect on bradykinin-stimulated efflux (Fig. 21 and data not shown).

Effect of Decreasing pH_o on Total Cell Ca^{2+} in Human Skin Fibroblasts. To test the effect of pH_o on total cell Ca^{2+}, 30 μl of 1 N HCl was added to cultures that had been incubated overnight with $^{45}Ca^{2+}$. Lowering pH_o markedly decreased total cell Ca^{2+} (Figs. 34, 35). The maximal decrease (60-70%) occurred 1-2 min after decreasing pH_o (Fig. 35) which is similar to that produced by bradykinin (Fig. 30). Lowering pH_o to 6.5 half-maximally decreased cell Ca^{2+} (Fig. 34).

The cells slowly regained much of the lost Ca^{2+} even when pH_o was kept at 6.0 (Fig. 35A). Changing pH_o back to 7.4 markedly increased the rate of recovery of total cell Ca^{2+} (Fig. 35A). After cell Ca^{2+} had returned to the basal level, a second 2 min acid pulse decreased total cell Ca^{2+} similarly to the first pulse (Fig. 35A). Next, pH_o was cycled between 7.4 and 6.0 to find out if total Ca^{2+} would repeatedly rise and fall in response to the pH_o changes. Five successive changes in pH_o repeatedly increased and decreased total cell Ca^{2+} by substantial amounts (Fig. 35B). Furthermore, bradykinin decreased cell Ca^{2+} after a partial recovery from an acid pulse (Fig. 35B), indicating that Ca^{2+} had been reaccumulated by the hormone-sensitive pool.

Effect of Decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ Uptake in Human Skin Fibroblasts. The initial rate of $^{45}Ca^{2+}$ uptake (15-60 sec) was about 30% faster in the absence of Na^+_o (Fig. 36). Between 5 and 30 min, there was a 60% increase in the rate of $^{45}Ca^{2+}$ uptake in the absence of Na^+_o. The increase in the rate of

$^{45}Ca^{2+}$ uptake caused by decreasing Na^{+}_{o} was too small to compensate for the large increase in $^{45}Ca^{2+}$ efflux; therefore, total cell Ca^{2+} decreased (Fig. 29). Bradykinin (40 nM) had no effect on the initial rate (0 to 2 min) of $^{45}Ca^{2+}$ uptake (Fig. 37), indicating that Ca^{2+} influx made little or no contribution to the increase in $[Ca^{2+}]_i$ evoked by bradykinin (Figs. 3, 7). From 2 to 30 min, bradykinin stimulated the rate of $^{45}Ca^{2+}$ uptake about 2-fold (Fig. 37). This increase may be due to re-filling of intracellular Ca^{2+} stores after release (see ref. cited in Exton, 1988).

Effect of Decreasing $[Na^{+}]_o$ or Adding Bradykinin on $^{45}Ca^{2+}$ Uptake in Endothelial Cells. Replacing Na^{+}_{o} with choline stimulated $^{45}Ca^{2+}$ uptake 1.75-fold (Fig. 38); the initial rate of uptake (15-60 sec) was 0.37 nmol/min x mg protein in the presence of Na^{+}_{o} and 0.65 nmol/min x mg protein in its absence (Fig. 38). Loading the cells with Na^{+}, 30 min incubation with 0.1 mM ouabain, had virtually no effect on $^{45}Ca^{2+}$ uptake in the presence or absence of Na^{+}_{o} (Fig. 38).

The cells contained 9.4 ± 0.1 nmol Ca^{2+}/mg protein assuming that the $^{45}Ca^{2+}$ equilibrates with cell Ca^{2+} during the 20 hr incubation in Medium 199 containing 10% FBS and 10 μCi/ml $^{45}Ca^{2+}$. From this value of total Ca^{2+} and the increase in the first-order rate constant produced by removing Na^{+}_{o} (Table 2), the rate of stimulated efflux is 2.4 nmol Ca^{2+}/min x mg protein. Therefore, the stimulation of $^{45}Ca^{2+}$ uptake (0.3 nmol/min x mg protein) is not sufficient to account for the stimulation of $^{45}Ca^{2+}$ efflux, indicating that it probably does not occur by $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange.

Hypothesis II

The three stimuli, decreasing $[Na^+]_o$, addition of Cd^{2+} and certain other divalent metals, or decreasing pH_o trigger the release of stored Ca^{2+} by acting via an extracellular site.

Decreasing Extracellular, not Intracellular, $[Na^+]$ Triggers the Release of Stored Ca^{2+}. Decreasing $[Na^+]_o$ would be expected to decrease $[Na^+]_i$ as well. If a decrease in $[Na^+]_i$ causes the stimulation of $^{45}Ca^{2+}$ efflux by decreasing $[Na^+]_o$, then increasing $[Na^+]_i$ should inhibit the stimulation of $^{45}Ca^{2+}$ efflux. A 30 min incubation with 0.1 mM ouabain increased $[Na^+]_i$ from 15 ± 1 mM (n = 6) to 35 ± 3 mM (n = 4) in the fibroblasts. Increasing $[Na^+]_i$ had no effect on the stimulation of $^{45}Ca^{2+}$ efflux produced by replacing PSS containing 0.1 mM ouabain with choline PSS containing 0.1 mM ouabain 60 sec after starting the assay (data not shown). Na^+ loading slightly decreased the basal rate of $^{45}Ca^{2+}$ efflux (data not shown).

In the endothelial cells, cell Na^+ was 0.078 ± 0.011 μmol/mg protein or about 21 mM assuming a homogeneous distribution in cell water space (n = 3 experiments on triplicate cultures). Incubating the cells with 0.1 mM ouabain for 30 min increased $[Na^+]_i$ by 2.5-fold to 0.194 ± 0.022 μmol/mg protein, about 53 mM (n = 2 experiments on triplicate cultures). Na^+ loading had no effect on the stimulation of $^{45}Ca^{2+}$ efflux evoked by removing Na^+_o (data not shown). Similarly, in the neuroblastoma cells, a 30 min incubation with 0.1 mM ouabain did not inhibit $^{45}Ca^{2+}$ stimulated by decreasing $[Na^+]_o$ although $[Na^+]_i$ was not

measured after the ouabain incubation (data not shown). These findings suggest that a decrease in the concentration of extracellular, not intracellular, Na^+ stimulates $^{45}Ca^{2+}$ efflux in the fibroblasts, endothelial and neuroblastoma cells.

$^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ was also assayed in the presence of 10 μM bumetanide or 25 μM ethylisopropyl amiloride. At these concentrations, bumetanide and ethylisopropyl amiloride inhibit the $Na^+/K^+/Cl^-$ cotransporter and Na^+/H^+ exchange, respectively, by more than 95% (data not shown). Neither of these Na^+ transport inhibitors had any effect on $^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ 60 sec after starting the assay (data not shown). In addition, 100 μM SITS (4-isothiocyano-4'-acetamidostilbene-2,2'-disulfonic acid), which blocks anion exchange in human fibroblasts (Elgavish et al., 1985), had no effect on $^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ (data not shown). Therefore, Ca^{2+} mobilization triggered by decreasing $[Na^+]_o$ does not involve the activity of the Na^+ pump, $Na^+/K^+/Cl^-$ cotransporter, Na^+/H^+ exchanger, or anion exchanger.

Cd^{2+} Acts at an Extracellular Site. We used a cell-permeable chelator of heavy metals, TPEN, to find out if Cd^{2+} acts intracellularly or extracellularly. TPEN has a high affinity for Cd^{2+} ($10^{16.33}$ M^{-1}) and low affinities for Ca^{2+} or Mg^{2+} ($10^{1.7}$ and $10^{4.4}$ M^{-1}, respectively) (Aneregg et al., 1977; Arslan et al., 1985). As expected from the stability constant of TPEN for Cd^{2+}, a small excess of extracellular TPEN added simultaneously with Cd^{2+} completely blocked the stimulation of $^{45}Ca^{2+}$ efflux from fibroblasts (Fig. 39) and endothelial cells

(Fig. 40). However, incubating the cells with 50 μM TPEN for 40 min and assaying efflux in the presence of 50 μM TPEN only slightly inhibited the stimulation of efflux by 55 μM Cd^{2+}, 20 nM bradykinin (Fig. 39), or 150 μM ATP (Fig. 40). Under these conditions, intracellular TPEN would be expected to substantially exceed the extracellular concentration of free Cd^{2+}, so TPEN should have completely blocked the stimulation of efflux if Cd^{2+} acts intracellularly. These data suggest that extracellular, not intracellular, Cd^{2+} triggers Ca^{2+} mobilization.

Decreasing Extracellular, not Intracellular, pH Triggers the Release of Stored Ca^{2+} from Human Skin Fibroblasts. Decreasing pH_o from 7.4 to 6.0 decreases intracellular pH (pH_i) from about 7.3 to 7.0 (Fig. 7). Moderate decreases in pH_i are readily produced by exposing cells to the salt of a weak acid at constant pH_o as described previously for fibroblasts (Moolenaar et al., 1984) and endothelial cells (Kitazono et al., 1988). The addition of 5, 10, 20, or 40 mM sodium propionate (pK 4.87) decreases pH_i by 0.1-0.4 units (Moolenaar et al., 1984; Smith, unpublished data). The addition of these concentrations of sodium propionate had no effect on $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ efflux (data not shown). Because moderate decreases in pH_i at constant pH_o failed to evoke a $[Ca^{2+}]_i$ spike or stimulate $^{45}Ca^{2+}$ efflux, the protonation of an extracellular site probably triggers Ca^{2+}-mobilization.

Hypothesis III

The release of stored Ca^{2+} by Cd^{2+} and the other metals or by decreasing pH_o is not secondary to a general, nonspecific toxic effect.

Divalent Metals. In the fibroblasts, Ca^{2+}-mobilization by the metals is probably not the result of production of reactive oxygen species. First, H_2O_2 (0.1-1 mM) had no effect on basal or Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux (data not shown). Second, neither superoxide dismutase (50 units/ml), 20 mM mannitol, a hydroxyl radical scavenger, nor antioxidants (25 μM butylated hydroxyanisole or 100 μM butylated hydroxytoluene) had any effect on $^{45}Ca^{2+}$ efflux evoked by 1 μM Cd^{2+} or 10 μM Fe^{2+} (data not shown). Third, Co^{2+}, which is much less toxic than Cd^{2+} (Goyer, 1986), was almost as potent as Cd^{2+} in evoking Ca^{2+}_o release (Figs. 16B, 33). Fourth, the effects of Cd^{2+} and Co^{2+} on total cell Ca^{2+} were largely reversible (Figs. 32, 33). Fifth, 10 or 60 min incubations with 10 μM Cd^{2+}, Co^{2+}, or Fe^{2+} had no effect on cell morphology, permeability to propidium iodide, or cell K^+ (data not shown).

Similarly, in the neuroblastoma cells, a 10 min incubation with 10 μM Cd^{2+} had no effect on the intracellular concentrations of K^+ or Na^+; intracellular K^+ was 0.76 ± 0.04 μmol/mg protein (n = 5) in the absence, and 0.77 ± 0.02 μmol/mg protein (n = 5) in the presence, of Cd^{2+}. Intracellular Na^+ was 0.039 ± 0.003 μmol/mg protein (n = 5) in the absence, and 0.043 ± 0.001 μmol/mg protein (n = 5) in the presence, of Cd^{2+}. The differences are not statistically

significant, $p = 0.84$ and $p = 0.16$, for K^+ and Na^+, respectively.

Endothelial cells may be more sensitive to the toxic effects of Cd^{2+} than other cell types (Liu et al., 1989). A 10 min incubation with 10 μM Cd^{2+} had no effect on cell morphology as observed by phase contrast microscopy. However, total cell K^+ decreased by 19%, from 0.694 ± 0.012 to 0.564 ± 0.008 μmol/mg protein (n = 8), and cell Na^+ increased by 30%, from 0.095 ± 0.002 to 0.123 ± 0.002 μmol/mg protein (n = 8). These differences are statistically significant, $p < 0.001$. In the endothelial cells, Cd^{2+} may have a specific toxic effect on the Na^+/K^+ ATPase which could account for the decrease in cell Na^+ and the increase in cell K^+. However, the effect of Cd^{2+} on Na^+/K^+ ATPase was not investigated here. Furthermore, these changes were after a 10 min incubation. The effect of Cd^{2+} on cell K^+ or Na^+ during the 10 to 20 sec required to trigger Ca^{2+} mobilization was not measured. The similarity of the effects of Cd^{2+} on Ca^{2+} mobilization in endothelial cells compared to the fibroblasts and neuroblastoma cells suggests that the release of stored Ca^{2+} triggered by Cd^{2+} is not secondary to a general nonspecific toxic effect. Additionally, other metals that are much less toxic than Cd^{2+}, for example, Fe^{2+} and Co^{2+}, produce effects on endothelial cell Ca^{2+} regulation similar to Cd^{2+}.

Decreasing pH_o from 7.4 to 6.0. Decreasing pH_o had no effect on total cell K^+ ($p = 0.615$, Student's t test). Cell K^+ was 1.21 ± 0.06 μmol/mg protein (n = 8) after 10 min at pH_o 6.0 compared to 1.25 ± 0.04 μmol/mg protein (n = 12) in

control cultures incubated at pH_o 7.4. Changing pH_o to 6.0 for 10 min significantly increased cell Na^+ from 0.118 ± 0.002 (n = 12) to 0.155 ± 0.006 (n = 12, $p < 0.001$). The cultures were incubated for 1 hr in PSS containing glucose prior to changing pH_o as described for $^{45}Ca^{2+}$ efflux. The lack of effect of pH_o on cell K^+ indicates that pH_o selectively affects cell Ca^{2+} regulation and does not generally alter permeability of the cells to cations. Exposing the cells to pH 6 for 2-10 min had no effect on cell morphology, and there was no decrease in cell viability for at least 3 days after the acid treatment as judged by phase contrast microscopy or plating efficiency after detachment with trypsin. Finally, the effect of decreasing pH_o on cell Ca^{2+} was reversible (Fig. 35).

Hypothesis IV

Decreasing $[Na^+]_o$, adding Cd^{2+} or certain other divalent metals, or decreasing pH_o stimulates production of [^{3}H]inositol phosphates similarly to Ca^{2+}-mobilizing agonists.

Effect of Decreasing $[Na^+]_o$ on [^{3}H]Inositol Phosphate Production in Human Skin Fibroblasts. Decreasing $[Na^+]_o$ from 120 mM to 0 mM rapidly increased [^{3}H]inositol phosphate levels (Fig. 41). [^{3}H]inositol monophosphate levels increased more slowly than those of the polyphosphates (Fig. 41). Bradykinin produced much larger and faster increases in [^{3}H]inositol phosphate levels than decreasing $[Na^+]_o$; 15 sec after adding 100 nM bradykinin, the levels of IP, IP_2, and IP_3 plus IP_4 were 332, 2735, and 1376% of the control levels, respectively. The control levels were: GPI, 1169 ± 176; IP, 4754 ± 60; IP_2, 6864 ± 86; and IP_3 plus IP_4, 3413 ± 102. These values are the means

of duplicate cultures from one of two independent experiments which gave similar results. Neither bradykinin nor decreasing $[Na^+]_o$ affected [^{3}H]GPI levels (Figs. 41 and 43B).

Effect of Ca^{2+}-mobilizing Metals on [^{3}H]Inositol Phosphate Production in Human Skin Fibroblasts. Cd^{2+} (5 μM) rapidly increased [^{3}H]IP_3 and [^{3}H]IP_4 by about 4- and 2-fold, respectively (Fig. 42). Cd^{2+} also increased [^{3}H]IP and [^{3}H]IP_2 (Fig. 42). Peak increases in [^{3}H]IP and [^{3}H]IP_2 occurred after those in [^{3}H]IP_3 and [^{3}H]IP_4. Cd^{2+} had no effect on [^{3}H]GPI. The effects of Cd^{2+} on inositol phosphates are similar to those produced by stimulating receptors that are coupled to phosphoinositidase (Smith et al., 1989a; Smith and Smith, 1987, Berridge, 1987). Fe^{2+} or Co^{2+} increased [^{3}H]inositol phosphates similarly to Cd^{2+}. A 1 min incubation with 20 μM Fe^{2+} or Co^{2+} or 10 μM Cd^{2+} increased [^{3}H]inositol phosphates (IP + IP_2 + IP_3) by 286 ± 11, 464 ± 30, and 414 ± 22% control (mean ± SE, n = 3), respectively. Zn^{2+} (100 μM) had no effect on [^{3}H]inositol phosphates (106 ± 5% control) and completely inhibited the stimulation of [^{3}H]inositol phosphate production by 10 μM Cd^{2+} or 20 μM Fe^{2+} (98 ± 3 and 114 ± 7% control, n = 3, respectively).

Effect of Decreasing pH_o on [^{3}H]Inositol Phosphates in Human Skin Fibroblasts. Changing pH_o from 7.4 to 6.1 for 15 sec increased [^{3}H]IP_2 and [^{3}H]IP_3 by 10- and 5-fold, respectively (Fig. 43A). [^{3}H]IP increased less rapidly than the polyphosphates. [^{3}H]IP_4 increased by about 90% 30 sec after decreasing pH_o. Bradykinin produced similar changes in

Table 5. Effects of Cd^{2+}, low pH_o, or acetylcholine on [^{3}H]inositol phosphates in neuroblastoma cells.

	[^{3}H]inositol phosphate, cpm/culture				
	GPI	IP	IP_2	IP_3	n
Additions					
None	1375±83	1420±79	536±52	134±42	10
Cd^{2+}	1367±84	1818±75*	674±61	175±24	9
pH 6	1455±170	1926±101*	748±62*	182±19	9
Ach, 15nM	1161±12*	1651±7*	529±17	273±12*	2
Ach, 20nM	1551±208	2077±64*	711±27*	315±49*	2
Ach, 10μM	1630±174	9956±230*	5158±435*	1346±49*	8

*Statistically different ($p < 0.02$) from controls (no additions) by Student's t-test (unpaired samples). Values are mean ± SE The cultures (100 mm diameter) were rinsed twice with medium 199 one day after they were subcultured 1:2. They were incubated for 2 days with 5 ml medium 199 containing 2% fetal bovine serum and 5 μCi [^{3}H]inositol as described (Smith et al., 1989). The cultures were rinsed 5 times with PSS and incubated with 10 ml PSS containing 10 mM glucose for 2 hr. Then the medium was removed and replaced with 5 ml of PSS containing the additions indicated. After 2 min, the medium was removed and 3 ml ice-cold 10% trichloroacetic acid was added. [^{3}H]inositol phosphates were extracted and analyzed as described (Smith et al., 1989). Each culture contained about 4 mg cell protein.

[^{3}H]IP's as lowering pH_o (Fig. 43B). Neither the hormone nor the change in pH_o affected [^{3}H]GPI (Figs. 43A, B).

Effect of Acetylcholine, Cd^{2+}, or Decreasing pH_o on [^{3}H]Inositol Phosphates in the Neuroblastoma Cells. Acetylcholine (10 μM), a concentration that maximally stimulates $^{45}Ca^{2+}$ efflux, markedly increased production of [^{3}H]inositol phosphates in a 2 min incubation: IP, IP_2, and IP_3 increased to 701, 964, and 1004% of the control values, respectively (Table 5). Addition of 10 μM Cd^{2+} increased the [^{3}H]inositol phosphates 128, 126, and 131%, respectively (Table 5). Similarly, decreasing pH_o from 7.4 to 6.0 increased the [^{3}H]inositol phosphates 136, 140 and 136%, respectively (Table 5); the increases in IP and IP_2 were statistically significant ($p < 0.02$). Low concentrations of acetylcholine (15-20 nM) evoked similar increases in $^{45}Ca^{2+}$ efflux (data not shown) and [^{3}H]inositol phosphates (Table 5) as 10 μM Cd^{2+} or decreasing pH_o to 6.0.

Hypothesis V

If decreasing $[Na^+]_o$ triggers [^{3}H]inositol phosphate production similarly to Ca^{2+}-mobilizing hormones, then a G-protein may mediate the activation of phospholipase C.

Effect of Pertussis Toxin on the Increase in $[Ca^{2+}]_i$ and the Stimulation of $^{45}Ca^{2+}$ Efflux by Decreasing $[Na^+]_o$ in Human Skin Fibroblasts. Pertussis toxin is a pentameric enzyme produced by the bacterium, Bordetella pertussis. The toxin ADP-ribosylates a 41 kd membrane bound protein in human fibroblasts that is a member of the G-protein family (Moss et

Table 6. Effect of pertussis toxin on $^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ or adding 40 nM bradykinin in human skin fibroblasts.

Treatment	[PT]μg/ml	K (x 10^{-3} sec^{-1})	%inhibit	n
Minus Na^+_o	-	13.18 ± 1.21	-	10
	10	5.48 ± 1.75	58.4	4
	20	3.37 ± 1.44	74.4	4
	30	3.57 ± 0.62	72.9	2
40 nM BK	-	17.26 ± 0.64	-	5
	20	14.60 ± 0.63	15.4	2
	30	8.25 ± 1.16	52.2	2

Cultures were incubated for 20 hr in DME + 10% fetal bovine serum containing 10μCi $^{45}Ca^{2+}$ and the indicated concentration of pertussis toxin or an equal volume of vehicle (10 mM sodium phosphate, 50 mM NaCl). Cultures were incubated for 1 hr prior to the efflux assay in PSS containing 10 mM glucose and $^{45}Ca^{2+}$ at the same specific radioactivity as the overnight incubation medium. To start the efflux, cultures were rinsed 8 times with PSS. At 60 sec, 40 nM bradykinin (BK) was added or PSS was replaced with choline-PSS until the end of the experiment. Data presented are the stimulated rate of efflux determined by subtracting the basal rate of efflux from the rate evoked by removing external Na^+ or adding bradykinin.

Table 7. Effect of pertussis toxin on the $[Ca^{2+}]_i$ response to bradykinin or decreasing $[Na^+]_o$ in human skin fibroblasts.

	$[Ca^{2+}]_i$ (nM)		% inhibition
Control - 19 hour			
Bradykinin	615 ± 54	(8)	-
Decreasing $[Na^+]_o$	594 ± 26	(8)	-
Pertussis toxin - 19 hour			
Bradykinin	365 ± 74	(3)	50
Decreasing $[Na^+]_o$	248 ± 8	(3)	72
Control - 25 hour			
Bradykinin	538 ± 24	(8)	-
Decreasing $[Na^+]_o$	544 ± 26	(10)	-
Pertussis toxin - 25 hour			
Bradykinin	390 ± 16	(3)	35
Decreasing $[Na^+]_o$	123 ± 3	(3)	98

Values are means ± SE (n). Treatment with the pertussis toxin vehicle (10 mM sodium phosphate, 50 mM NaCl) had no effect on basal $[Ca^{2+}]_i$ or the peak increase produced by decreasing $[Na^+]_o$ or 100 nM bradykinin. Hence, the data from the vehicle controls were combined with those from coverslips not treated with toxin or vehicle. None of the treatments affected the basal $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was 116 ± 4 nM (n = 46). It was subtracted from peak $[Ca^{2+}]_i$ to calculate % inhibition by the toxin. Measurements of $[Ca^{2+}]_i$ were made by Tao Zheng.

al., 1988). ADP-ribosylation impairs G-protein transduction of cell-surface receptor signals (Gilman, 1987).

Cultures were incubated overnight with 10, 20, or 30 μg/ml pertussis toxin. Incubation with pertussis toxin had no effect on $^{45}Ca^{2+}$ labeling. Pertussis toxin caused a concentration dependent inhibition of $^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ (Table 6). Similarly, pertussis toxin incubation inhibited $^{45}Ca^{2+}$ efflux evoked by a saturating concentration of bradykinin (40 nM). However, bradykinin appeared to be much less sensitive to inhibition by pertussis toxin (Table 6).

Pertussis toxin also inhibited the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$. Incubation of the cells with 30 μg/ml pertussis toxin for 19 hr markedly inhibited, and incubation for 25 hr completely blocked, the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$ (Fig. 44, Table 7). These results suggest that pertussis toxin inhibits the release of stored Ca^{2+} evoked by decreasing $[Na^+]_o$. However, we have no evidence that the pertussis toxin inhibition is a direct effect of ADP-ribosylation of a G-protein. In addition, the concentration of pertussis toxin required to significantly inhibit the stimulation of $^{45}Ca^{2+}$ efflux or the increase in $[Ca^{2+}]_i$ produced by decreasing Na^+ or bradykinin is high compared to that used to inhibit $G_{i\alpha}$ in human fibroblasts (Moss et al., 1988). Thus, nonspecific effects of the toxin may have produced the inhibition of Ca^{2+} mobilization by decreasing $[Na^+]_o$.

Hypothesis VI

Extracellular Ca^{2+} and Mg^{2+} potentiate the release of stored Ca^{2+} by decreasing $[Na^+]_o$ or adding Cd^{2+}.

Extracellular Ca^{2+} and Mg^{2+} Potentiate the Increase in $[Ca^{2+}]_i$ and the Stimulation of $^{45}Ca^{2+}$ Efflux by Decreasing $[Na^+]_o$ or adding Cd^{2+} in Human Skin Fibroblasts.

Decreasing $[Na^+]_o$ from 120 mM to 3 mM rapidly and transiently increased $[Ca^{2+}]_i$ from a resting level of 137 ± 4 nM (n = 9) to 678 ± 27 nM (n = 4) (Fig. 45A) as previously reported (Smith et al., 1989). In contrast, decreasing $[Na^+]_o$ in the absence of Ca^{2+} and Mg^{2+} had no effect on $[Ca^{2+}]_i$ (Fig. 45B). After incubation in Ca^{2+}/Mg^{2+}-free solutions, 3 μM A23187 transiently increased $[Ca^{2+}]_i$ to 756 ± 40 nM (n = 3), indicating that stored Ca^{2+} was not depleted by the brief incubation in the absence of external Ca^{2+} (Fig. 45B). Decreasing $[Na^+]_o$ to 3 mM in the presence of 2 mM Mg^{2+} and absence of Ca^{2+}, or vice versa, produced an increase in $[Ca^{2+}]_i$ that was 50-80% (n = 2) of the increase produced in the presence of both divalents, 1 mM each (data not shown). Therefore, the combination of Ca^{2+} plus Mg^{2+} (1 mM each) was slightly more effective for releasing stored Ca^{2+} when $[Na^+]_o$ was decreased than either divalent alone.

Decreasing $[Na^+]_o$ from 120 to 3 mM stimulated $^{45}Ca^{2+}$ efflux 9.8-fold (Fig. 46A); the first-order rate constant increased from 1.1 ± 0.1 x 10^{-3} sec^{-1} to 10.8 ± 0.9 x 10^{-3} sec^{-1} (n = 13). In contrast, in the absence of Ca^{2+} and Mg^{2+}, decreasing $[Na^+]_o$ increased the first-order rate constant less than 2-fold from 1.3 ± 0.1 x 10^{-3} sec^{-1} to 2.3 ± 0.2 x 10^{-3} sec^{-1} (n = 17) (Figs. 46A, B). If $[Na^+]_o$ was decreased to 3 mM in the absence of

Ca^{2+} and Mg^{2+}, adding Ca^{2+} or Mg^{2+} back produced a concentration dependent stimulation of $^{45}Ca^{2+}$ efflux (Figs. 46A, B). The concentration of Ca^{2+} that half-maximally increased the first-order rate constant for $^{45}Ca^{2+}$ efflux was 38 μM ($r = 0.975$) as determined by the method of Chou (1976) using software from Elsevier_BIOSOFT (Cambridge, U.K.). Mg^{2+} was somewhat less potent than Ca^{2+} (Fig. 46B).

Decreasing $[Na^+]_o$ to 3 mM also triggered the release of stored Ca^{2+} in WI-38 lung fibroblasts; the first-order rate constant of $^{45}Ca^{2+}$ efflux increased 8.3-fold from $1.3 \pm 0.1 \times 10^{-3}$ sec^{-1} to $10.8 \pm 1.0 \times 10^{-3}$ sec^{-1} ($n = 4$). In the absence of Ca^{2+} and Mg^{2+}, decreasing $[Na^+]_o$ to 3 mM increased the first-order rate constant less than 3-fold, from $1.1 \pm 0.1 \times 10^{-3}$ sec^{-1} to $2.9 \pm 0.3 \times 10^{-3}$ sec^{-1} ($n = 7$). Therefore, Ca^{2+} or Mg^{2+} potentiates Ca^{2+}-mobilization evoked by decreasing $[Na^+]_o$ in the lung as well as the skin fibroblasts.

Cd^{2+} was a more potent stimulus for Ca^{2+}-mobilization in the presence than in the absence of Ca^{2+} and Mg^{2+} (Fig. 47). At sub-saturating concentrations of Cd^{2+} (31-47 nM), the presence of Ca^{2+} and Mg^{2+} (1 mM each) potentiated Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux more than 4-fold. The potentiation was statistically significant at 31, 47, and 63 nM Cd^{2+}, $p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively. In contrast, a saturating concentration of Cd^{2+} (10 μM) was equally effective in the presence and absence of extracellular Ca^{2+} and Mg^{2+}. Ca^{2+} and Mg^{2+} potentiated the increase in $[Ca^{2+}]_i$ evoked by 47 nM Cd^{2+} (Table 8). At 47 nM, Cd^{2+} produced a 3-fold greater increase in $[Ca^{2+}]_i$ in the presence compared to the absence of

Table 8. Effect of external Ca^{2+} and Mg^{2+} on the increase in $[Ca^{2+}]_i$ produced by low and high concentrations of Cd^{2+}.

	Increase in $[Ca^{2+}]_i$ (nM)			
Stimulus	Control	No Ca^{2+}/Mg^{2+}	% control	P
47 nM Cd^{2+}	218 ± 26 (9)	74 ± 10 (15)	34	<0.001
10 μM Cd^{2+}	905 ± 160 (8)	742 ± 75 (9)	82	0.373

Values are mean ± SE (n). Basal $[Ca^{2+}]_i$ (no Cd^{2+}) was 113 ± 12 (9) and 101 ± 8 (15) in the presence and absence of external divalent cations, respectively. The means are not significantly different (p = 0.46). p values were obtained by Student's t test for unpaired samples. Measurements of $[Ca^{2+}]_i$ were made by Robert Lyu.

Ca^{2+} and Mg^{2+} (Table 8). Extracellular Ca^{2+} and Mg^{2+} did not potentiate $^{45}Ca^{2+}$ efflux evoked by sub-saturating concentrations of bradykinin (Fig. 48).

Effect of La^{3+} or Zn^{2+} on the Increase in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ Efflux Produced by Decreasing $[Na^+]_o$. La^{3+} (100 μM) completely prevented the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$ (Fig. 49). La^{3+} inhibited the peak increase by 50% at a concentration of 6 μM (Fig. 50) determined as described above by the method of Chou (1976). In contrast, La^{3+} had no effect on the peak increase in $[Ca^{2+}]_i$ produced by bradykinin; $[Ca^{2+}]_i$ increased from 197 ± 15 nM to 1303 ± 49 nM (n = 6) in the absence of La^{3+} (Fig. 49A), and to 1605 ± 329 (n = 9) in the presence of 1 mM La^{3+} (Fig. 49B). However, La^{3+} converted the transient increase in $[Ca^{2+}]_i$ produced by bradykinin into a long-lasting step-increase (Fig. 49B). $[Ca^{2+}]_i$ probably remained high because La^{3+} prevents transmembrane Ca^{2+} movements and net Ca^{2+} efflux causes the decrease in $[Ca^{2+}]_i$ from the peak produced by bradykinin (Smith et al., 1989). Additionally, a sustained increase in IP_3 produced by bradykinin may prevent the endoplasmic reticulum from decreasing $[Ca^{2+}]_i$. Thus, $[Ca^{2+}]_i$ remains elevated in the presence of both bradykinin and La^{3+}.

Zn^{2+}, which competitively inhibits Cd^{2+}-evoked release of stored Ca^{2+} (Smith et al., 1989a) also inhibited $^{45}Ca^{2+}$ efflux stimulated by decreasing $[Na^+]_o$ (Fig. 51). At 100 μM, Zn^{2+} almost completely inhibited $^{45}Ca^{2+}$ efflux (Fig. 51) without affecting basal efflux. Zn^{2+} half-maximally inhibited $^{45}Ca^{2+}$ efflux at 36 μM as determined by the method of Chou (1976)

described above. By contrast, 100 μM Zn^{2+} has no effect on bradykinin-evoked $^{45}Ca^{2+}$ efflux (Smith et al., 1989a).

The specific inhibitory effects of La^{3+} and Zn^{2+} further support the hypothesis that Ca^{2+}-mobilization by decreasing $[Na^+]_o$ is potentiated by divalent cations. La^{3+} selectively blocks Ca^{2+} transport across the plasma membrane (Dos Remedios, 1981; Wendt-Gallitelli and Isenberg, 1985; Smith et al., 1989c). La^{3+} also displaces Ca^{2+} from cell surface binding sites (Langer and Frank, 1972) and does not readily enter cells (Wendt-Gallitelli and Isenberg, 1985; Gylfe et al., 1986; Negulescu and Machen, 1988). La^{3+} abolished the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$ (Figs. 49, 50). Decreasing $[Na^+]_o$ releases stored Ca^{2+} in the absence of extracellular Ca^{2+} (Smith et al., 1989); therefore, La^{3+} does not prevent the increase in $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx. In contrast, La^{3+} has no effect on the peak increase in $[Ca^{2+}]_i$ produced by bradykinin, indicating that La^{3+} does not inhibit inositol polyphosphate production or release of stored Ca^{2+}. La^{3+} probably prevents the increase in $[Ca^{2+}]_i$ evoked by decreasing $[Na^+]_o$ by displacing Ca^{2+} and Mg^{2+} from an external domain, the Ca^{2+}/Mg^{2+} site, of the Cd^{2+} receptor. Zn^{2+} probably inhibits $^{45}Ca^{2+}$ efflux by a mechanism similar to La^{3+}: displacement of Ca^{2+} and Mg^{2+} from the Ca^{2+}/Mg^{2+} site. A model of the Cd^{2+} receptor proposed to account for the results described in this study contains three cation binding sites: a Cd^{2+} site, a Na^+ site, and a Ca^{2+}/Mg^{2+} site (see Discussion and illustration on page 64).

Several lines of evidence support the hypothesis that the Cd^{2+} receptor contains three distinct cation binding sites. First, Zn^{2+} competitively inhibits the release of stored Ca^{2+} by Cd^{2+} with a K_i of 0.4 μM (Fig. 32). Zn^{2+} also inhibits the release of stored Ca^{2+} by decreasing $[Na^+]_o$, however, 50% inhibition requires about 36 μM Zn^{2+} (Fig. 51). These data indicate that Zn^{2+} inhibits the release of stored Ca^{2+} by the two stimuli from different sites. Second, Ca^{2+} or Mg^{2+} are required for the release of stored Ca^{2+} by decreasing $[Na^+]_o$ but not Cd^{2+} (Figs. 45, 46). It is not likely that Ca^{2+} and Mg^{2+} are acting at the Cd^{2+} site because, at 120 mM $[Na^+]_o$, increasing the concentration of these cations to 16 mM does not stimulate $^{45}Ca^{2+}$ efflux. This suggests that Ca^{2+} and Mg^{2+} are binding at a separate site, the Ca^{2+}/Mg^{2+} site. Zn^{2+} probably inhibits the release of stored Ca^{2+} by decreasing $[Na^+]_o$ by displacing Ca^{2+} and Mg^{2+} from the Ca^{2+}/Mg^{2+} site, which could also account for the difference in Zn^{2+} concentrations required to inhibit, respectively, Cd^{2+} and decreasing $[Na^+]_o$. Third, the existence of distinct Cd^{2+} and Ca^{2+}/Mg^{2+} sites is supported by the observation that Ca^{2+} and Mg^{2+} potentiate the release of stored Ca^{2+} by very low concentrations of Cd^{2+} (Fig. 47, Table 8). This suggests that, to release stored Ca^{2+} in the absence of external Ca^{2+} and Mg^{2+}, Cd^{2+} binding is necessary at both the Cd^{2+} site and the Ca^{2+}/Mg^{2+} site. Thus, at very low Cd^{2+} concentrations, the presence of Ca^{2+} and Mg^{2+} makes Cd^{2+} binding at the Ca^{2+}/Mg^{2+} site unnecessary. In contrast, Ca^{2+} and Mg^{2+} are not necessary at higher Cd^{2+} concentrations (Fig. 47, Table 8).

DISCUSSION

The effect of bradykinin and three unusual stimuli, decreasing $[Na^+]_o$, decreasing pH_o, or adding Cd^{2+} or certain other metals, on cell Ca^{2+} regulation in human skin fibroblasts is strikingly similar: (1) a rapid 4- to 8-fold increase in $[Ca^{2+}]_i$, (2) a several-fold increase in the rate of $^{45}Ca^{2+}$ efflux, (3) a 40-60% decrease in total cell Ca^{2+}, and (4) a rapid stimulation of [^{3}H]inositol phosphate production (Smith et al., 1989, 1989a, 1989b). The three stimuli evoke changes in endothelial cell Ca^{2+} regulation similar to ATP (Figs. 4, 6, 7). ATP triggers the production of [^{3}H]inositol phosphates and releases stored Ca^{2+} in endothelial cells via purinergic receptors (Pirotton et al., 1987). Muscarinic cholinergic agonists stimulate [^{3}H]inositol phosphate production and increase $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} in SK-N-SH neuroblastoma cells (Fisher et al., 1989). We found that the three stimuli also trigger [^{3}H]inositol phosphate production and release stored Ca^{2+} in SK-N-SH neuroblastoma cells (Fig. 14, Table 8). Thus, the three stimuli and Ca^{2+}-mobilizing hormones have similar effects on the PI second messenger system and cell Ca^{2+} regulation.

Two lines of evidence suggest that a single receptor mediates the Ca^{2+}-mobilizing effects. First, the Ca^{2+}-

mobilization response to the three stimuli is cell type specific. Among the cell types that we have tested, each responded to all three stimuli or to none of them (Table 4, Smith et al., 1989b). Five cell types respond to all three stimuli: human skin fibroblasts, human lung fibroblasts (WI-38), human umbilical artery smooth muscle cells, human neuroblastoma cells, and canine coronary artery endothelial cells. Two cell types do not respond to any of the three stimuli: rat aortic smooth muscle cells and rat embryo fibroblasts. A third cell type, A-431 human epidermoid carcinoma cells, is unusual; decreasing $[Na^+]_o$ stimulated $^{45}Ca^{2+}$ efflux in these cells, but adding Cd^{2+} or decreasing pH_o had no effect (Smith et al., 1989b). However, increasing $[Na^+]_i$ by incubation with ouabain completely blocked the stimulation of efflux in A-431 cells. In contrast, increasing $[Na^+]_i$ in the fibroblasts, endothelial cells, and neuroblastoma cells has no effect on the release of stored Ca^{2+} (Smith et al., 1989). Therefore, a decrease in intracellular $[Na^+]$, not extracellular, triggers $^{45}Ca^{2+}$ efflux in the A-431 cells (Smith et al., 1989b). Second, fibroblasts (data not shown) and endothelial cells (Table 3) incubated for 2 hr with 10 μM $ZnSO_4$ lose the Ca^{2+}-mobilization response to Cd^{2+}, decreasing $[Na^+]_o$, and decreasing pH_o. The desensitization is reversible. In contrast, bradykinin- and ATP-evoked release of stored Ca^{2+} is unaffected by incubation of the fibroblasts (data not shown) or the endothelial cells, respectively, in $ZnSO_4$ (Table 3). These data support the hypothesis that a single receptor

mediates the Ca^{2+}-mobilization response to Cd^{2+}, decreasing $[Na^+]_o$, and decreasing pH_o.

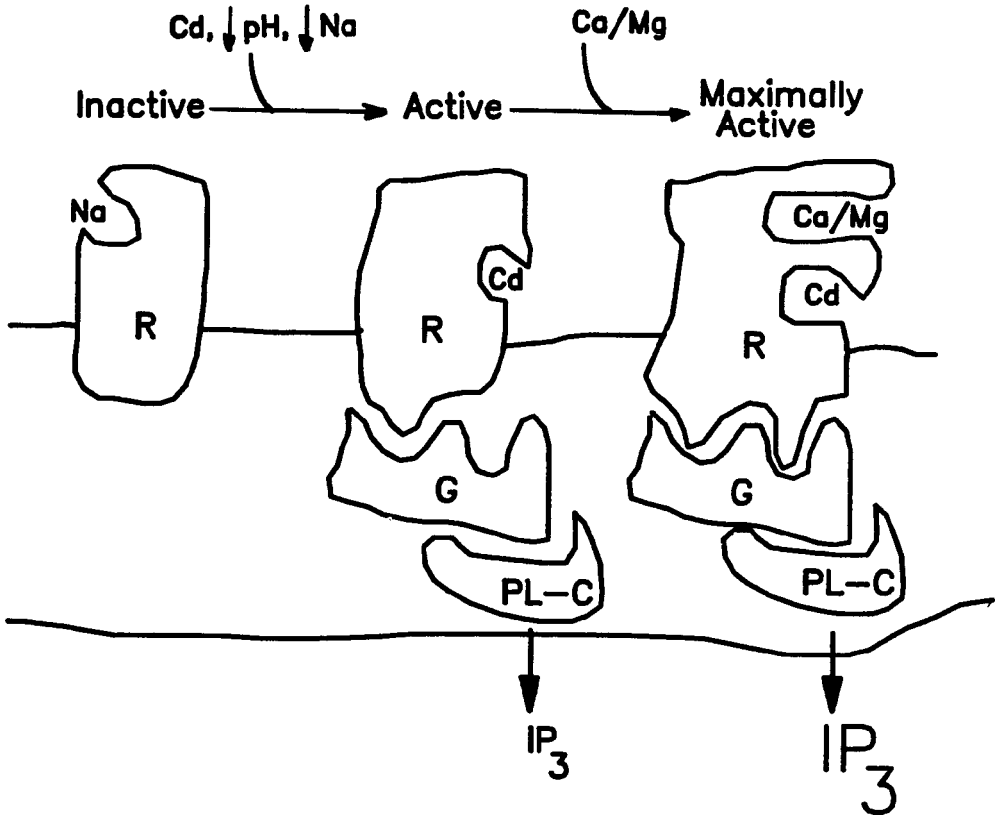
Three lines of evidence are consistent with the hypothesis that not only is a single receptor involved but that the same receptor is present on the responsive cell types. The cell type specificity and Zn^{2+} desensitization data, described above, support this hypothesis. In addition, approximately the same $K_{1/2}$ for the stimulation of $^{45}Ca^{2+}$ efflux by decreasing pH_o was observed in the human skin fibroblasts, endothelial cells, and the neuroblastoma cells (Smith et al., 1989b). At pH_o 6.3-6.4, the rate of $^{45}Ca^{2+}$ efflux is half-maximally increased (Figs. 27, 28). The observed $K_{1/2}$ for the decrease in total cell Ca^{2+} produced by decreasing pH_o was 6.5 (Fig. 34) which agrees well with the $K_{1/2}$ for $^{45}Ca^{2+}$ efflux. Furthermore, the same potency order of the stimulatory metals, $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$, was observed in the fibroblasts, endothelial cells, neuroblastoma cells, and human umbilical artery smooth muscle cells (Figs. 16, 23, 25, 26).

We suggest that the Ca^{2+}-mobilization response to the three stimuli is receptor-mediated; the Cd^{2+} receptor satisfies many of the criteria used to pharmacologically define a receptor. First, the receptor has high affinity for the ligand: the $K_{1/2}$ for Cd^{2+}-evoked decreases in total cell Ca^{2+} is 0.1 μM; the $K_{1/2}$ for the stimulation of $^{45}Ca^{2+}$ efflux by decreasing pH_o in the fibroblasts is 6.4 which corresponds to a hydrogen ion concentration of 0.43 μM. We observed approximately the same $K_{1/2}$ for decreasing pH_o in the endothelial and neuroblastoma cells (see above). Second, the

effect of Cd^{2+} and decreasing pH_o is saturable: Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux saturates at 1-10 μM Cd^{2+} (Fig. 16); higher concentrations do not produce a greater effect. The stimulation of $^{45}Ca^{2+}$ efflux by decreasing pH_o saturates at pH 5.7-6.0 (Fig. 27). Third, the receptor demonstrates agonist and antagonist specificity, and the antagonism is reversible: the same potency order of the stimulatory metals is observed in the fibroblasts, endothelial, neuroblastoma, and umbilical artery smooth muscle cells. Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux is inhibited by $Zn^{2+} > Cu^{2+} > Pb^{2+}$ (Fig. 20). Zn^{2+} and Cu^{2+} have no effect on bradykinin- or ATP-evoked $^{45}Ca^{2+}$ efflux in the fibroblasts and endothelial cells, respectively (Figs. 21, 24). Fourth, the receptor apparently couples the agonist to an enzyme that produces an intracellular second messenger: (a) all three stimuli trigger an increase in [^{3}H]inositol phosphates; (b) the three stimuli increase [^{3}H]inositol phosphates and release stored Ca^{2+} via an extracellular site; (c) other cell types that have the PI second messenger system do not respond to the three stimui; (d) Zn^{2+} selectively desensitizes the responsive cell types to the three stimuli but has no effect on bradykinin- or ATP-evoked Ca^{2+} mobilization in the fibroblasts and endothelial cells, respectively. It is not likely that the three stimuli act directly on phospholipase C because Cd^{2+} either has no effect or markedly inhibits, respectively, the two phosphoinositide-specific phospholipases C purified from bovine brain (Ryu et al., 1987) while Co^{2+}, Mn^{2+}, and Ni^{2+} have no effect on either form of phospholipase C (Ryu et al., 1987). Finally,

phospholipase C apparently does not span the plasma membrane (Katan et al., 1988). Therefore, it appears that the receptor is intrinsic to the propagation of the signal. Fifth, all three stimuli evoke a characteristic cellular response. One criterion for the definition of a receptor that has not been satisfied is that an agonist should be present in the cellular environment at a concentration that stimulates the receptor. The natural agonist for the Cd^{2+} receptor, if one exists, is not known.

The data presented here are consistent with the following model (illustrated on page 60) for a Cd^{2+} receptor. The receptor contains three cation binding sites: a "Cd^{2+} site", a "Ca^{2+}/Mg^{2+} site", and a "Na^{+} site". Binding of Cd^{2+} or certain other divalent metals at the Cd^{2+} site converts the receptor from an inactive N-state to an active A-state which then interacts with a G-protein and, in turn, triggers inositol phospholipid hydrolysis by activating phospholipase C. Similarly, decreasing $[Na^{+}]_o$ or decreasing pH_o converts the receptor from the N-state to the A-state. Extracellular Na^{+} may stabilize the N-state. Binding of Ca^{2+} and Mg^{2+} at the Ca^{2+}/Mg^{2+} site may potentiate the effect of Cd^{2+} or decreasing $[Na^{+}]_o$ by facilitating the conversion of the receptor from the N-state to the A-state. La^{3+} and Zn^{2+} probably displace Ca^{2+} and Mg^{2+} from the Ca^{2+}/Mg^{2+} site which, in turn, blocks the conversion of the receptor to the A-state (Figs. 49, 50). The mechanism by which decreasing pH_o releases stored Ca^{2+} is not clear; however, the presence of a critical histidine residue in the Cd^{2+} site may be inferred. Histidine is the



Proposed Model for the Cd^{2+} Receptor. Adding Cd^{2+}, decreasing pH_o, or decreasing $[Na^+]_o$, activates the receptor. When decreasing $[Na^+]_o$ or sub-saturating concentrations of Cd^{2+} is the stimulus, the presence of external Ca^{2+} and Mg^{2+} potentiate the Ca^{2+} mobilization response. The effect of Ca^{2+} and Mg^{2+} on Ca^{2+} mobilization evoked by decreasing pH_o is uncertain because low pH reduces the binding affinity of cations. R = Cd^{2+} receptor; G = G-protein; PL-C = phospholipase C; IP_3 = inositol 1,4,5-trisphosphate.

most common amino acid in the Zn^{2+} binding sites of proteins, and Zn^{2+} and Cd^{2+} are chemically similar. Histidine is also the only amino acid with a pK_a near 6.5 which is the pH value at which the release of stored Ca^{2+} triggered by decreasing pH_o is half-maximal (Fig. 34). Therefore, when pH_o is decreased, protons may bind at the histidine residue of the Cd^{2+} binding site to trigger the release of stored Ca^{2+}.

$[Na^+]_o$ removal increases $[Ca^{2+}]_i$ in monkey kidney (Snowdowne and Borle, 1985), toad bladder epithelial (Jacobs and Mandel, 1987), and smooth muscle cells from guinea pig taenia coli (Pritchard and Ashley, 1987). Although Ca^{2+} influx via Na^+/Ca^{2+} antiporter was suggested to cause the rise in $[Ca^{2+}]_i$, additional experiments are needed to further assess the possibility that Ca^{2+} mobilization causes or contributes to the rise in Ca^{2+} in some of these cell types. The human skin fibroblasts and endothelial cells used here apparently do not have Na^+/Ca^{2+} antiport activity. First, replacement of Na^+_o with choline in the fibroblasts had little effect on $^{45}Ca^{2+}$ influx during the 10-30 sec in which the $[Ca^{2+}]_i$ spike occurred and $^{45}Ca^{2+}$ efflux was maximally stimulated (Figs. 1, 3, 37, 38). Treatments which markedly enhance Ca^{2+} influx by Na^+/Ca^{2+} antiport in smooth muxcle cells had no effect in the fibroblasts (Smith, unpublished data). Second, decreasing $[Na^+]_o$ stimulated $^{45}Ca^{2+}$ efflux to the same rate and extent in the presence and absence of extracellular Ca^{2+}, which indicates the stimulation of efflux is not secondary to increased influx (Fig. 10, Table 2). Third, in the endothelial cells, decreasing $[Na^+]_o$ increased $^{45}Ca^{2+}$ uptake by only about 0.3

nmol/min x mg protein. By contrast, $^{45}Ca^{2+}$ uptake is 8 nmol/min x mg protein greater at 0 than at 120 mM $[Na^+]_o$ in smooth muscle cells that have abundant Na^+/Ca^{2+} antiport activity (Smith et al., 1987). The small increase in endothelial cell $^{45}Ca^{2+}$ uptake caused by decreasing $[Na^+]_o$ may be due to a pathway other than Na^+/Ca^{2+} antiport, for example, a Ca^{2+} channel that is activated by the release of stored Ca^{2+} (Sage and Rink, 1987; refs. cited in Exton, 1988). Fourth, Na^+-loading the endothelial cells did not potentiate the effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ influx (Fig. 38) as occurs in smooth muscle cells (Smith, Zheng, and Smith, 1989c). On the other hand, the smooth muscle cells that we cultured from umbilical arteries have Na^+/Ca^{2+} antiport activity (Smith, unpublished data) as well as a Cd^{2+} receptor that triggers the release of stored Ca^{2+} (Figs. 15, 26). Therefore, $[Na^+]_o$ removal may increase $[Ca^{2+}]_i$ by both mechanisms in cell types that have both.

Winquist et al. (1985) and Ku and Ann (1987) have suggested that Na^+/Ca^{2+} antiport is present in endothelial cells because dichlorobenzamil inhibited endothelium-dependent relaxation of isolated blood vessels produced by acetylcholine, A23187, or decreasing extracellular Ca^{2+}, whereas the Ca^{2+} channel blockers, nifedipine and verapamil, did not. However, dichlorobenzamil is a more potent inhibitor of Ca^{2+} channels than of Na^+/Ca^{2+} antiport (Bielefeld et al., 1986; Garcia et al., 1987). The inhibitory effect of dichlorobenzamil on endothelium-dependent relaxation may be caused by the inhibition of Ca^{2+} entry via channels that are

relatively insensitive to nifedipine or verapamil. Alternatively, dichlorobenzamil may have pharmacologic actions on endothelium-dependent relaxation that are unrelated to Ca^{2+} transport.

Decreasing $[Na^+]_o$ would be expected to decrease $[Na^+]_i$. A decrease in $[Na^+]_i$ is probably not involved in Ca^{2+}-mobilization evoked by decreasing $[Na^+]_o$ because loading the cells with Na^+ had no effect on the stimulation of $^{45}Ca^{2+}$ efflux produced by decreasing $[Na^+]_o$. Although choline was the usual substitute for external Na^+, replacing NaCl with NMG, K^+, or sucrose stimulated $^{45}Ca^{2+}$ efflux by the same extent as replacement with choline chloride (Table 1). Muallem et al. (1988) showed that choline evoked the release of stored Ca^{2+} in pancreatic acinar, paritetal, and peptic cells by stimulating cholinergic-muscarinic receptors. Because atropine had no effect on Ca^{2+}-mobilization produced by replacing Na^+ with choline (except in neuroblastoma cells, Fig. 17) and because Na^+ replacements with no structural resemblance to acetylcholine increased $^{45}Ca^{2+}$ efflux, cholinergic receptors are not involved in the response to decreasing $[Na^+]_o$.

Recently, Sasaguri and Watson (1988) reported that decreasing $[Na^+]_o$ to 20 mM by equimolar substitution with K^+ or sucrose increased [^{3}H] inositol phosphates about 2-fold after a 30 min incubation of chopped slices or dispersed cells from guinea pig ileum smooth muscle. The increase in [^{3}H] inositol phosphates evoked by decreasing $[Na^+]_o$ may be unrelated to the receptor we have reported because such a long

incubation in low $[Na^+]_o$ could significantly alter many transport and metabolic processes. Furthermore, Sasaguri and Watson (1988) did not identify the inositol phosphates produced nor the time course of their production. Thus it is not known if IP_3 was produced by decreasing $[Na^+]_o$. Moreover, Sasaguri and Watson (1988) did not examine the effect of decreasing $[Na^+]_o$ on cell Ca^{2+} regulation. Exposure to low $[Na^+]_o$ solution (choline substitution, with or without Ca^{2+}) increases $[Ca^{2+}]_i$ in cultured chick heart cells (Kim et al., 1987). One mM La^{3+} or EGTA prevented decreasing $[Na^+]_o$, but not caffeine, from increasing $[Ca^{2+}]_i$. Kim et al. (1987) suggested that La^{3+} or EGTA displaces cell surface bound Ca^{2+} and prevents Ca^{2+}-induced Ca^{2+} release. However, the potential role of other divalent cations (Mg^{2+}) or cholinergic receptors was not addressed, nor were changes in inositol phosphates or cell Ca^{2+} regulation measured. Thus, the mechanism of low $[Na^+]_o$-evoked increases in $[Ca^{2+}]_i$ in the chick heart cells and its relationship to the $[Na^+]_o$-sensitive receptor that we describe are unknown.

The physiological relevance of the response to decreasing $[Na^+]_o$ is uncertain because such a large decrease in $[Na^+]_o$ is required to appreciably mobilize cell Ca^{2+} (Fig. 9). Smaller changes in $[Na^+]_o$ might have physiological significance, however, under certain conditions. For example, Walsh-Reitz (1986) reported that decreasing $[Na^+]_o$ from 155 to 130 mM evoked the release of an autocrine polypeptide growth factor from monkey kidney epithelial cells; however, it is not known if decreasing $[Na^+]_o$ mobilizes Ca^{2+} in epithelial cells. In

the cell types we have found that respond to the three stimuli (Table 4), modest decreases in $[Na^+]_o$ might interact synergistically with hormones and neurotransmitters that release stored Ca^{2+}. However, this possibility has not yet been tested. Na^+ and other monovalent cations also appear to modulate receptor signaling by hormones and neurotransmitters, including dopamine (Watanabe et al., 1985), epinephrine (Michel et al., 1980; Aktories et al., 1979; Connolly and Limbird, 1983; Motulsky and Insel, 1983), opioids (Pert and Snyder, 1974; Wuster et al., 1984; Jauzac et al., 1986), and PGE_1 (Steer and Wood, 1981). However, these modulatory effects of Na^+ on hormone-receptor signaling are distinctly different from Ca^{2+} mobilization triggered by decreasing $[Na^+]_o$ in the absence of any other stimuli as described here. Some of the previously reported effects of $[Na^+]_o$ removal may be due to the release of sequestered Ca^{2+}. For example, Churchill and co-workers (1985) found that decreasing extracellular Na^+ inhibited renin secretion from juxtaglomerular cells by a mechanism not involving Na^+/Ca^{2+} exchange or cholinergic receptors. Increasing $[Ca^{2+}]_i$ is known to inhibit renin secretion (Churchill et al., 1985). Thus, decreasing $[Na^+]_o$ may inhibit renin secretion by releasing stored Ca^{2+} in the juxtaglomerular cell. The release of stored Ca^{2+} in response to decreasing $[Na^+]_o$ may be widespread in mammalian cells.

There appears to be no precedent for the release of stored Ca^{2+} by Cd^{2+} and the other stimulatory divalent metals. Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} are often used to investigate cell Ca^{2+} regulation (Smith et al., 1987; Cobbold and Rink,

1987; Merritt and Rink, 1987, Nilsson et al., 1987; Hirning et al., 1988; Verbost et al., 1989). In general, the metals block Ca^{2+} transport systems including Ca^{2+} channels, the Na^{+}/Ca^{2+} antiport system, and Ca^{2+}-ATPase (Smith et al., 1987; Merritt and Rink, 1987; Verbost et al., 1989). Nemeth and Scarpa (1987) reported evidence for a putative cell surface Ca^{2+} receptor in bovine parathyroid cells. They found that increasing the concentration of external Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} transiently increased $[Ca^{2+}]_i$. Similarly, Nilsson et al., (1987) observed that Ca^{2+}, Sr^{2+}, and Ba^{2+} transiently increased $[Ca^{2+}]_i$ in pancreatic β cells. Both groups suggested that inositol trisphosphate was the second messenger, although measurements of inositol polyphosphates were not made. At 120 mM $[Na^{+}]_o$, Ca^{2+} and Mg^{2+} are not agonists for the Cd^{2+} receptor that we describe because increasing external Ca^{2+} or Mg^{2+} up to 16 mM had no effect on cell Ca^{2+} (Smith et al., 1989a). Similarly, Sr^{2+} and Ba^{2+} at concentrations up to 4 mM had no effect on cell Ca^{2+} (Fig. 19; Smith et al., 1989a). Therefore, the putative Ca^{2+} receptor proposed by these two groups (Nemeth and Scarpa, 1987; Nilsson et al., 1987) is distinct from the Cd^{2+} receptor that we have described (Smith et al., 1989; Smith et al., 1989a; Smith et al., 1989b).

The finding that decreasing pH_o mobilizes stored Ca^{2+} also appears to be without precedent. Much effort has been focused on agents that both mobilize Ca^{2+} and alter intracellular pH (Hatori et al., 1987; Berk et al., 1987a; Berk et al., 1987b; Ives and Daniel, 1987). However, few studies regarding the relationship between pH_o and cell Ca^{2+} regulation have

appeared. Kim and Smith (1987) reported that changing pH_o from 7.4 to 6.0 decreased $^{45}Ca^{2+}$ uptake by 29% and increased $^{45}Ca^{2+}$ efflux by 17% from cultured chick embryo ventricular cells. The authors suggested that the shift in pH_o may affect Na^+/Ca^{2+} antiport activity and Ca^{2+} binding on the cell surface. Iijima et al. (1986) examined the effects of external pH on gating and permeation in Ca^{2+} channels with the whole-cell configuration of the patch-clamp technique. They concluded that protonation reduces the amplitude of the negative surface potential which is sensed by the gating mechanism. Drapeau and Nachshen (1988) examined the effects of lowering internal and/or external pH on Ca^{2+} regulation in synaptosomes. They found that changing internal pH to 5.8 or external pH to 5.5, which decreased internal pH to 6.4 in 30 sec, had no effect on $[Ca^{2+}]_i$ which was measured with fura-2. Changes in intracellular pH produce relatively small changes in $[Ca^{2+}]_i$ which are inconsistent with respect to the direction of the $[Ca^{2+}]_i$ change among different cell types (Moody, 1984). We found that changing pH_o to 6 decreased cell pH to only about 7 (Fig. 7) and that moderate decreases in intracellular pH had no effect on $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ efflux in the fibroblasts (Smith et al., 1989b) and endothelial cells (Dwyer, Zhuang, and Smith, submitted).

In our studies, decreasing intracellular pH failed to mobilize cell Ca^{2+}. Therefore, acidifying the extracellular medium apparently triggers Ca^{2+} mobilization by protonating a functional group, possibly imidizolium, in a cell-surface protein. The imidizole group (pK_a 6 to 7) of histidine is

the most common protein functional group with a pK_a near the pH_o (6.5) which half-maximally induced Ca^{2+} mobilization.

Cell-surface receptors that mediate the endocytosis of specific macromolecules cycle continuously between the plasma membrane and intracellular organelles (Goldstein et al., 1985). After internalization, the receptors encounter mild acidity (pH 5.0 to 6.5) in endocytic vesicles and lysosomes (Yamashiro et al., 1984). The low pH of the endocytic compartments usually causes the macromolecule to dissociate from the receptor, which is essential for receptor sorting (Brown, et al., 1983). DiPaola and Maxfield (1984) observed that mild acidity induces conformational changes in the receptor for epidermal growth factor in A431 cells and in the purified asialoglycoprotein receptor reconstituted in liposomes. Turkewitz et al. (1988) showed recently that a soluble fragment of the transferrin receptor that contains 95% of its external domain undergoes a reversible conformational transition and self-association below pH 6. Lowering pH_o may trigger inositol polyphosphate production by inducing a conformational change in the ectodomain of a cell-surface protein that normally encounters low pH only after endocytosis.

Decreasing $[Na^+]_o$, pH_o, or adding Cd^{2+} maximally releases stored Ca^{2+} similarly to bradykinin in the fibroblasts, or ATP in the endothelial cells. In contrast, in the neuroblastoma cells, the maximal effect of acetylcholine on the stimulation of $^{45}Ca^{2+}$ efflux is at least 2-fold greater than the maximal effect of the three unusual stimuli based on the first-order

rate constants. There are at least two possible explanations for this observation. First, the neuroblastoma cells may express fewer Cd^{2+} receptors than are necessary to maximally stimulate inositol phosphate production. Cantau et al. (1988) report an analogous observation on vasopressin receptors in WRK1 cells. This group demonstrated that a decrease in the number of vasopressin receptors due to receptor internalization correlated well with a decrease in vasopressin-stimulated [^{3}H]inositol phosphates. An increase in the number of vasopressin receptors likewise correlated with an increase in vasopressin-stimulated [^{3}H]inositol phosphates (Cantau et al., 1988). Second, the Cd^{2+} receptor may be less efficiently coupled to its G-protein. Vasopressin receptors from rat pulmonary artery smooth muscle spontaneously couple to their G-protein which increases the affinity for vasopressin (Bielinski et al., 1988). In contrast, rat liver vasopressin receptors require agonist binding to couple with G-proteins (Bielinski et al., 1988). The neuroblastoma cell acetylcholine receptor may be more efficiently coupled to its G-protein than is the Cd^{2+} receptor. However, it seems more likely that fewer receptors, not less efficient coupling to G-proteins, explains our observations. Increasing agonist concentration might overcome less efficient coupling and a maximal effect would be observed. However, fewer receptors would limit the maximal effect of an agonist regardless of its concentration.

The loss of cell Ca^{2+} following decreasing $[Na^+]_o$, decreasing pH_o, or adding Cd^{2+} is remarkably large and fast:

about 2 nmol/30 sec per mg cell protein (about 2 x 10^6 cells). Presumably the rise in $[Ca^{2+}]_i$ activates the plasma membrane Ca^{2+} pump which removes Ca^{2+} from the cytoplasm. About 200,000 pumps per cell would be required to expel Ca^{2+} at this rate assuming a turnover number of 50 ATP/pump/sec and a stoichiometry of 2 Ca^{2+}/ATP (Rega and Garrahan, 1985). If the cells contain 5 to 10 mM ATP, the expulsion of this amount of Ca^{2+} would require only a few percent of the cellular ATP. Thus, it is reasonable to suggest that the plasma membrane Ca^{2+} ATPase drives the rapid net exodus of Ca^{2+} after its release from the intracellular compartment by IP_3.

The function of the Cd^{2+} receptor is not known. It may normally be a hormone receptor. For example, in washed rabbit platelets, Zn^{2+} is a potent inhibitor of platelet activating factor-induced platelet aggregation and serotonin secretion (Huo et al., 1988). Kramhoft et al. (1988) showed that Cu^{2+} induces cytoplasmic alkalinization by activating Na^+/H^+ exchange in Ehrlich ascites tumor cells. Many hormones are known to produce the same effects in a number of cell types (Grinstein and Rothstein, 1986). However, it is not known if Cu^{2+} acts directly on the exchanger or via a separate receptor (Kramhoft et al., 1988). Alternatively, the receptor might normally interact with Fe^{2+} or other essential metal. Cd^{2+} causes iron deficiency anemia by competing with Fe^{2+} for the iron transport system in the intestines (Sakata et al., 1988). The Cd^{2+} receptor might, on the other hand, function as a type of stress receptor; it may participate in cellular handling of toxic compounds such as heavy metals. Additionally, at

sites of infection, abscess, or ischemia, pH is known to drop precipitously; a decrease in pH_o may be a signal to initiate the wound healing process. Cerebral and cardiac ischemia also cause tissue pH to decrease. Huang and Sun (1986) have reported that cerebral ischemia evokes a rapid decrease in rat brain PIP_2 and concomitant increase in DAG. It is possible that the decrease in tissue pH may have triggered the PI second messenger system.

While this study has characterized in detail the effects of Cd^{2+} or certain other metals, decreasing $[Na^+]_o$, or decreasing pH_o on cell Ca^{2+} regulation, many questions remain unanswered. Identification of the receptor and definite proof that a single receptor mediates the effects of all three stimuli is lacking. This will require receptor purification and reconstitution or gene cloning and expression. This study suggests that all three stimuli activate the PI second messenger system; to demonstrate that this is indeed true will require measurements of changes in cellular PIP_2 levels, changes in DAG levels, and changes in protein kinase C activity. The effects of the three stimuli on other second messenger systems is also not known; therefore, it could prove fruitful to investigate alterations in cellular cyclic AMP and cyclic GMP levels.

Elucidation of the amino acid sequence of the Cd^{2+} receptor may lead to the identification of a novel receptor. Similarly, a search for the endogenous agonist could reveal a new hormone. Further study could also result in a better

understanding of pathological events associated with infection, ischemia, or exposure to toxic metals.

Figure 1. Effect of decreasing $[Na^+]_o$ on $[Ca^{2+}]_i$. PSS was removed and replaced with PSS at the first arrow and with choline-PSS at the $-Na^+_o$ arrow. A, fluorescence intensity minus autofluorescence at each excitation wavelength: 345 nm in the upper tracing and 380 nm in the lower tracing. B, the ratio of fluorescence at the two wavelengths, which was converted to $[Ca^{2+}]_i$, shown in panel C, as described by Grynkiewicz et al. (1985). $[Ca^{2+}]_i$ measurements were made by Tao Zheng.

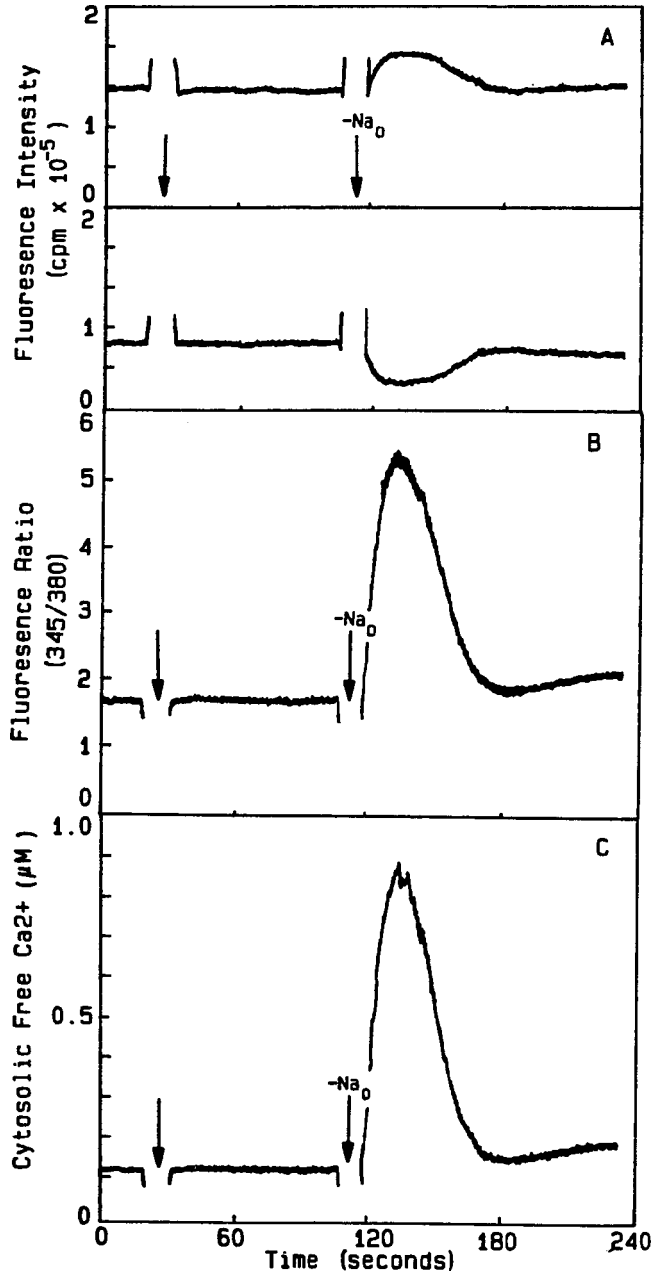

A
B
C
Fluoresence Intensity (cpm x 10^{-5})
Fluoresence Ratio (345/380)
Cytosolic Free Ca2+ (μM)
$-Na_o$
Time (seconds)
0
60
120
180
240

Figure 2. Extracellular Na^+ dependence of the peak increase in $[Ca^{2+}]_i$. The experiment was done as described for Fig. 1. The sum of the choline chloride and NaCl concentrations always equaled 120 mM. Values are means ± SE with the number of replicate cover glasses in parentheses. $[Ca^{2+}]_i$ measurements were made by Tao Zheng.

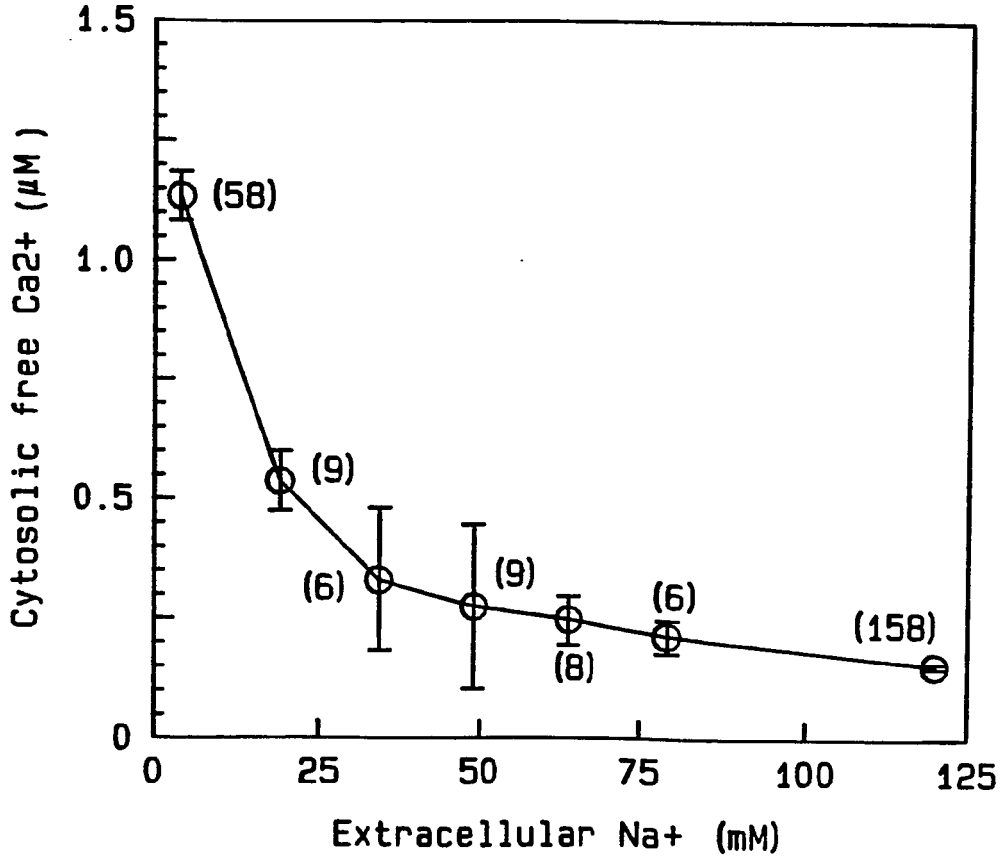

1.5
1.0
0.5
0
Cytosolic free Ca2+ (μM)
(58)
(9)
(6)
(9)
(8)
(6)
(158)
0
25
50
75
100
125
Extracellular Na+ (mM)

Figure 3. Effects of prior addition of bradykinin or A23187 on the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$. PSS was removed and replaced at the first arrow in each panel. In A, 100 nM bradykinin (BK) was added before removing Na^+_o. In B, 100 nM bradykinin was added after eliciting a response to removing Na^+_o. In C, 3 μM A23187 was followed by 100 nM bradykinin and Na^+_o removal. $[Ca^{2+}]_i$ measurements were made by Tao Zheng.

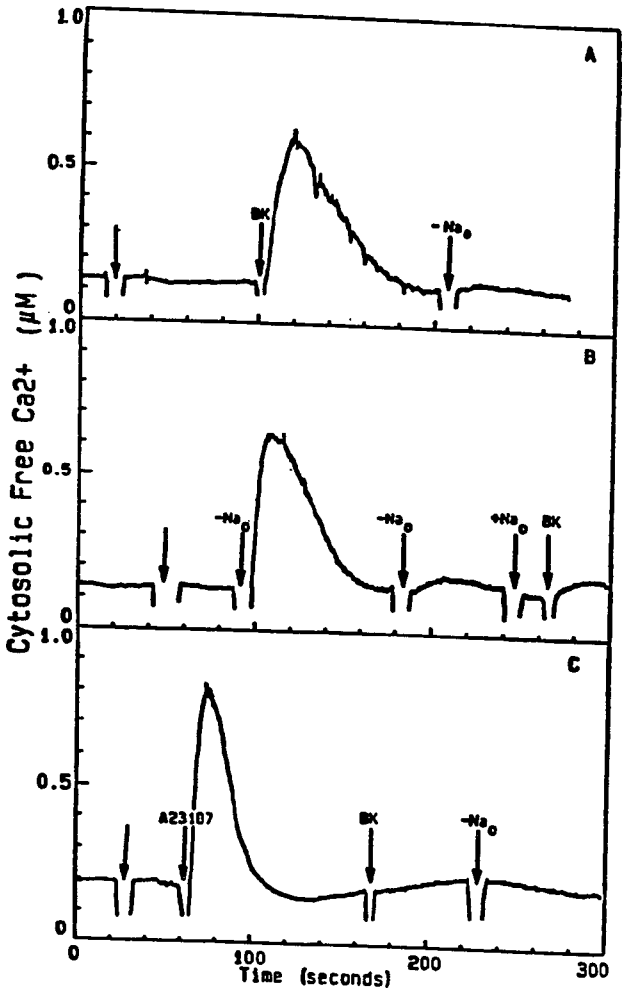
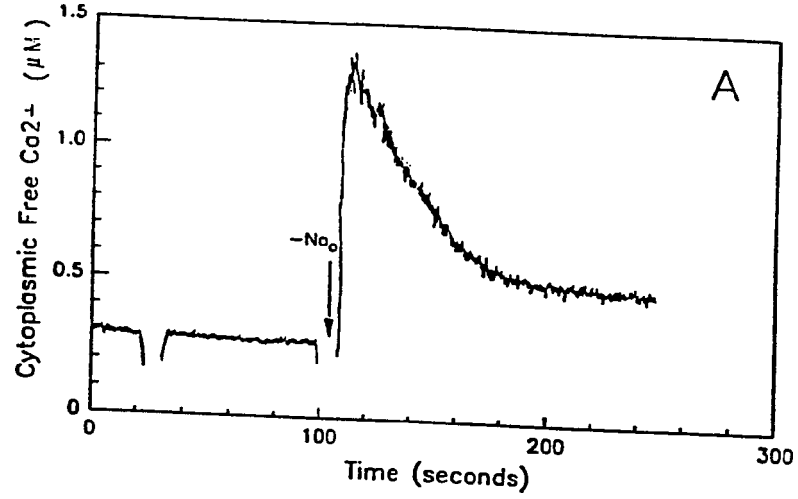

1.0
0.5
0
A
BK
-Na$_o$
1.0
0.5
0
B
-Na$_o$
-Na$_o$
+Na$_o$
BK
1.0
0.5
0
C
A23187
BK
-Na$_o$
Cytosolic Free Ca2+ (μM)
0
100
200
300
Time (seconds)

Figure 4. Effect of removing external Na^+ and adding ATP on $[Ca^{2+}]_i$ in the coronary endothelial cells. A, At the first interruption in the tracing, PSS was removed and replaced. At the $-Na^+_o$ arrow, PSS was removed and replaced with choline PSS. B, At the first two arrow, ATP (100 μM) was added to the cuvette which contained 1 ml PSS. At the third arrow, the solution in the cuvette was replaced with PSS, and at the $-Na^+_o$ arrow, choline PSS was exchanged for PSS. All solutions contained 10 mM glucose. The values of R_{min}, R_{max}, and Sf_2/Sb_2 were 0.6, 9.1, and 5.3, respectively. $[Ca^{2+}]_i$ measurements were made by Tao Zheng.


A
Cytoplasmic Free Ca2+ (μM)
1.5
1.0
0.5
0
$-Na_o$
0
100
200
300
Time (seconds)

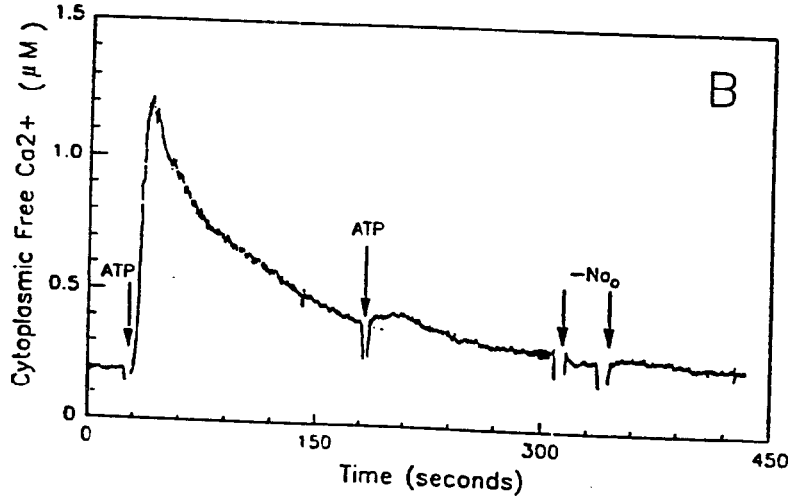

B
Cytoplasmic Free Ca2+ (μM)
1.5
1.0
0.5
0
ATP
ATP
$-Na_o$
0
150
300
450
Time (seconds)

Figure 5. Dependence of $[Ca^{2+}]_i$ on $[Na^+]_o$ in coronary endothelial cells. Peak values of $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$ were measured as indicated in Fig. 4. The sum of the Na^+ and choline concentrations was always 120 mM. Values are means ± SE (n). Error bars are shown when SE was larger than the marker. The apparent $K_{1/2}$ was determined from the peak $[Ca^{2+}]_i$ using dose-effect analysis software (J. Chou and T.-C. Chou, Elsevier_BIOSOFT, Amsterdam). $[Ca^{2+}]_i$ measurements were made by Tao Zheng.

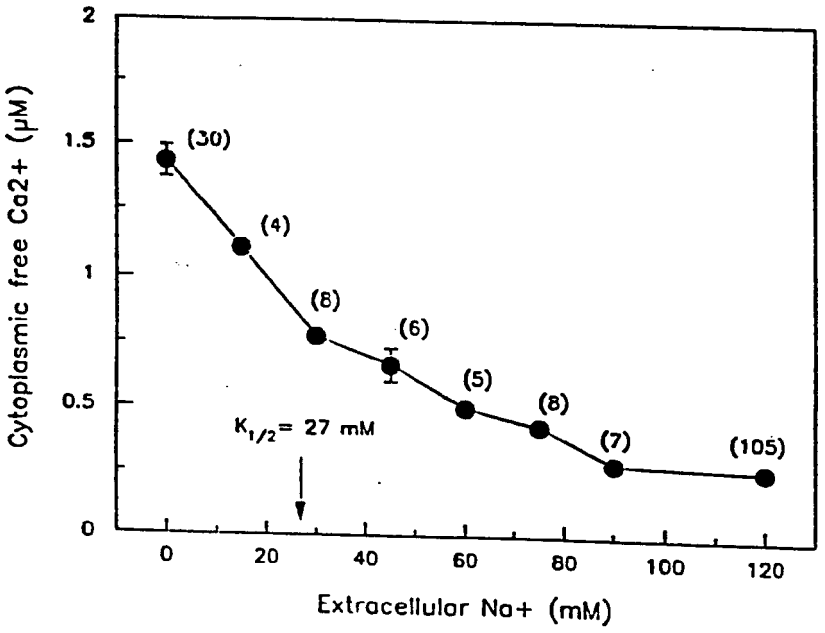
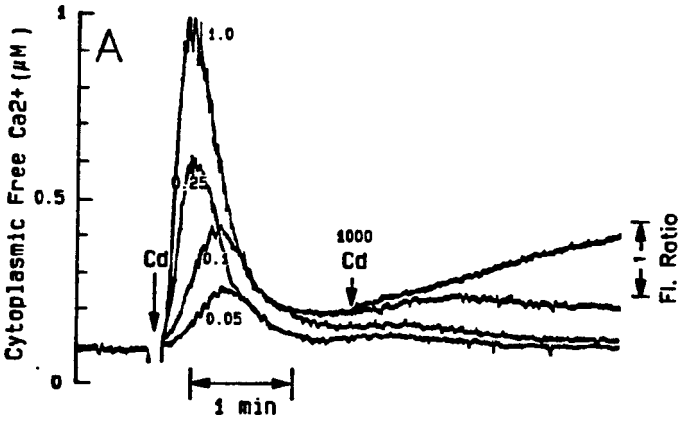
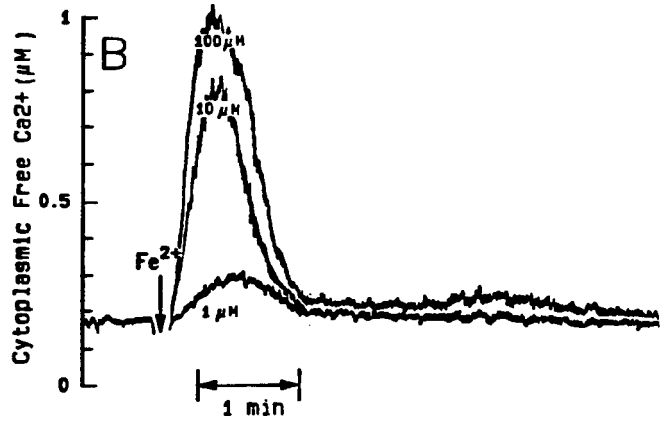

Cytoplasmic free Ca2+ (μM)
2
1.5
1
0.5
0
(30)
(4)
(8)
(6)
(5)
(8)
(7)
(105)
$K_{1/2}$= 27 mM
0
20
40
60
80
100
120
Extracellular Na+ (mM)

Figure 6. Increases in $[Ca^{2+}]_i$ evoked by Cd^{2+} or Fe^{2+} in human skin fibroblasts. $[Ca^{2+}]_i$ was measured in monolayers of fibroblasts that were loaded with fura-2. Each tracing is from a separate cover glass of cells treated with the indicated concentration of Cd^{2+} or Fe^{2+} in PSS. The tracings are representative of three experiments done in duplicate. C, the excitation spectra of 0.1 μM fura-2 in a solution containing 120 mM NaCl, 5 mM KCl, and 20 mM HEPES-Tris, pH 7.4. Divalent metals were removed by passing the solution through a column containing Chelex 100 (Bio-Rad). The solution also contained 0.1 mM Cd^{2+} or Ca^{2+} or 0.5 mM EGTA (No Ca^{2+} or Cd^{2+}). Fluorescence was measured at 510 nm. Bandwidths for excitation and emission were 5 and 14 nm, respectively. $[Ca^{2+}]_i$ were made by Tao Zheng and Robert Lyu.


A
Cytoplasmic Free Ca2+ (μM)
1
0.5
0
1.0
0.25
0.1
0.05
Cd
1000
Cd
Fl. Ratio
1 min


B
Cytoplasmic Free Ca2+ (μM)
1
0.5
0
100 μM
10 μM
Fe2+
1 μM
1 min

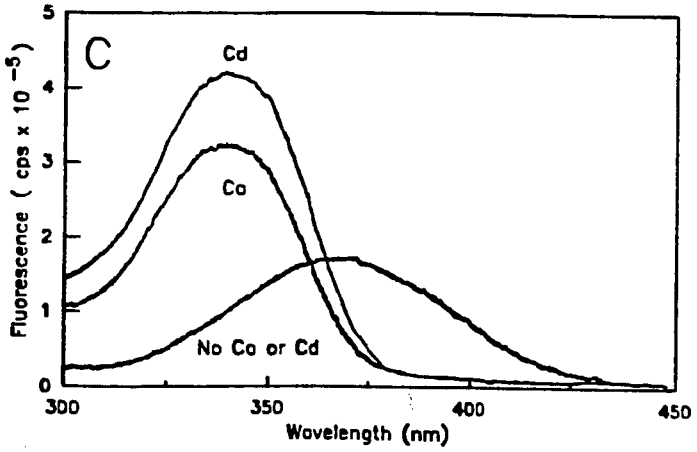
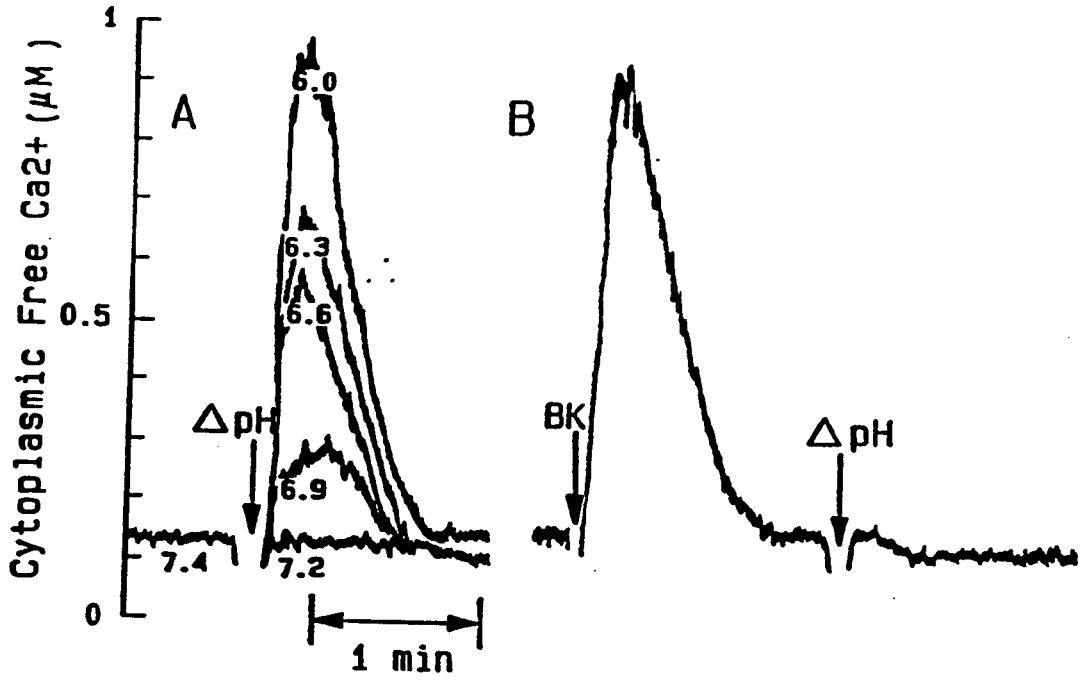

C
Fluorescence (cps x 10 -5)
5
4
3
2
1
0
Cd
Co
No Co or Cd
300
350
400
450
Wavelength (nm)

Figure 7. Effect of decreasing pH_o on $[Ca^{2+}]_i$ and pH_i in human skin fibroblasts. A, pH_o was shifted at the "delta" pH arrow by removing pH 7.4 PIPES PSS containing 10 mM glucose and replacing it with PIPES PSS containing glucose at the indicated pH. A separate cover glass was used for each pH value. B, bradykinin (100 nM) was added at the BK arrow, and pH_o was decreased to 6.1 at the "delta" pH arrow. C, the extracellular medium was removed and replaced each time the tracing of the ratio of BCECF fluorescence at 500 to 440 nm was interrupted. At the first interruption, PSS was removed and replaced; at the second interruption, PSS was replaced with pH 6.0 PIPES PSS. From the point indicated by $pH_i = pH_o$, the extracellular medium was high K^+ PSS containing 10 μg/ml nigericin at the pH indicated. Fluorescence at 440 nm was 200,000 counts/sec and changed by <10%. The tracings are representative of three independent experiments on duplicate cover glasses. $[Ca^{2+}]_i$ and pH_i measurements were made by Tao Zheng and Robert Lyu.


Cytoplasmic Free Ca2+ (μM)
1
0.5
0
A
B
6.0
6.3
6.6
6.9
7.2
7.4
ΔpH
BK
ΔpH
1 min

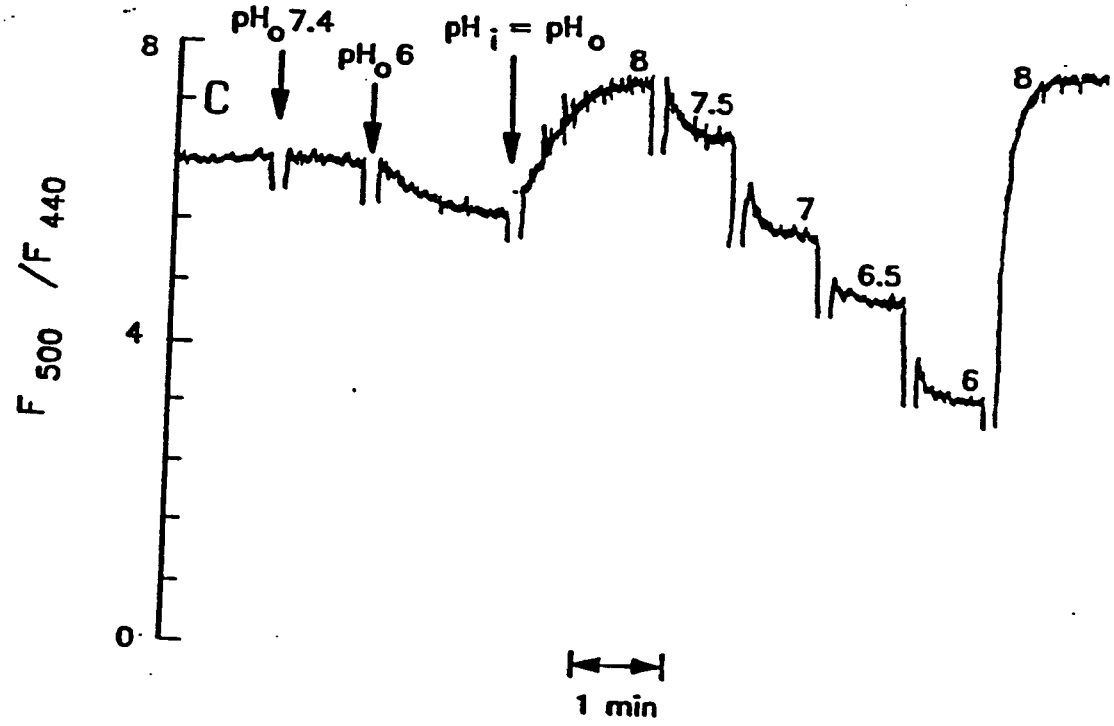

F 500 /F 440
8
4
0
C
pH o 7.4
pH o 6
pH i = pH o
8
7.5
7
6.5
6
8
1 min

Figure 8. Effect of decreasing pH_o on $[Ca^{2+}]_i$ in coronary endothelial cells in the presence and absence of extracellular Ca^{2+}. The tracings are representative of seven cover glasses in the presence of external Ca^{2+} and five in the absence of Ca^{2+}. The Ca^{2+}-free PIPES PSS contained 0.1 mM EGTA and no added Ca^{2+}. $[Ca^{2+}]_i$ were made by Dr. Jeff Smith.

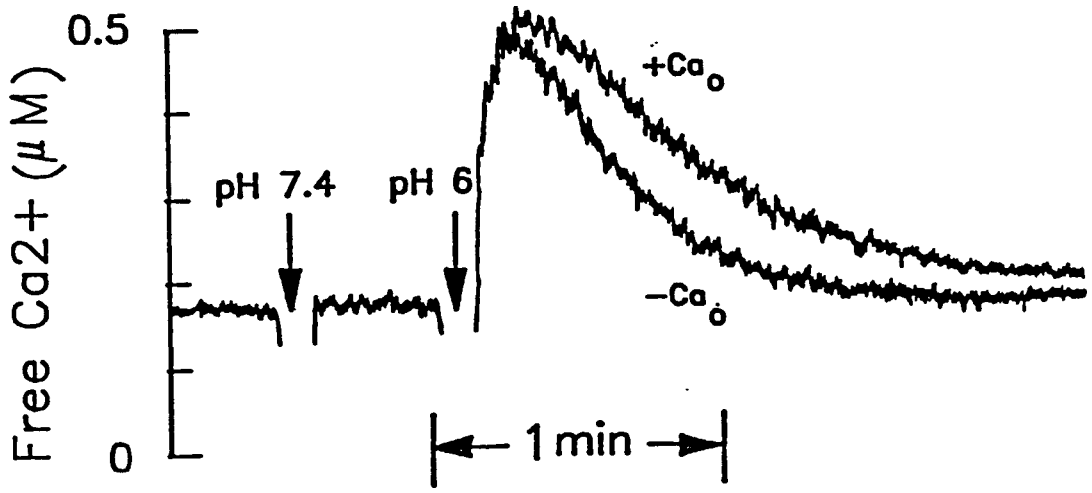

0.5
Free Ca2+ (μM)
0
pH 7.4
pH 6
+Ca$_o$
−Ca$_o$
1 min

Figure 9. Effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ efflux in human skin fibroblasts. The cultures were labeled with 20 μCi of $^{45}Ca^{2+}$ in 1 ml of PSS containing 10 mM glucose for 2 hr. The cultures were rinsed with PSS containing the indicated Na^+ concentration to start the efflux. The sum of the NaCl and choline chloride concentrations always equaled 120 mM. Values are the means of four experiments.

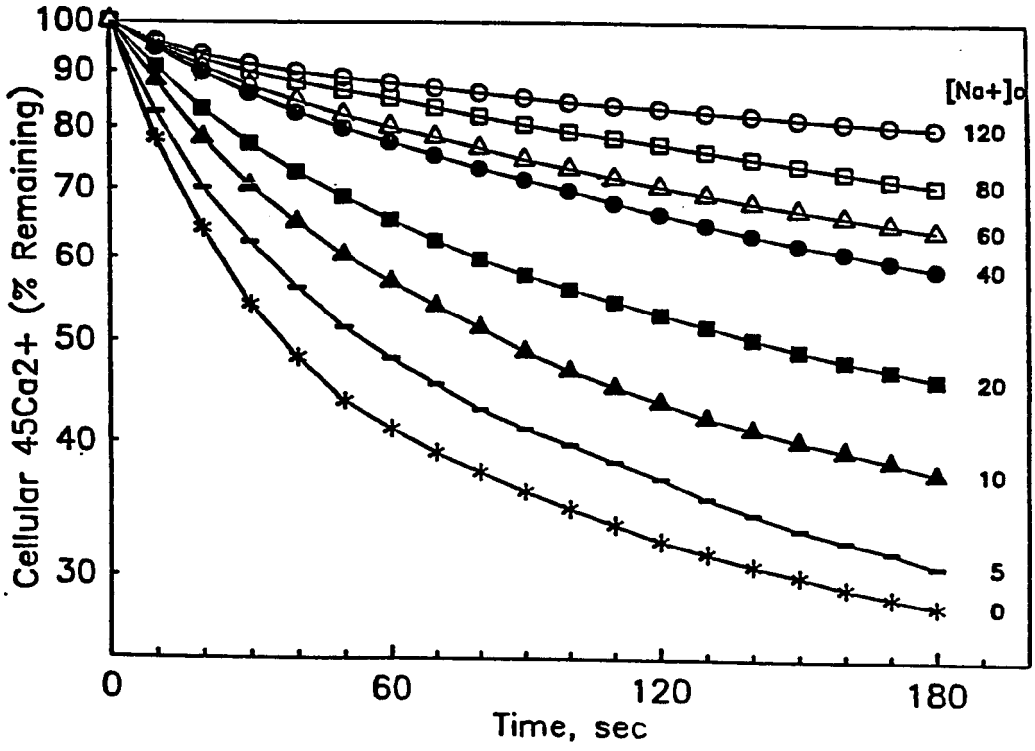

100
90
80
70
60
50
40
30
Cellular 45Ca2+ (% Remaining)
0
60
120
180
Time, sec
[Na+]o
120
80
60
40
20
10
5
0

Figure 10. Effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ efflux in the absence of external Ca^{2+} in human skin fibroblasts. To start efflux, cultures were rinsed 8 times with PSS (unfilled circles), PSS containing 0.1 mM EGTA and no added Ca^{2+} (unfilled squares, and filled triangles), choline PSS (filled circles), or choline PSS containing 0.1 mM EGTA and no added Ca^{2+} (filled squares). Efflux was assayed from 0 to 180 sec in PSS (unfilled circles), PSS containing 0.1 mM EGTA and no added Ca^{2+} (unfilled squares), choline PSS (filled circles), or in PSS containing 0.1 mM EGTA and no added Ca^{2+} from 0 to 60 sec and then switched to choline PSS containing 0.1 mM EGTA and no added Ca^{2+} (filled triangles). Data are from one of two experiments that gave similar results.

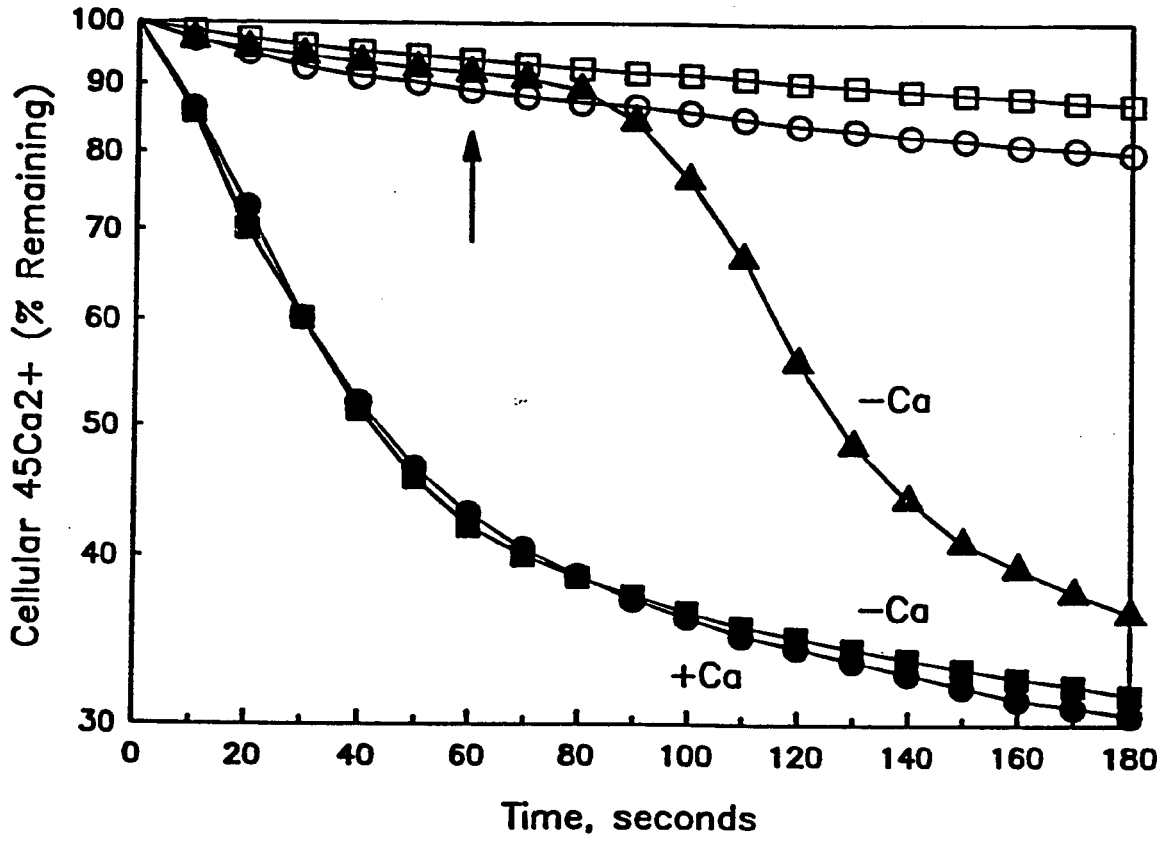

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
0
20
40
60
80
100
120
140
160
180
Time, seconds
−Ca
−Ca
+Ca

Figure 11. Comparison of Bradykinin-evoked $^{45}Ca^{2+}$ efflux and that produced by decreasing $[Na^+]_o$. Efflux was assayed in PSS, or initiated in PSS and switched to choline PSS at 60 sec (filled circles), or 160 sec (filled squares). Bradykinin (100 nM) was added at 60 sec (unfilled squares) or 160 sec (unfilled triangles). Bradykinin was present from the time of addition to the end of the assay. Values are means of duplicates. Another experiment gave similar results.

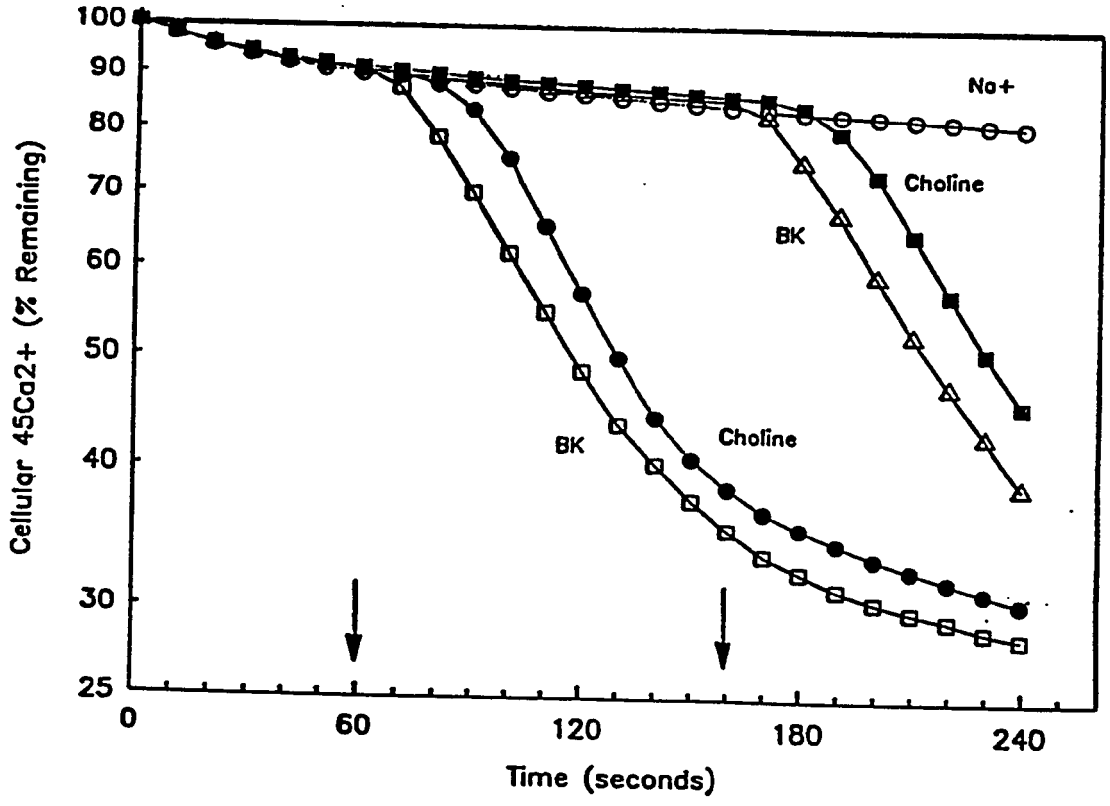

100
90
80
70
60
50
40
30
25
0
60
120
180
240
Time (seconds)
Cellular 45Ca2+ (% Remaining)
Na+
Choline
BK
BK
Choline

Figure 12. Effect of histamine on $^{45}Ca^{2+}$ efflux in human skin fibroblasts. Efflux was assayed in PSS from 0 to 60 sec. At 60 sec (arrow), the indicated concentration of histamine (μM) was added in PSS and was present until the end of the experiment.

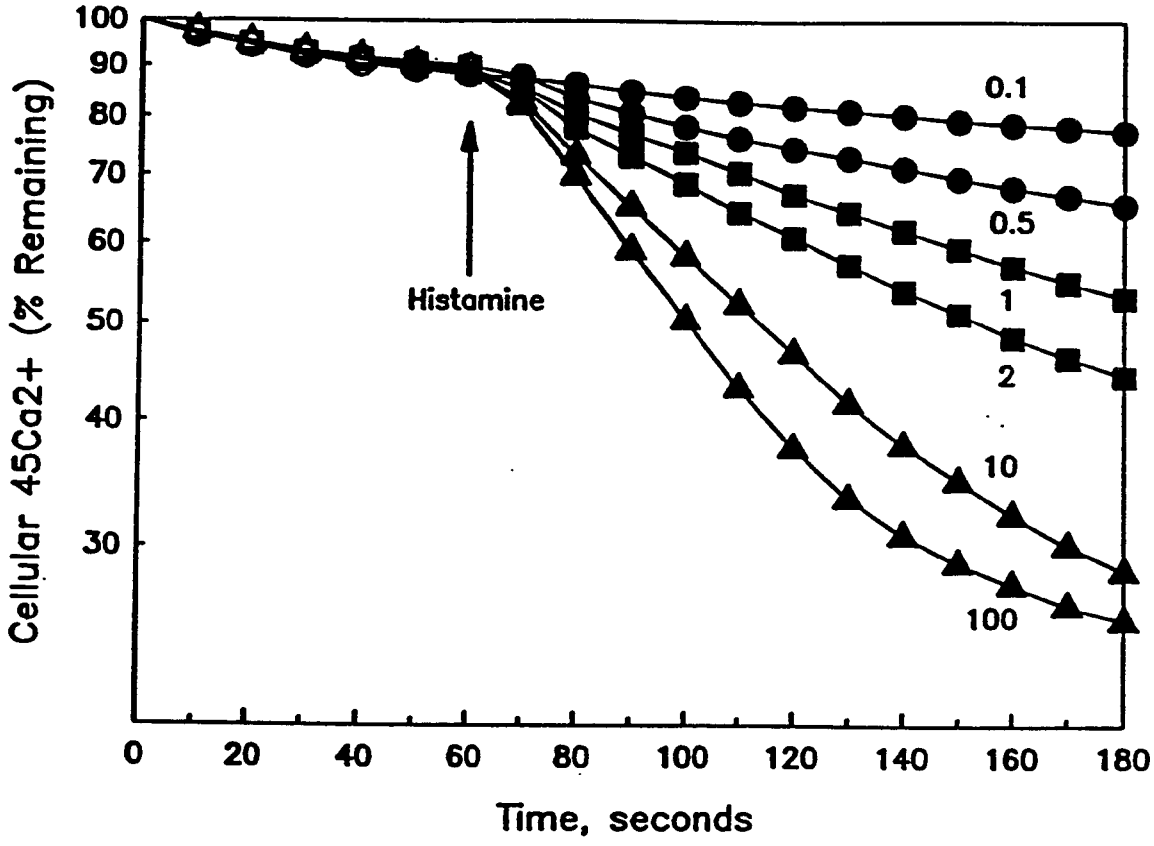
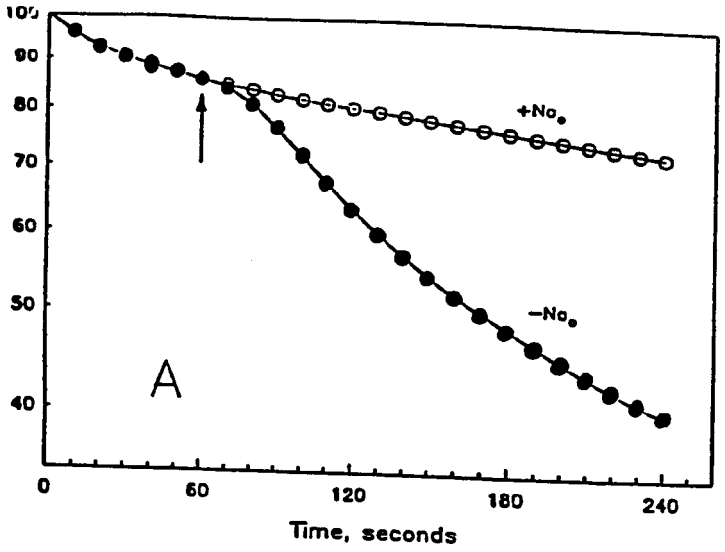

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
0
20
40
60
80
100
120
140
160
180
Time, seconds
Histamine
0.1
0.5
1
2
10
100

Figure 13. Effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ efflux in coronary endothelial cells. A, efflux from some cultures (unfilled circles) was assayed in PSS from 0 to 180 sec. Efflux from other cultures (filled circles) was started in PSS (0 to 60 sec) and switched to choline PSS from 60 to 180 sec. Data are representative of more than 10 experiments. B, efflux was started by rinsing the cultures with the solution used to assay efflux: PSS (unfilled circles), sucrose PSS (triangles), NMG PSS (squares), or choline PSS (filled circles). Two additional experiments gave similar results.


100
90
80
70
60
50
40
+Na
-Na
A
0
60
120
180
240
Time, seconds

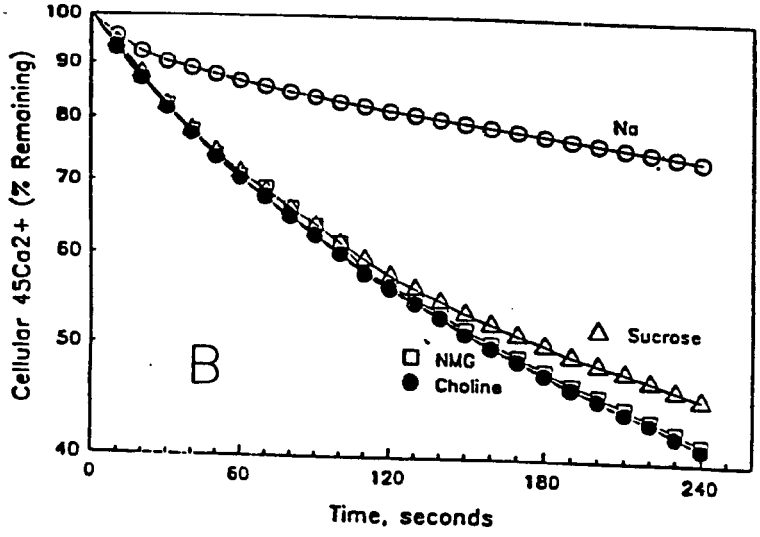

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
Na
Sucrose
NMG
Choline
B
0
60
120
180
240
Time, seconds

Figure 14. Effect of decreasing $[Na^+]_o$ or adding acetylcholine on $^{45}Ca^{2+}$ efflux in neuroblastoma cells. Cultures were rinsed 8 times with PSS, and $^{45}Ca^{2+}$ efflux was assayed with (filled symbols) or without (unfilled symbols) 20 μM atropine. At 60 sec (arrow), 10 μM acetylcholine was added (squares), the efflux buffer was changed to choline PSS (triangles) or NMG-PSS (half-filled circles), or efflux was maintained in PSS (control, unfilled circles). Data represent two experiments with choline PSS and three with acetylcholine that gave similar results.

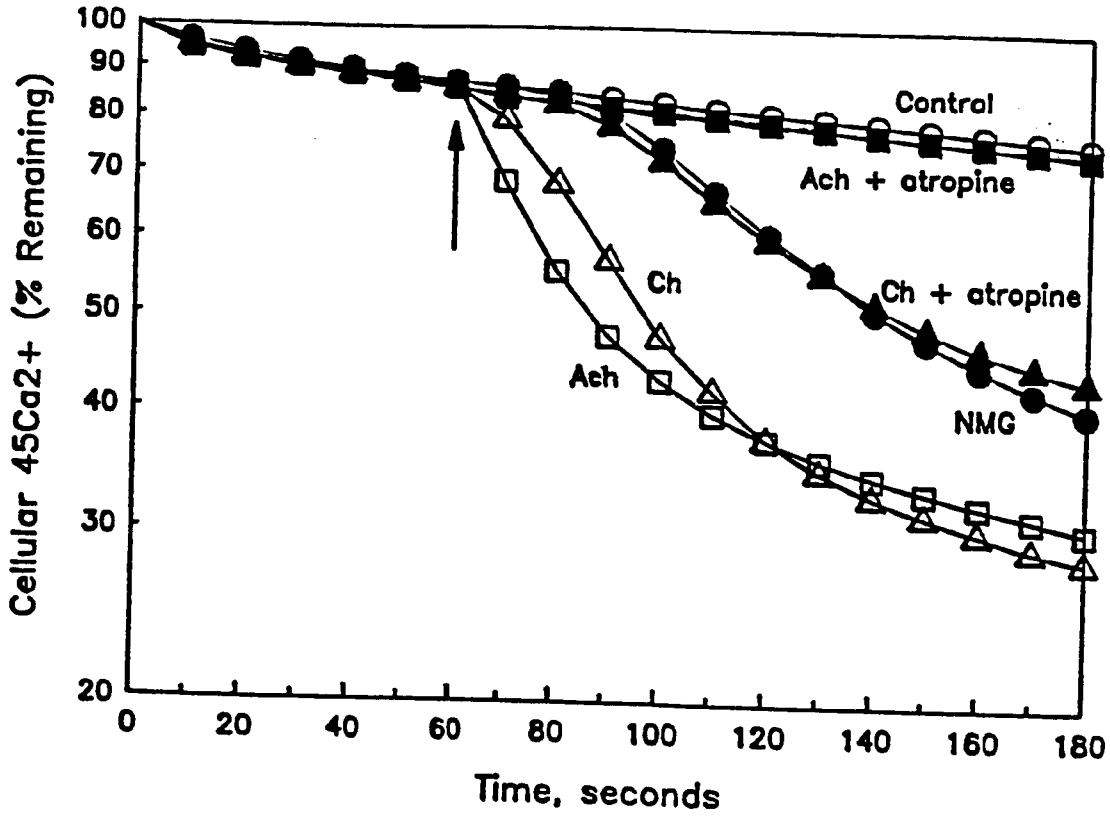

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
180
Time, seconds
Control
Ach + atropine
Ch
Ch + atropine
Ach
NMG

Figure 15. Effect of replacing external Na^+ with NMG, K^+, or choline on $^{45}Ca^{2+}$ efflux in human umbilical artery smooth muscle cells (HUA). Cultures were labeled for 2 hr with $^{45}Ca^{2+}$ in PSS containing 10 mM glucose and then rinsed 8 times with PSS to start efflux. From 0 to 60 sec, efflux was assayed in PSS. At 60 sec (arrow), PSS was switched to NMG-PSS (filled circles), K^+-PSS (filled triangles), or choline-PSS (filled squares) until the end of the experiment. Control cultures (unfilled circles) were maintained in PSS from 0 to 180 sec.

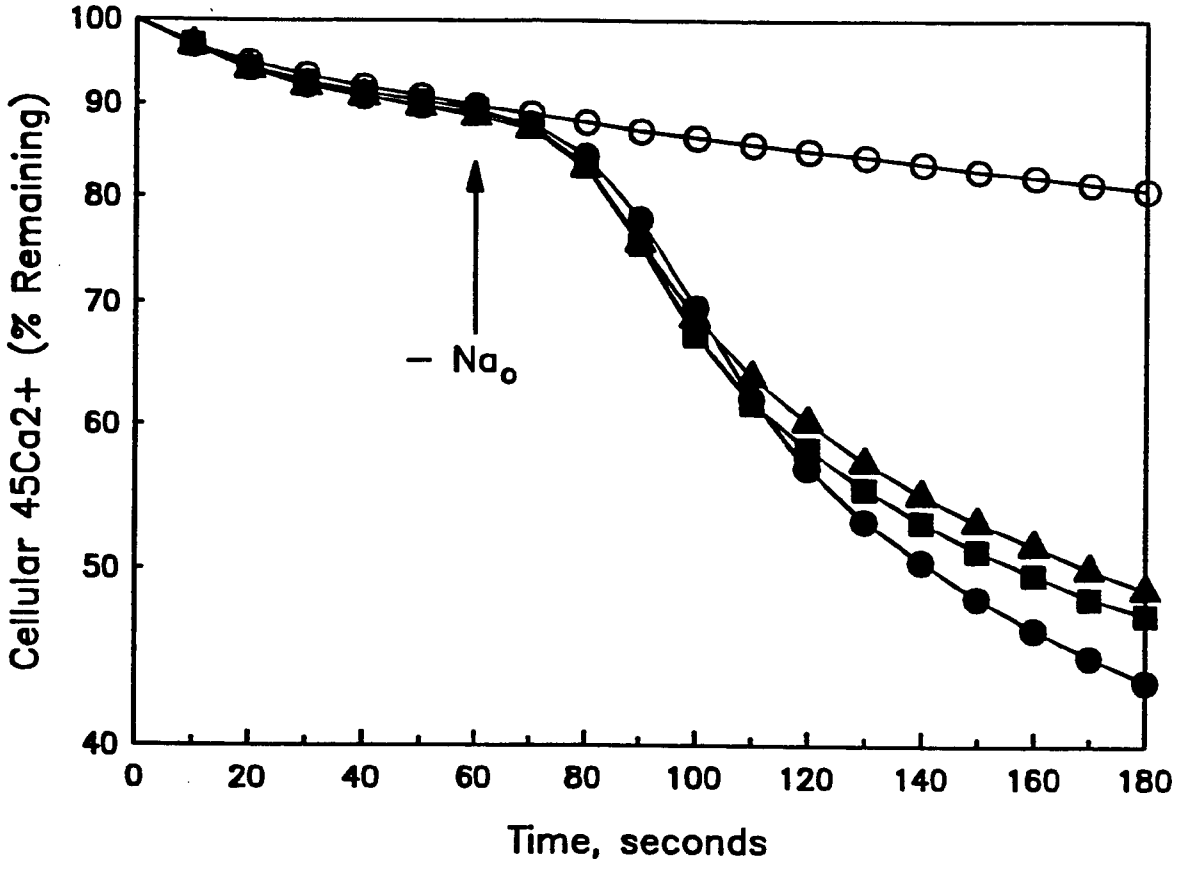
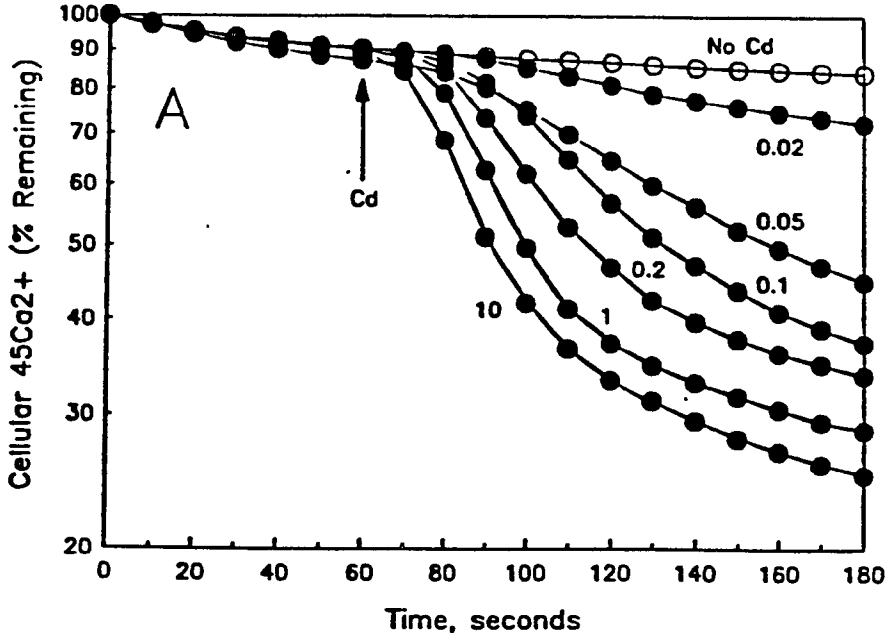

100
90
80
70
60
50
40
Cellular 45Ca2+ (% Remaining)
0
20
40
60
80
100
120
140
160
180
Time, seconds
— Na$_o$

Figure 16. Effect of Cd^{2+} and certain other divalent metals on $^{45}Ca^{2+}$ efflux in human skin fibroblasts. A and B, increases in $^{45}Ca^{2+}$ efflux produced by Cd^{2+} and other divalent metals. The chloride salt (except $FeSO_4$) at the indicated concentration (μM) was added 60 sec after starting efflux in PSS and was present until the end of the experiment. The concentration of Cd^{2+} that half-maximally increased the initial first-order rate constant of efflux was 0.11 μM (regression coefficient = 0.9847) which was determined as described by Chou (1976) with software from Elsevier_BIOSOFT (Amsterdam).


A
Cd
No Cd
0.02
0.05
0.2
0.1
10
1
Cellular 45Ca2+ (% Remaining)
Time, seconds

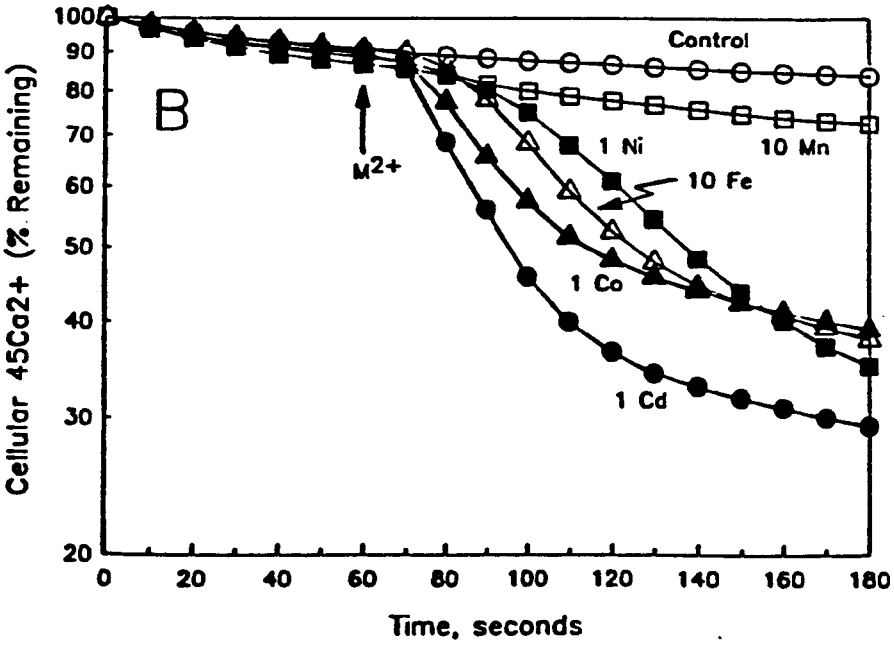

B
M2+
Control
1 Ni
10 Mn
10 Fe
1 Co
1 Cd
Cellular 45Ca2+ (% Remaining)
Time, seconds

Figure 17. Comparison of Fe^{2+}- and Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux and the inhibition of Fe^{2+}-stimulated $^{45}Ca^{2+}$ efflux by Zn^{2+} in human skin fibroblasts. Efflux was assayed in PSS from 0 to 60 sec. At 60 sec (arrow), 10 μM Cd^{2+} or the indicated concentration of Fe^{2+} (μM) was added and was present until the end of the experiment. Where indicated, 100 μM Zn^{2+} was present from 0 to 180 sec.

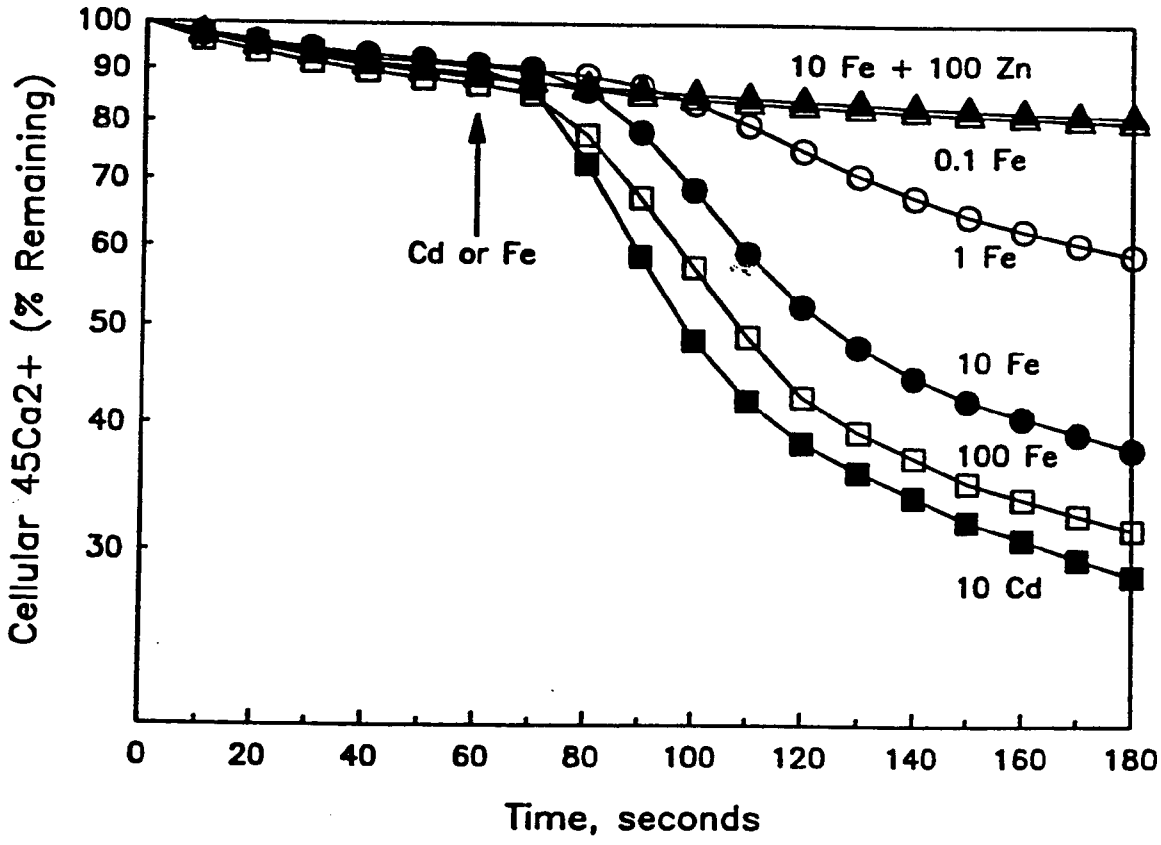
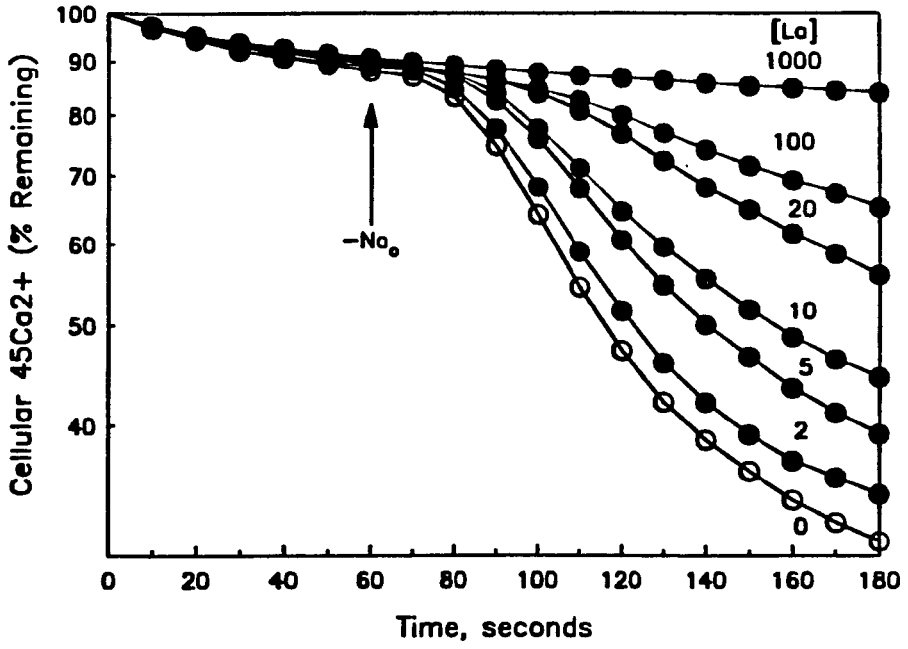

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
0
20
40
60
80
100
120
140
160
180
Time, seconds
Cd or Fe
10 Fe + 100 Zn
0.1 Fe
1 Fe
10 Fe
100 Fe
10 Cd

Figure 18. Effect of certain trivalent metals on $^{45}Ca^{2+}$ efflux stimulated by decreasing $[Na^+]_o$ in human skin fibroblasts. A, La^{3+} was present from 0 to 180 sec at the indicated concentrations. At 60 sec ($-Na^+_o$ arrow), PSS was replaced with choline PSS containing the indicated La^{3+} concentrations until the end of the experiment. A similar concentration-response profile was obtained with Cd^{2+} (0.5 μM). B, Sm^{3+}, Gd^{3+}, Tb^{3+}, or La^{3+} were present (1 μM) from 0 to 180 sec. At 60 sec ($-Na^+_o$ arrow), PSS was replaced with choline-PSS containing the indicated trivalent metal at 1 μM until the end of the experiment.


[La]
1000
100
20
10
5
2
0
$-Na_o$
Cellular 45Ca2+ (% Remaining)
Time, seconds

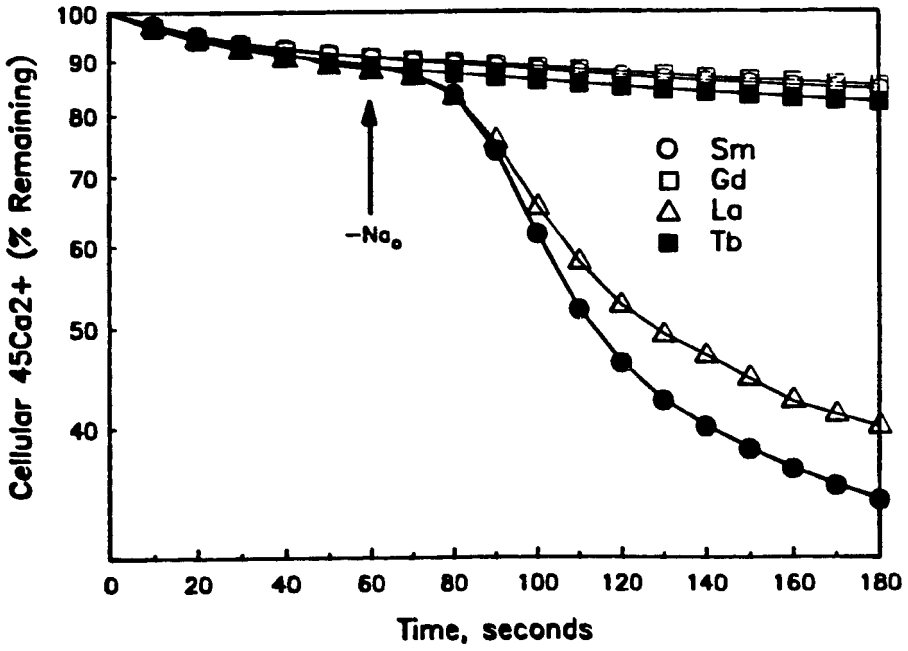
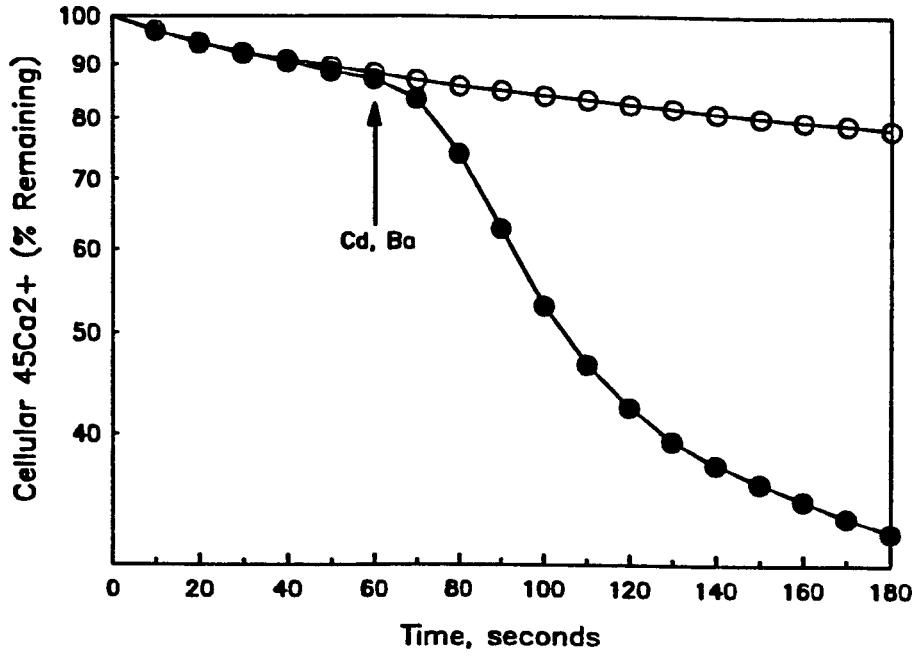

Sm
Gd
La
Tb
$-Na_o$
Cellular 45Ca2+ (% Remaining)
Time, seconds

Figure 19. Ba^{2+} and Sr^{2+} do not stimulate $^{45}Ca^{2+}$ efflux in human skin fibroblasts. Efflux was assayed in PSS from 0 to 180 sec. At 60 sec (Cd, Ba arrow, top panel; Cd, Sr arrow, bottom panel), either 1 μM Cd^{2+} (filled circles) or 4 mM Ba^{2+} or Sr^{2+} was added and was present until the end of the experiment. One and 2 mM Ba^{2+} or Sr^{2+} were also tested and had no effect.


100
90
80
70
60
50
40
Cellular 45Ca2+ (% Remaining)
Cd, Ba
0
20
40
60
80
100
120
140
160
180
Time, seconds

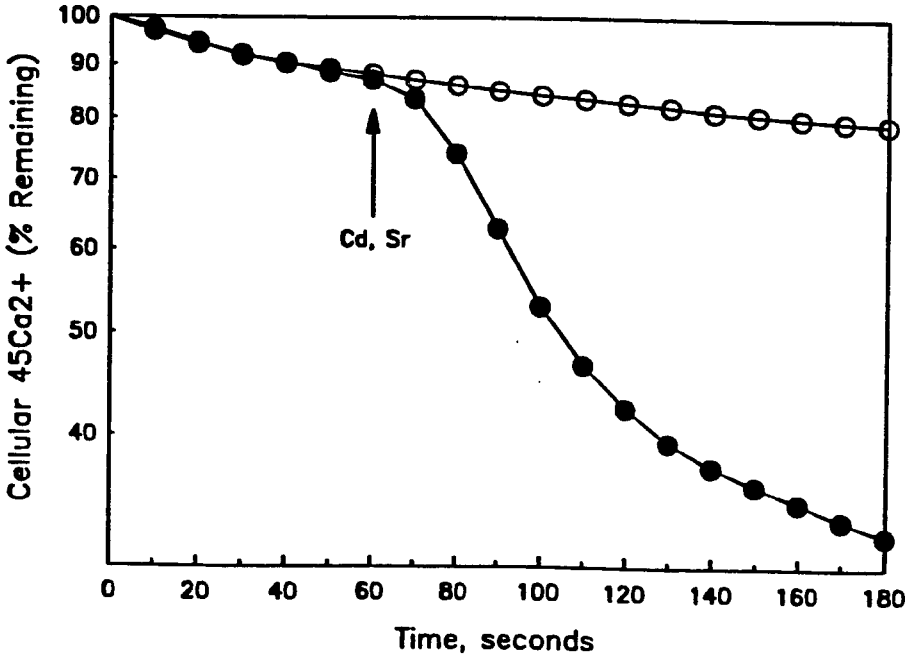
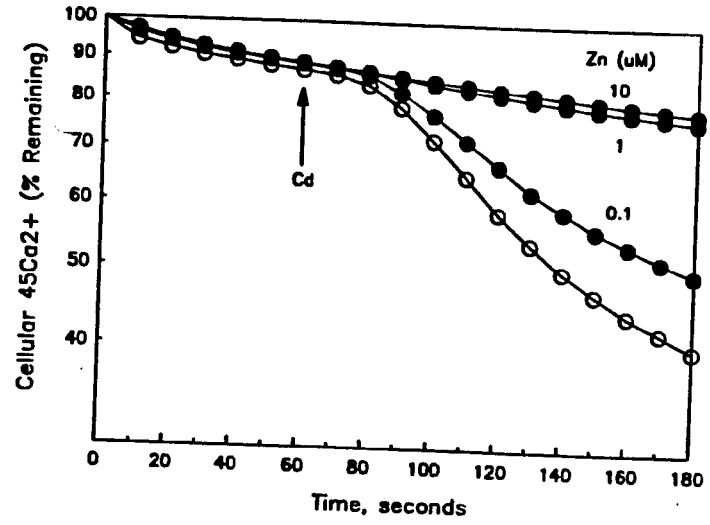
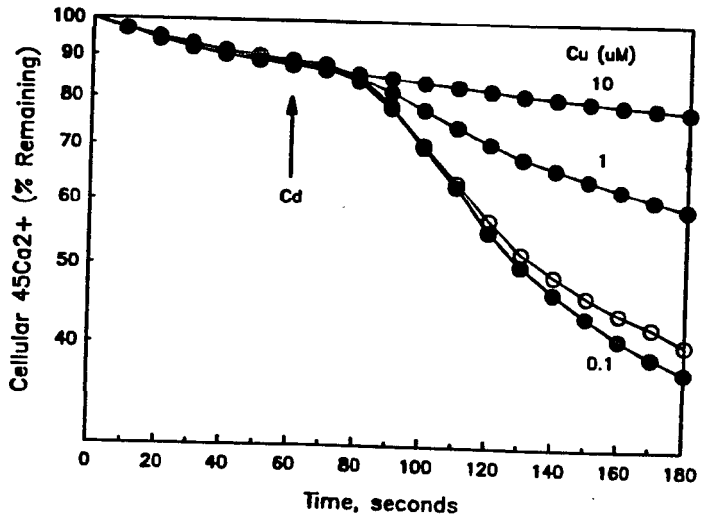

100
90
80
70
60
50
40
Cellular 45Ca2+ (% Remaining)
Cd, Sr
0
20
40
60
80
100
120
140
160
180
Time, seconds

Figure 20. Inhibition of Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux by Zn^{2+}, Cu^{2+}, and Pb^{2+} in human skin fibroblasts. Zn^{2+}, Cu^{2+}, or Pb^{2+} were present from 0 to 180 sec at the indicated concentrations. Cd^{2+} (0.1 μM) was added at 60 sec (arrow) and was present until the end of the experiment. Data are representative of two similar experiments.


Zn (uM)
10
1
0.1
Cd
Cellular 45Ca2+ (% Remaining)
Time, seconds


Cu (uM)
10
1
0.1
Cd
Cellular 45Ca2+ (% Remaining)
Time, seconds

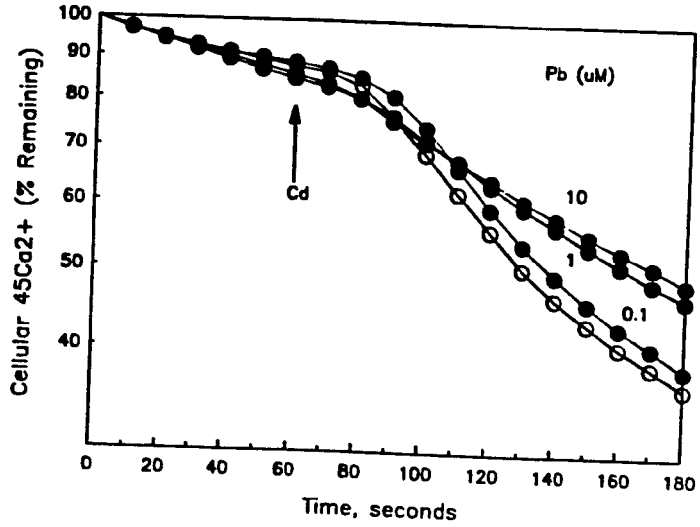

Pb (uM)
10
1
0.1
Cd
Cellular 45Ca2+ (% Remaining)
Time, seconds

Figure 21. Effect of Zn^{2+} on Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux in human skin fibroblasts. Zn^{2+} (100 μM) was present from 0 to 180 sec. Cd^{2+} (2 μM) or bradykinin (20 nM) was added at 60 sec and was present until the end of the experiment. Data shown are representative of six similar experiments.

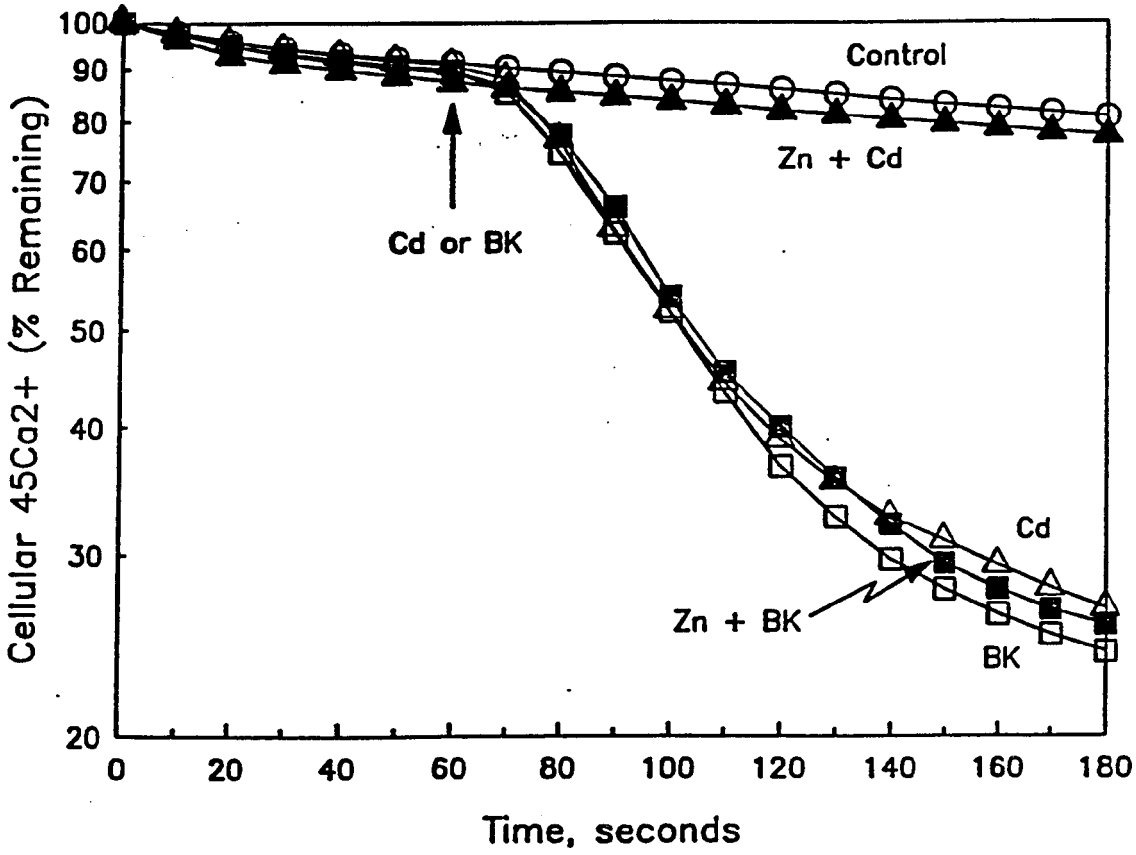

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
180
Time, seconds
Control
Zn + Cd
Cd or BK
Cd
Zn + BK
BK

Figure 22. Effect of Cd^{2+} on $^{45}Ca^{2+}$ efflux in coronary endothelial cells. Efflux was started in PSS and switched at 60 sec to PSS containing the indicated concentration of Cd^{2+}. Data are representative of three experiments that gave similar results.

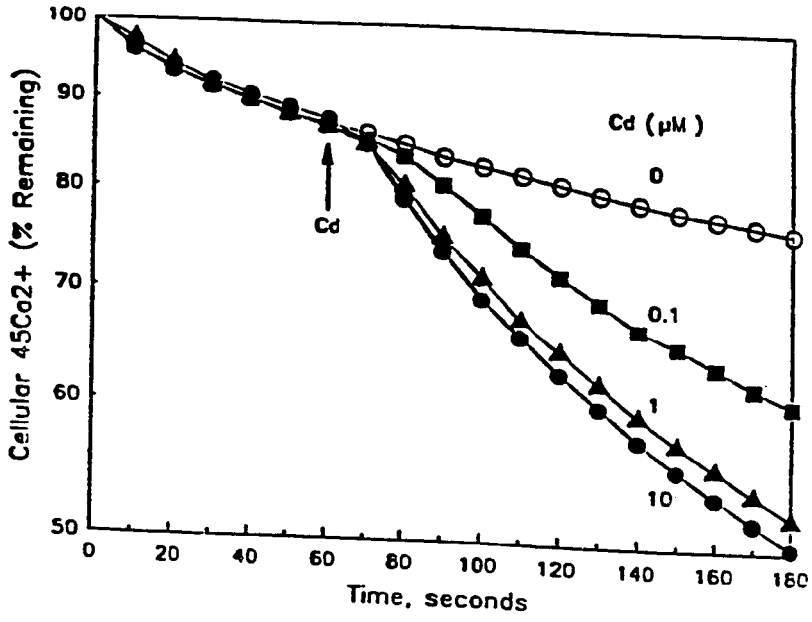

Cellular 45Co2+ (% Remaining)
100
90
80
70
60
50
0
20
40
60
80
100
120
140
160
180
Time, seconds
Cd
Cd (μM)
0
0.1
1
10

Figure 23. Effects of Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} on $^{45}Ca^{2+}$ efflux in coronary endothelial cells. Efflux was started in PSS and switched at 60 sec to PSS containing the indicated metal at the concentration indicated (μM). Data are representative of two experiments.

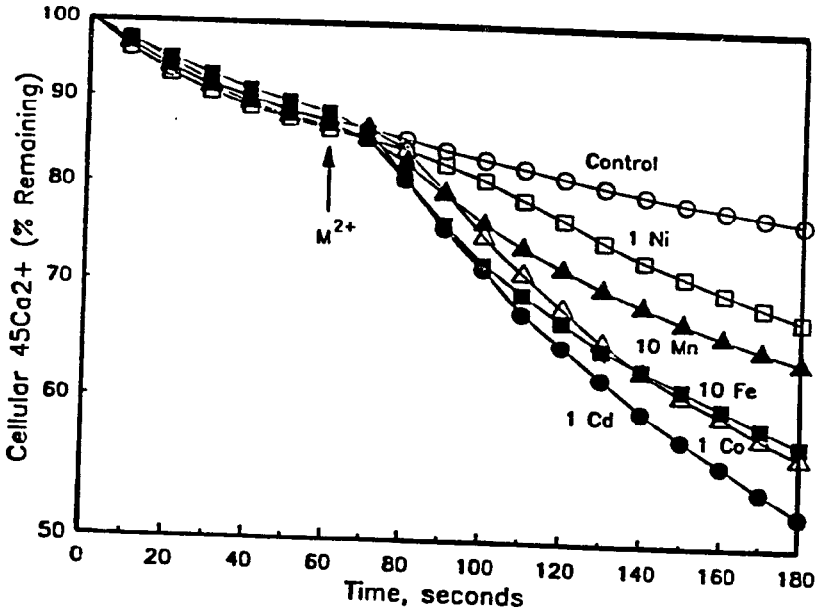

100
90
80
70
60
50
Cellular 45Ca2+ (% Remaining)
0
20
40
60
80
100
120
140
160
180
Time, seconds
M2+
Control
1 Ni
10 Mn
10 Fe
1 Co
1 Cd

Figure 24. Effect of Zn^{2+} and Cu^{2+} on the stimulation of $^{45}Ca^{2+}$ efflux by Cd^{2+} or ATP in coronary endothelial cells. Efflux was assayed in PSS containing 100 μM $ZnSO_4$ or $CuSO_4$ (0 to 180 sec) as indicated. Cd^{2+} (1 μM) or ATP (150 μM) was present from 60 to 180 sec as indicated. Data are representative of two experiments.

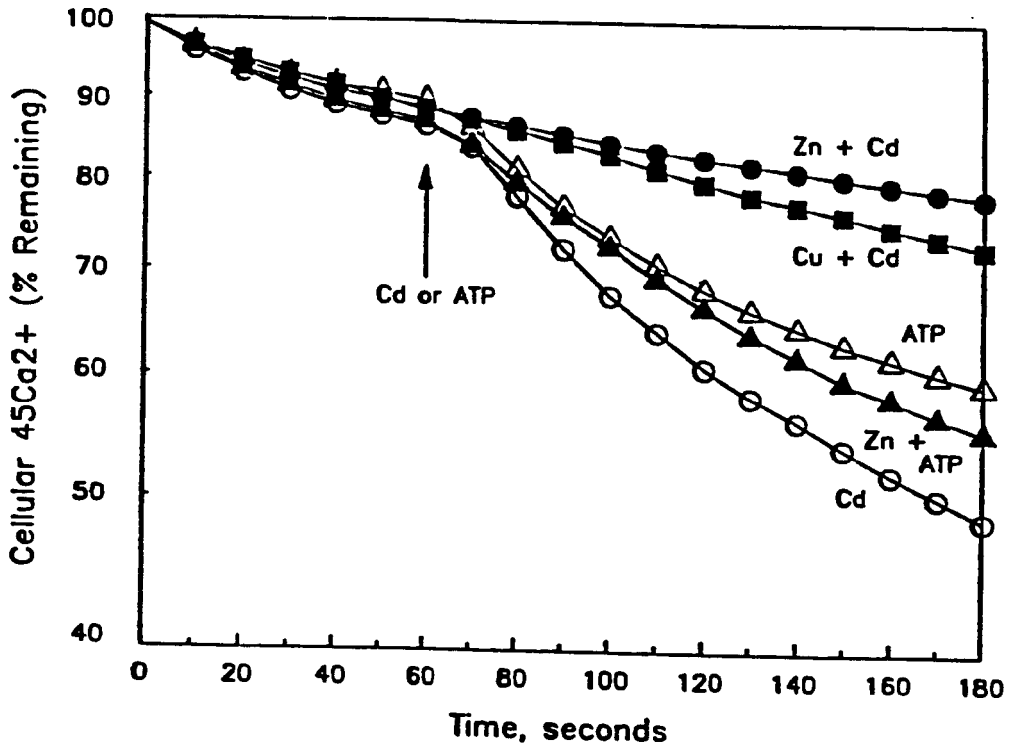
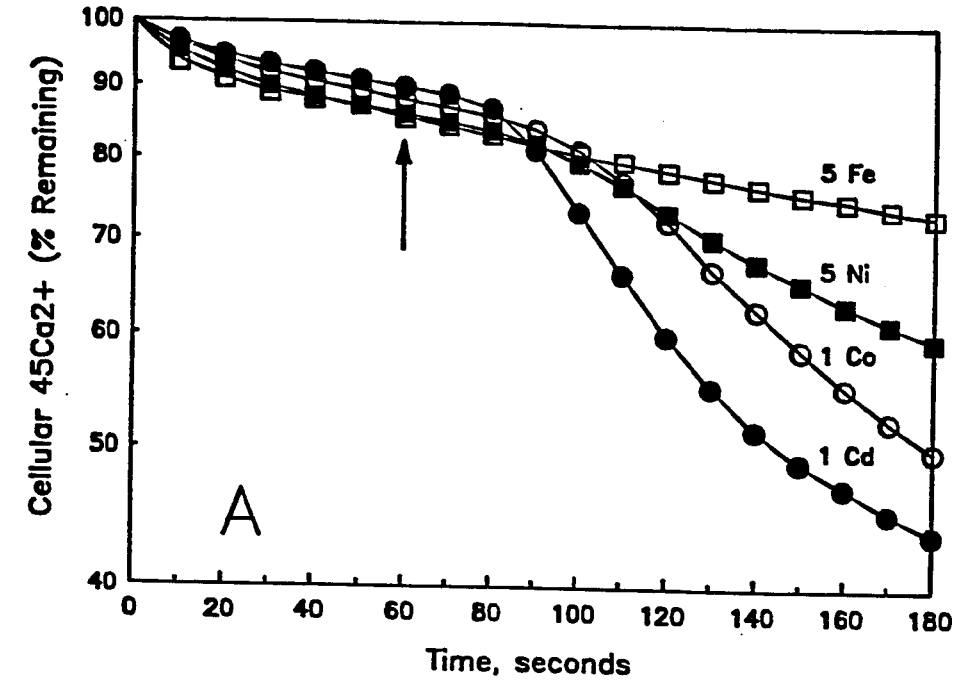

100
90
80
70
60
50
40
Cellular 45Ca2+ (% Remaining)
0
20
40
60
80
100
120
140
160
180
Time, seconds
Zn + Cd
Cu + Cd
Cd or ATP
ATP
Zn + ATP
Cd

Figure 25. Effect of Cd^{2+} and other divalent metals on $^{45}Ca^{2+}$ efflux in neuroblastoma cells. A, from 0 to 60 sec, efflux was assayed in PSS. At 60 sec (arrow), the indicated concentration (μM) of divalent metal was added in PSS and was present through 180 sec. B, at 60 sec (arrow), the indicated divalent metal was added (10 μM).


Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
0
20
40
60
80
100
120
140
160
180
Time, seconds
5 Fe
5 Ni
1 Co
1 Cd
A

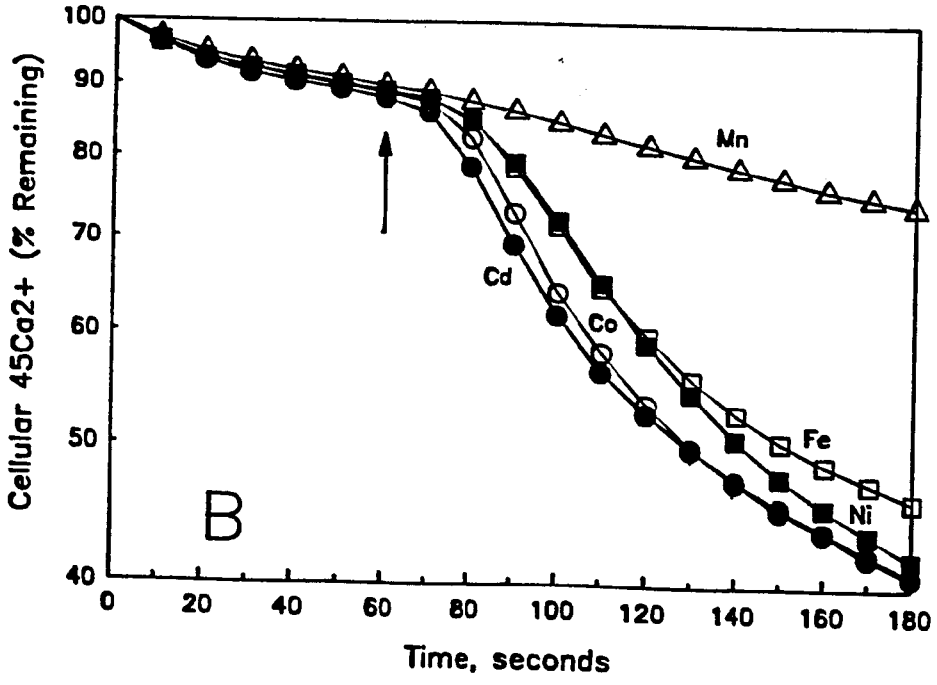

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
0
20
40
60
80
100
120
140
160
180
Time, seconds
Mn
Cd
Co
Fe
Ni
B

Figure 26. Effect of Cd^{2+} and certain other divalent metals on $^{45}Ca^{2+}$ efflux in HUA cells. From 0 to 60 sec, efflux was assayed in PSS. At 60 sec, (arrow), the indicated concentration (μM) of divalent metal was added in PSS and was present through 180 sec.

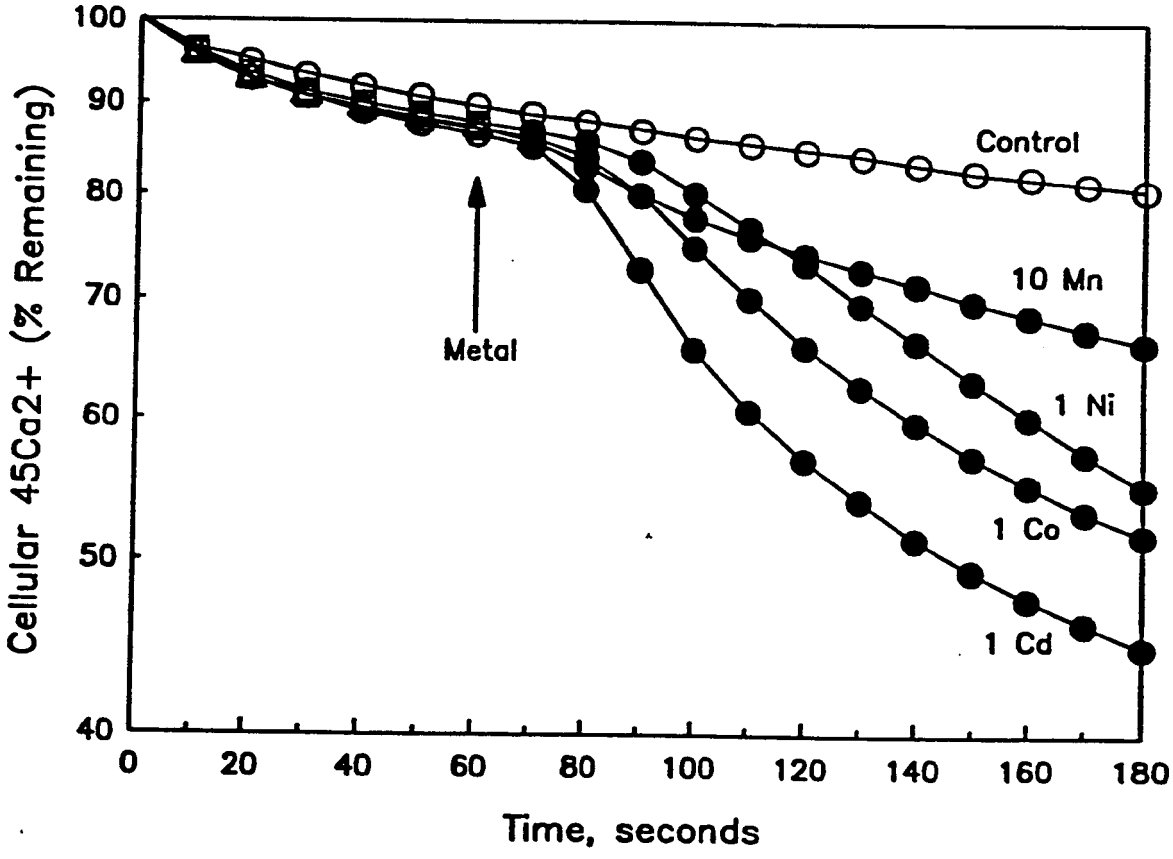
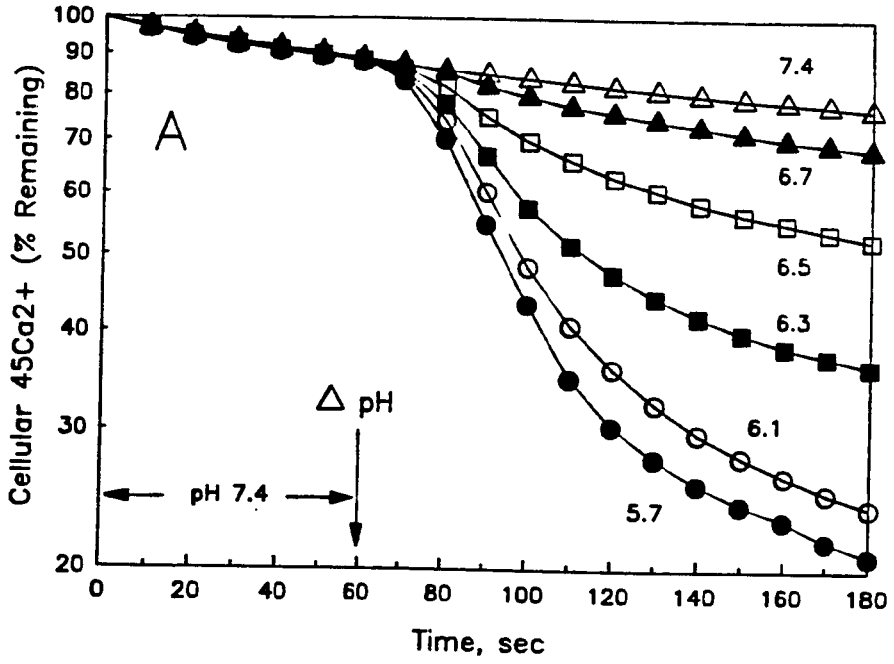

100
90
80
70
60
50
40
Cellular 45Ca2+ (% Remaining)
0
20
40
60
80
100
120
140
160
180
Time, seconds
Metal
Control
10 Mn
1 Ni
1 Co
1 Cd

Figure 27. Effect of decreasing pH_o on $^{45}Ca^{2+}$ efflux in human skin fibroblasts. A, the cultures were labeled for 2 hr in PSS containing 15 μCi $^{45}Ca^{2+}$ and rinsed 8 times with PSS to start efflux. Efflux was assayed in PIPES-PSS (pH 7.4) from 0 to 60 sec and in PIPES-HCl (pH 5.7 - 6.7) from 60 to 180 sec. Two additional experiments showed a similar dependence of $^{45}Ca^{2+}$ efflux on pH_o. B, the first-order rate constant of the initial portion of efflux was determined by exponential curve fitting with a Hewlett-Packard 11C calculator. The pH_o that half-maximally increased efflux was determined from a log-log plot of the data that was done with commercially available software (Dose-Effect Analysis with Microcomputers, by J. Chou and T.-C. Chou, Elsevier_BIOSOFT, Amsterdam). Fa/Fu (Chou, 1976) is fraction affected/fraction unaffected with 100% affected equal to the difference between the first-order rate constants at pH 5.7 and 7.4. The regression coefficient of the line was 0.9963.


A
Cellular 45Ca2+ (% Remaining)
Time, sec
pH 7.4
Δ pH
7.4
6.7
6.5
6.3
6.1
5.7
100
90
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
180

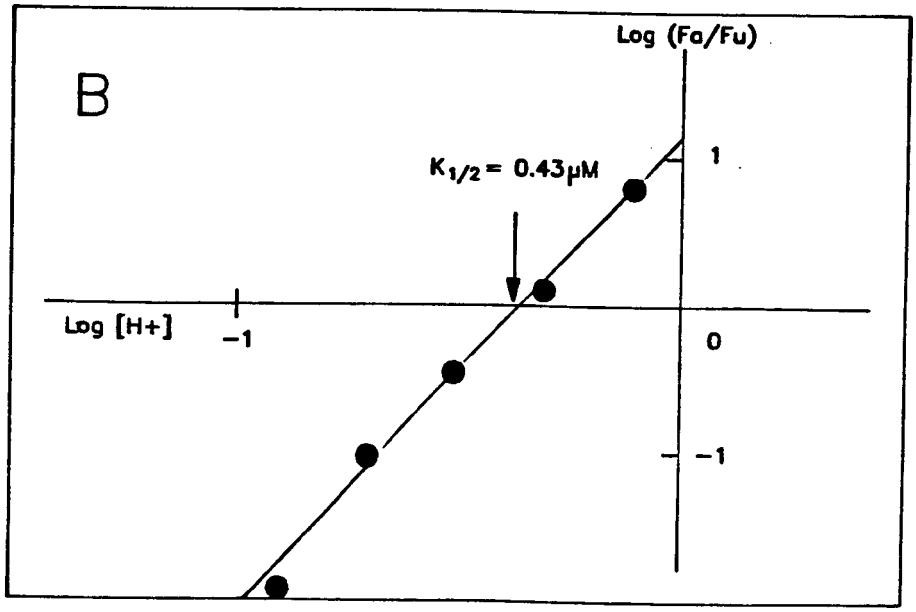
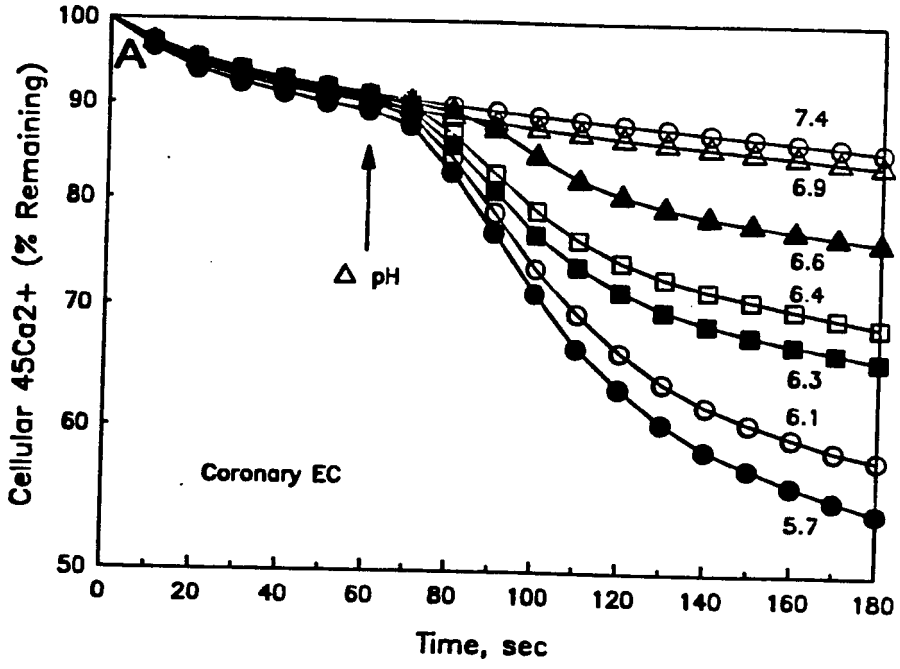

B
Log (Fa/Fu)
K1/2 = 0.43μM
Log [H+]
−1
1
0
−1

Figure 28. Effect of changing pH_o on $^{45}Ca^{2+}$ efflux from coronary endothelial (coronary EC) and neuroblastoma (SK-N-SH) cells. A, data are representative of two experiments at pH 5.7 and 6.0-6.9, and at least six experiments at pH 7.4 and 6.0. B, efflux from SK-N-SH cells was assayed in PSS containing 20 mM maleate adjusted to pH 5.7 - 6.7 with Tris or in PSS containing 20 mM Tris and adjusted to pH 6.9 - 7.4 with maleate. Three additional experiments gave similar results. The pH_o that half-maximally increased efflux was determined as indicated in the legend for (Fig. 30). The regression coefficients of the lines in endothelial cells and SK-N-SH cells were 0.9796 and 0.9988, respectively.


A
Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
0
20
40
60
80
100
120
140
160
180
Time, sec
Δ pH
Coronary EC
7.4
6.9
6.6
6.4
6.3
6.1
5.7

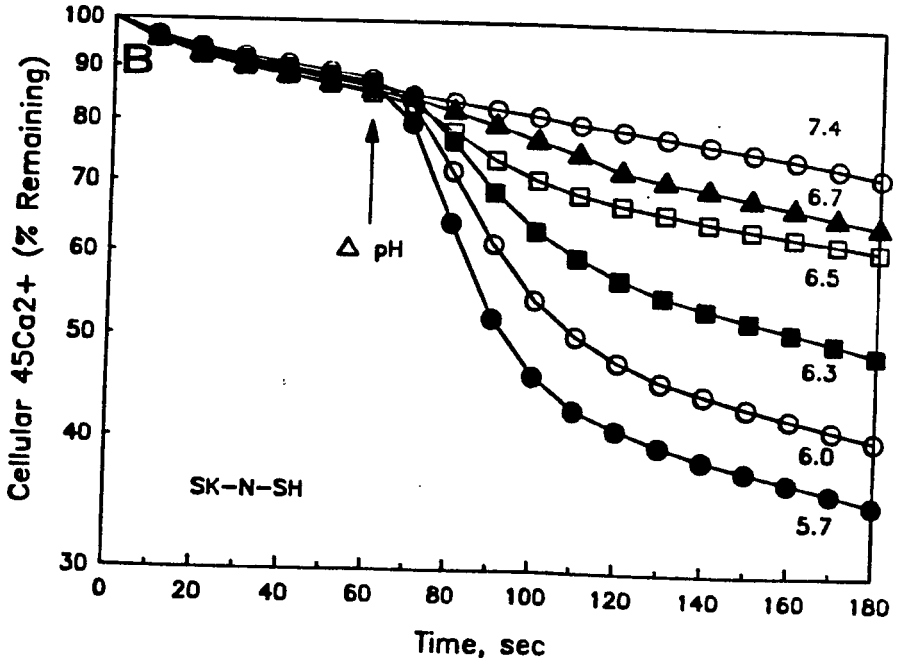

B
Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
0
20
40
60
80
100
120
140
160
180
Time, sec
Δ pH
SK-N-SH
7.4
6.7
6.5
6.3
6.0
5.7

Figure 29. Effect of decreasing $[Na^+]_o$ on total cell Ca^{2+} in human skin fibroblasts. Total cell Ca^{2+} was measured after equilibrium labeling with $^{45}Ca^{2+}$. The cultures were incubated for the indicated intervals in PSS (circles) or choline PSS (squares) containing $^{45}Ca^{2+}$ and 10 mM glucose. Some cultures (triangles) were incubated for 5 min in choline PSS to decrease total cell Ca^{2+} in order to follow the change in total Ca^{2+} after the cells were returned to PSS. All incubations were with 10 mM glucose and $^{45}Ca^{2+}$ at the same specific radioactivity as in the DME used for equilibrium labeling. Values are means of four experiments. The number of replicates is indicated in parentheses. Error bars are not shown because the SE values (0.03-0.34) did not exceed the size of the markers.

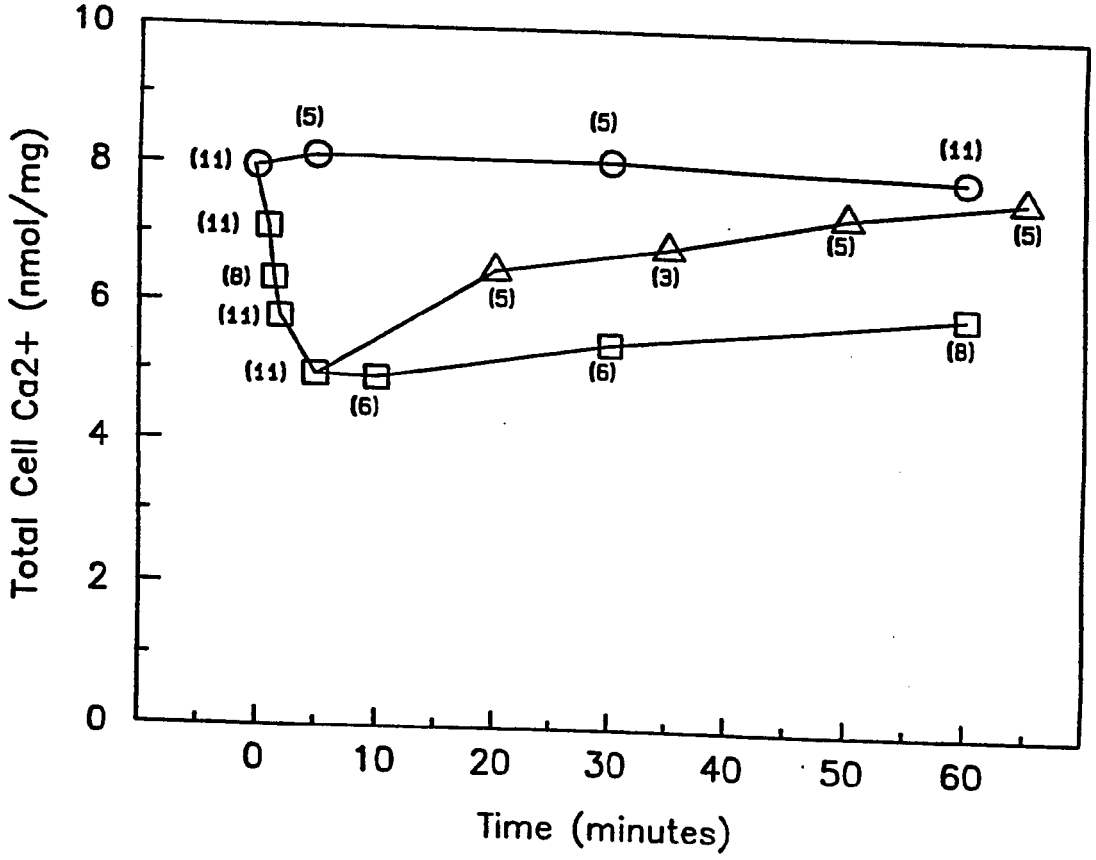
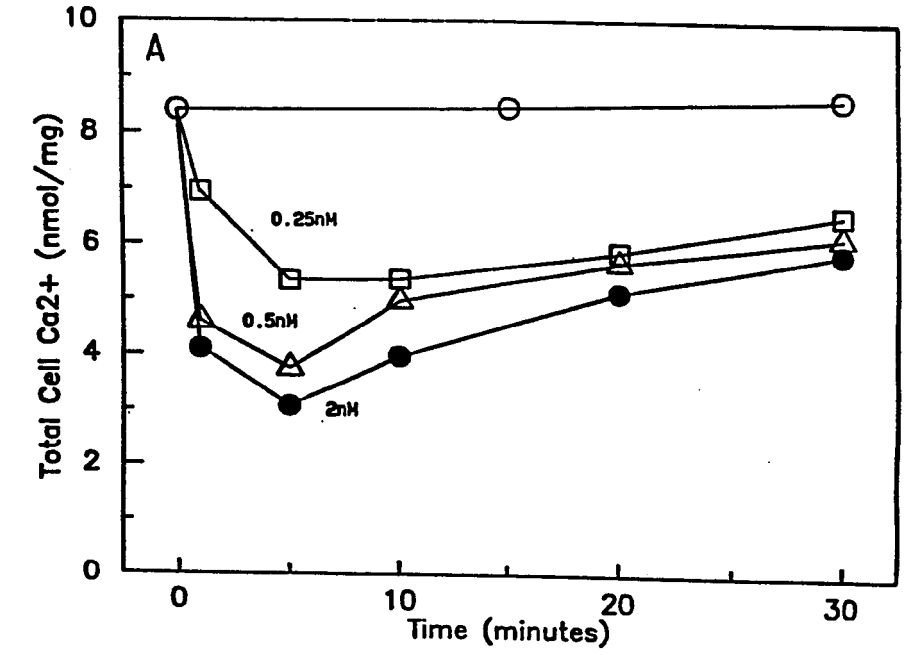

10
8
6
4
2
0
Total Cell Ca2+ (nmol/mg)
0
10
20
30
40
50
60
Time (minutes)
(11)
(5)
(5)
(11)
(11)
(8)
(11)
(11)
(6)
(5)
(3)
(5)
(5)
(6)
(8)

Figure 30. Effect of bradykinin on total cell Ca^{2+} in human skin fibroblasts. Bradykinin was added directly to the overnight labeling medium from a 50 times concentrated solution in water. The incubation was stopped by rinsing the cultures with MLB (see methods). A, values are means of triplicates. SE values ranged from 0.017 to 0.262. B, the concentration of bradykinin was 100 nM. Values are means of 2 or 3 experiments. The number of replicates is shown in paretheses. Error bars were omitted because SE values (0.07 to 0.26) were not larger than the markers. The inset shows a time course of the effect of 100 nM bradykinin on total cell Ca^{2+}. Values are means of triplicates.


A
Total Cell Ca2+ (nmol/mg)
Time (minutes)
0.25nM
0.5nM
2nM
0
2
4
6
8
10
0
10
20
30

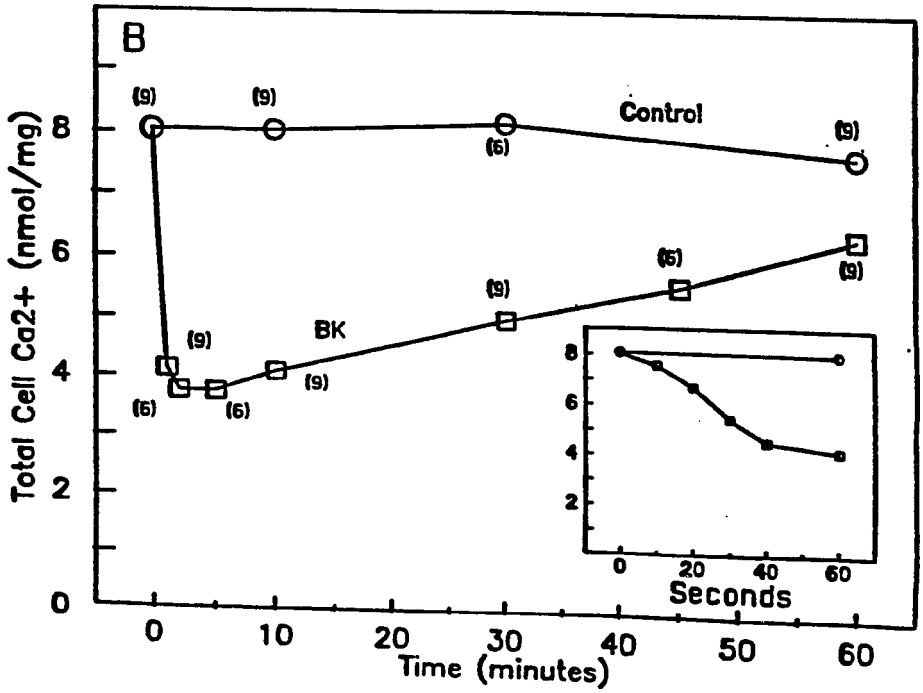

B
Total Cell Ca2+ (nmol/mg)
Time (minutes)
Control
BK
Seconds
(9)
(6)
0
2
4
6
8
10
20
30
40
50
60

Figure 31. Effect of bradykinin concentration on total cell Ca^{2+} in human skin fibroblasts. Bradykinin was added directly to the labeling medium from a 50 times concentrated solution. Values are means of triplicates. SE values ranged from 0.140 to 0.361. The concentration of bradykinin that half-maximally decreased total cell Ca^{2+} was 0.6 nM. This value was obtained from a log-log plot of the data using "Dose-Effect Analysis for Microcomputers" (Elsevier_BIOSOFT, Cambridge, UK) which gave a linear regression coefficient of 0.9986.

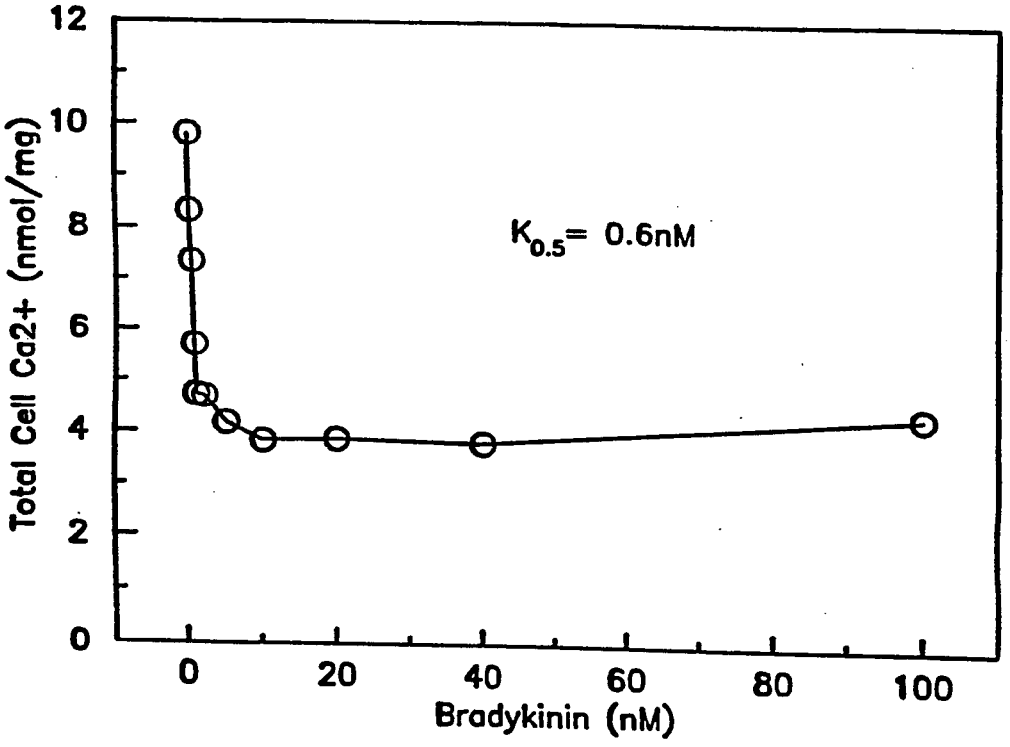
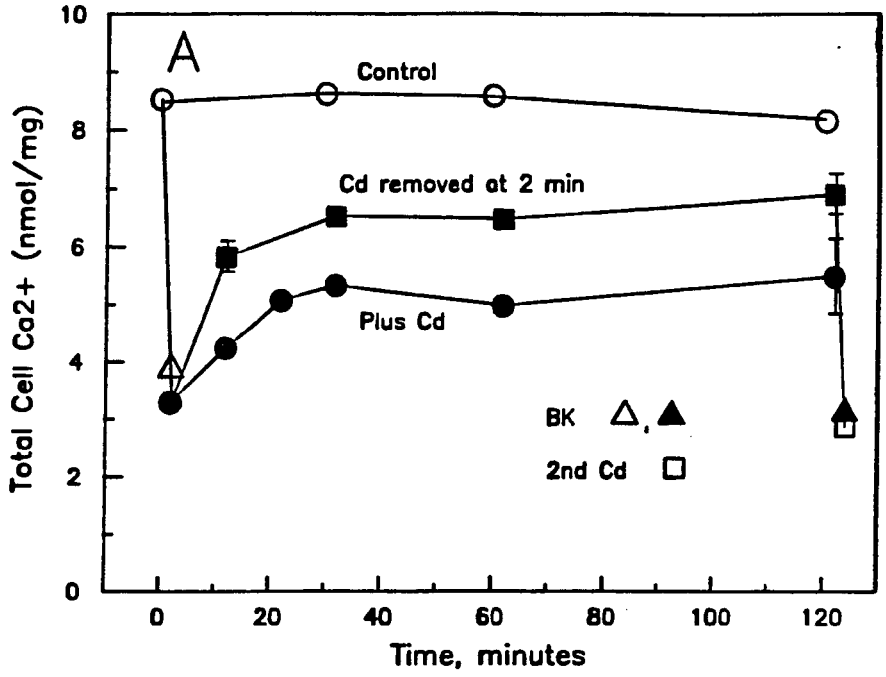

12
10
8
6
4
2
0
Total Cell Ca2+ (nmol/mg)
$K_{0.5}$= 0.6nM
0
20
40
60
80
100
Bradykinin (nM)

Figure 32. Cd^{2+} decreases total cell Ca^{2+} similarly to bradykinin (BK) and Zn^{2+} competitively antagonizes the response to Cd^{2+}. Total cell Ca^{2+} was measured after incubating the cultures overnight with 10 μCi of $^{45}Ca^{2+}$ in culture medium containing 2% FBS. The medium was removed and 1 ml of PSS containing 1.8 mM Ca^{2+}, 10 mM glucose, and 10 μCi of $^{45}Ca^{2+}$ was added 1 hr before incubation with Cd^{2+}. A, the cultures (filled circles) were incubated with 10 μM Cd^{2+} for the indicated time. Other cultures (filled squares) were incubated with 10 μM Cd^{2+} for 2 min, rinsed 3 times with ice-cold Ca^{2+}-free PSS containing 10 μM DTPA (a heavy metal chelator), and then incubated for the indicated time with PSS containing 1.8 mM Ca^{2+}, 10 mM glucose, and 10 μCi of $^{45}Ca^{2+}$. Bradykinin (20 nM) was added to some cultures (unfilled triangle) at zero time and to others (filled triangle) 2 hr after removing Cd^{2+}. Some cultures (unfilled square) received 10 μM Cd^{2+} 2 hr after removing Cd^{2+}. Values are means ± SE, n = 4 - 6, except n = 2 for bradykinin. B, Zn^{2+} was added at the indicated concentration (μM) 5 sec before adding Cd^{2+}. Control cultures contained 8.3 ± 0.1 nmol Ca^{2+}/mg protein (n = 4). Values are means of two to six replicates. The data were analyzed using the "Dose-Effect Analysis for Microcomputers" (Elsevier_BIOSOFT, Cambridge, UK). The program diagnosed the inhibition at 1 and 2 μM as competitive. Regression coefficients were all greater than 0.97.


A
Control
Cd removed at 2 min
Plus Cd
BK
2nd Cd
Total Cell Ca2+ (nmol/mg)
Time, minutes
0
2
4
6
8
10
0
20
40
60
80
100
120

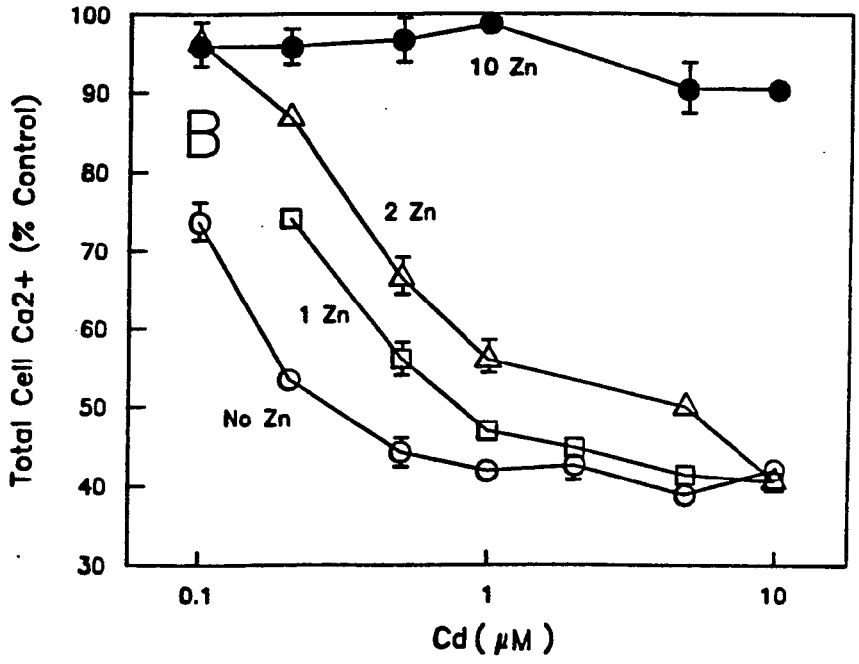

B
10 Zn
2 Zn
1 Zn
No Zn
Total Cell Ca2+ (% Control)
Cd (μM)
30
40
50
60
70
80
90
100
0.1
1
10

Figure 33. Effect of Co^{2+} on total cell Ca^{2+} in human skin fibroblasts. This experiment was conducted as described for Cd^{2+} in Fig. 33 except 10 μM Co^{2+} was substituted for Cd^{2+}. Values are means ± SE from five experiments (n = 6-8). Total cell Ca^{2+} measurements were made by Yingxin Zhuang.

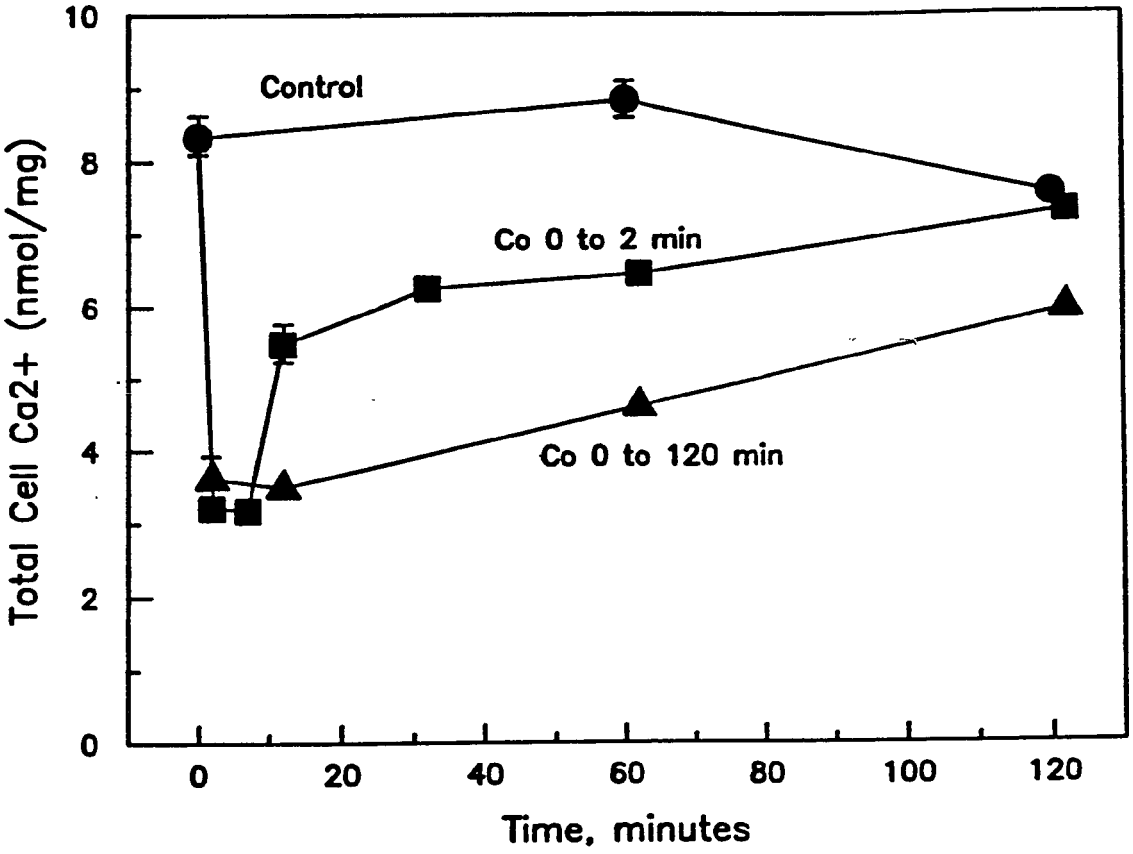

Control
Co 0 to 2 min
Co 0 to 120 min
Total Cell Ca2+ (nmol/mg)
Time, minutes
0
2
4
6
8
10
0
20
40
60
80
100
120

Figure 34. Effect of decreasing pH_o on total cell Ca^{2+} in human skin fibroblasts. Total cell Ca^{2+} was measured 1 min after decreasing pH_o from 7.4 to the indicated value. Values are means of two experiments on duplicate cultures. SE values were all smaller than the diameter of the markers (0.04-0.29; n = 4). The cultures were labeled overnight in 1 ml of DME containing 2% fetal bovine serum and 10 μCi of $^{45}Ca^{2+}$. The medium was aspirated and replaced with 1 ml of PIPES-PSS containing 10 mM glucose, 1.8 mM $CaCl_2$, and 10 μCi of $^{45}Ca^{2+}$. At zero time, the medium was aspirated and replaced with 1 ml of PIPES-PSS containing 1.8 mM $CaCl_2$ and 10 μCi of $^{45}Ca^{2+}$ at the indicated pH. The pH which half-maximally decreased total cell Ca^{2+}_o was determined from a log-log plot of the data as described in the legend to Fig. 27. The regression coefficient of the line was 0.9698.

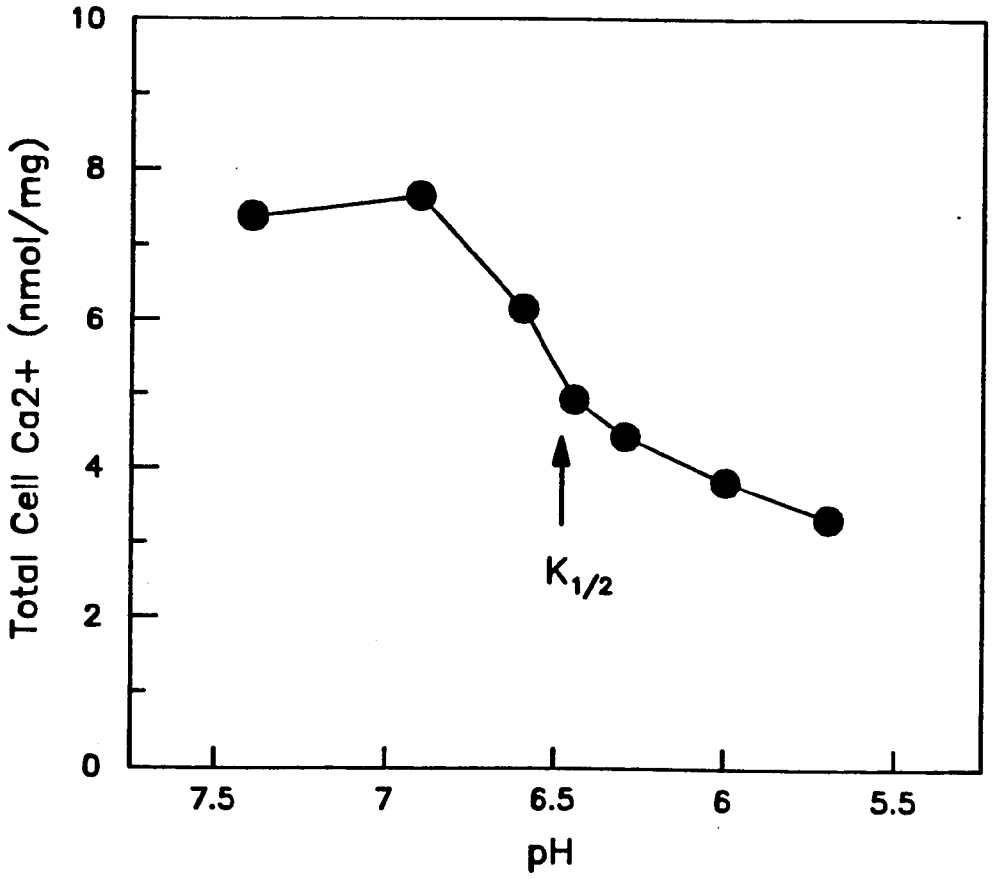
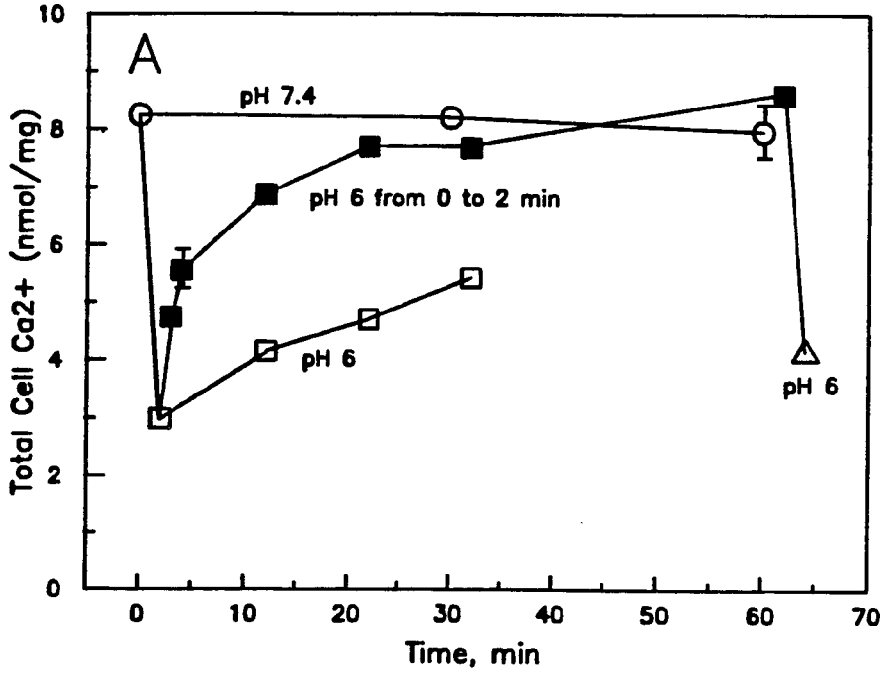

Total Cell Ca2+ (nmol/mg)
10
8
6
4
2
0
7.5
7
6.5
6
5.5
pH
K1/2

Figure 35. Recovery of cell Ca^{2+} after an acid pulse and repetitive decreases in total cell Ca^{2+} produced by acidifying the extracellular medium. The cultures were labeled overnight in 1 ml of DME containing 2% fetal bovine serum and 10 μCi of $^{45}Ca^{2+}$. A, 30 μl of 1 N HCl was added to the medium to decrease pH_o to 6.0. After 2 min, the medium was aspirated and replaced with 1 ml of DME containing 2% fetal bovine serum and 10 μCi of $^{45}Ca^{2+}$ and incubated for the indicated time. Values are the mean ± SE (n = 2-9) for two experiments. B, The overnight labeling medium was removed and replaced with freshly prepared medium of the same composition 1 hr before starting the experiment. The medium was acidified at the indicated times. After 2 min, the medium was aspirated and replaced with 1 ml of DME containing 2% fetal bovine serum and 10 μCi of $^{45}Ca^{2+}$ as in A. Values are mean ± SE (n = 6-19) for three experiments. Bradykinin was added (20 nM) to some cultures (triangles) at 0 or 12 min.


A
pH 7.4
pH 6 from 0 to 2 min
pH 6
pH 6
Total Cell Ca2+ (nmol/mg)
Time, min
0
2
4
6
8
10
0
10
20
30
40
50
60
70

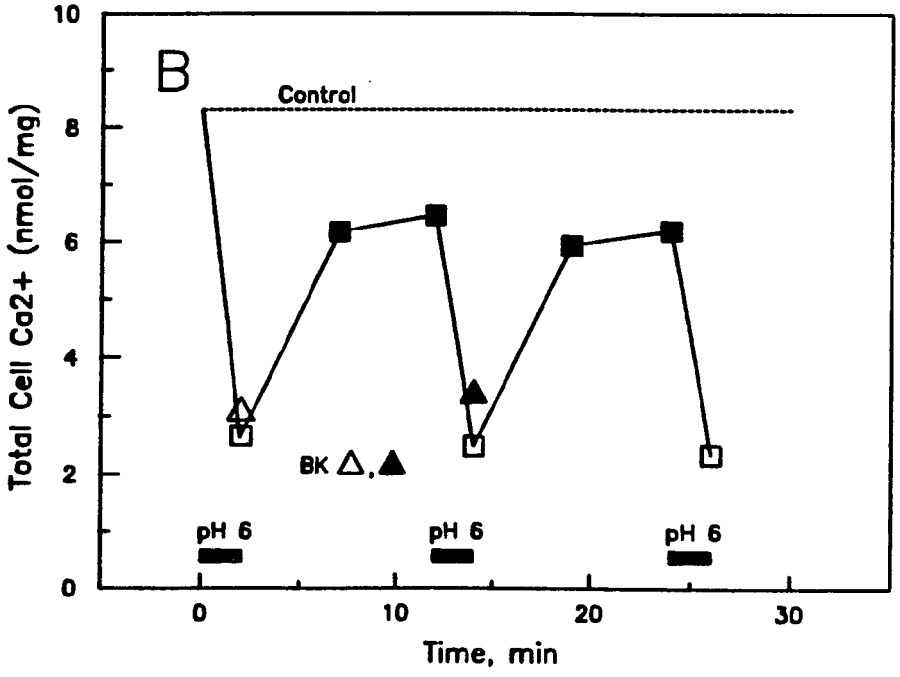

B
Control
BK △,▲
pH 6
pH 6
pH 6
Total Cell Ca2+ (nmol/mg)
Time, min
0
2
4
6
8
10
0
10
20
30

Figure 36. Effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ uptake in human skin fibroblasts. Each point is the mean of four independent experiments (n = 11). Error bars are absent because SE values were not larger than the markers. The slopes of the lines betwen 15 and 60 sec were 0.46 and 0.61 nmol/min x mg protein in the presence and absence of Na^+_o, respectively. The linear regression coefficients were 0.997 and 0.988, respectively. The slopes of the lines between 5 and 30 min were 0.048 and 0.078 nmol/min x mg protein in the presence and absence of Na^+_o, respectively. The regression coefficients were 0.997 and 0.993, respectively. Statistically significant differences are indicated as follows: a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$; and d, $p < 0.001$. The probabilities were obtained by unpaired Student's t-test.

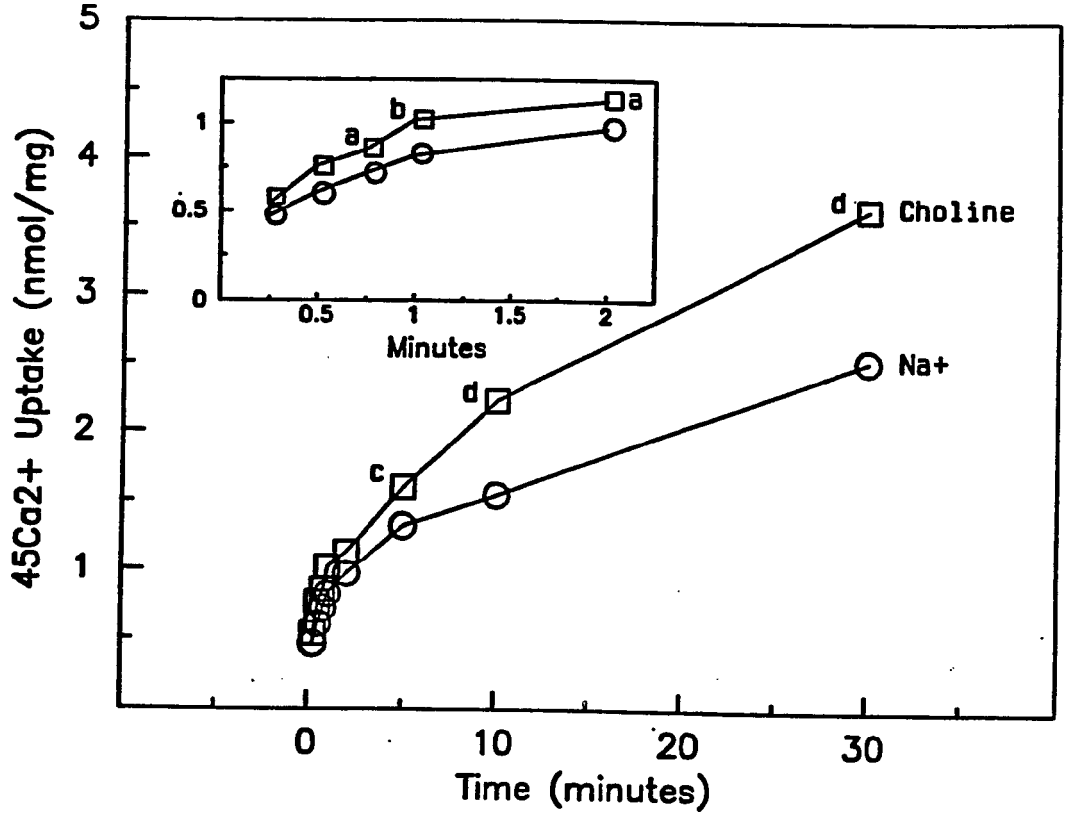

45Ca2+ Uptake (nmol/mg)
Time (minutes)
Minutes
Choline
Na+
a
b
c
d

Figure 37. Effect of bradykinin on $^{45}Ca^{2+}$ uptake in human skin fibroblasts. Each point is the mean of four experiments (n = 10). Error bars are not shown because the SE values (0.02-0.19) were not larger than the markers. Means were compared by Student's t-test. An "a" next to a marker indicates a statistically significant difference with $p < 0.05$.

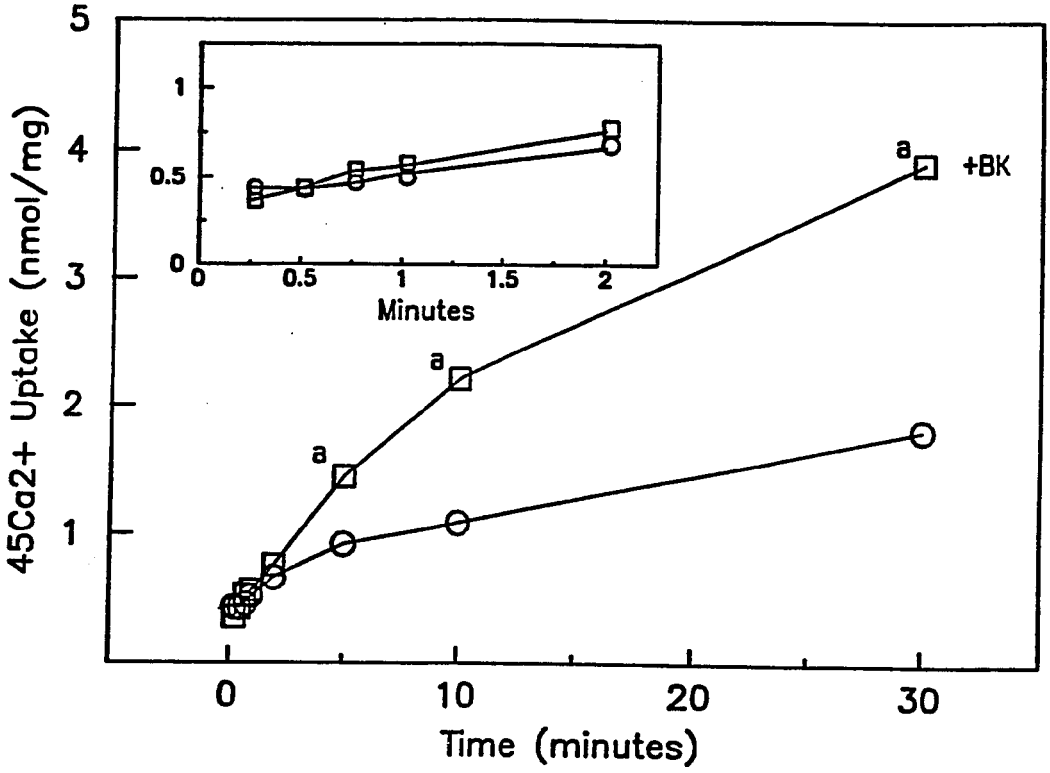

45Ca2+ Uptake (nmol/mg)
Time (minutes)
Minutes
+BK
a
a
a
0
10
20
30
1
2
3
4
5
0.5
1.5

Figure 38. Effect of decreasing $[Na^+]_o$ on $^{45}Ca^{2+}$ uptake by coronary endothelial cells with normal and increased $[Na^+]_i$. Uptake was assayed in the presence (circles) or absence of external Na^+ (triangles) by cells with normal (unfilled markers) or increased Na^+ (filled markers). Data are means of four experiments on triplicate cultures (n = 12). SE values ranged from 0.021 to 0.132; error bars are shown when SE was larger than the markers. The linear regression coefficient (15-60 sec) of both lines was 0.99.

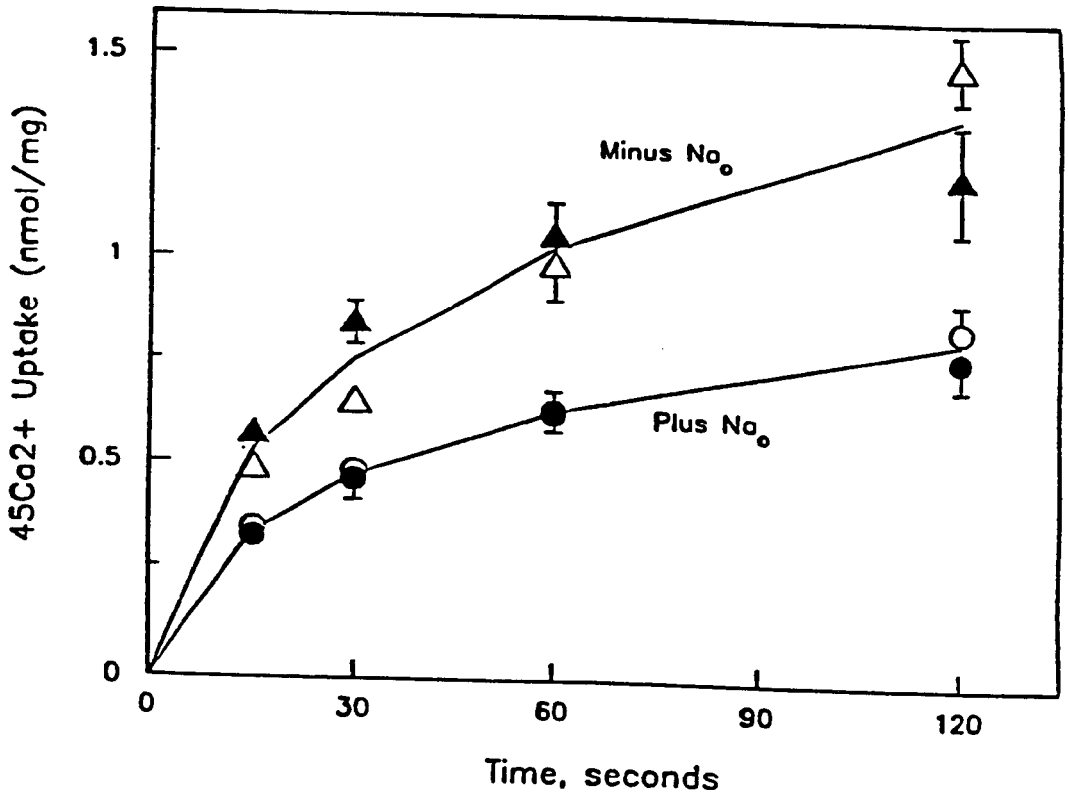

1.5
1
0.5
0
45Ca2+ Uptake (nmol/mg)
Minus No_o
Plus No_o
0
30
60
90
120
Time, seconds

Figure 39. Effect of TPEN on Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux in human skin fibroblasts. Extracellular, but not intracellular, TPEN blocks Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux. Some cultures (filled triangles) were incubated with 50 μM TPEN for 40 min before stating the assay by rinsing the cultures 8 times with PSS containing 50 μM TPEN and assaying efflux in this solution. The indicated concentration of Cd^{2+} was added at 60 sec. Some cultures (filled squares) received 4 μM TPEN and 2 μM Cd^{2+} at 60 sec. The data are representative of three to nine experiments.

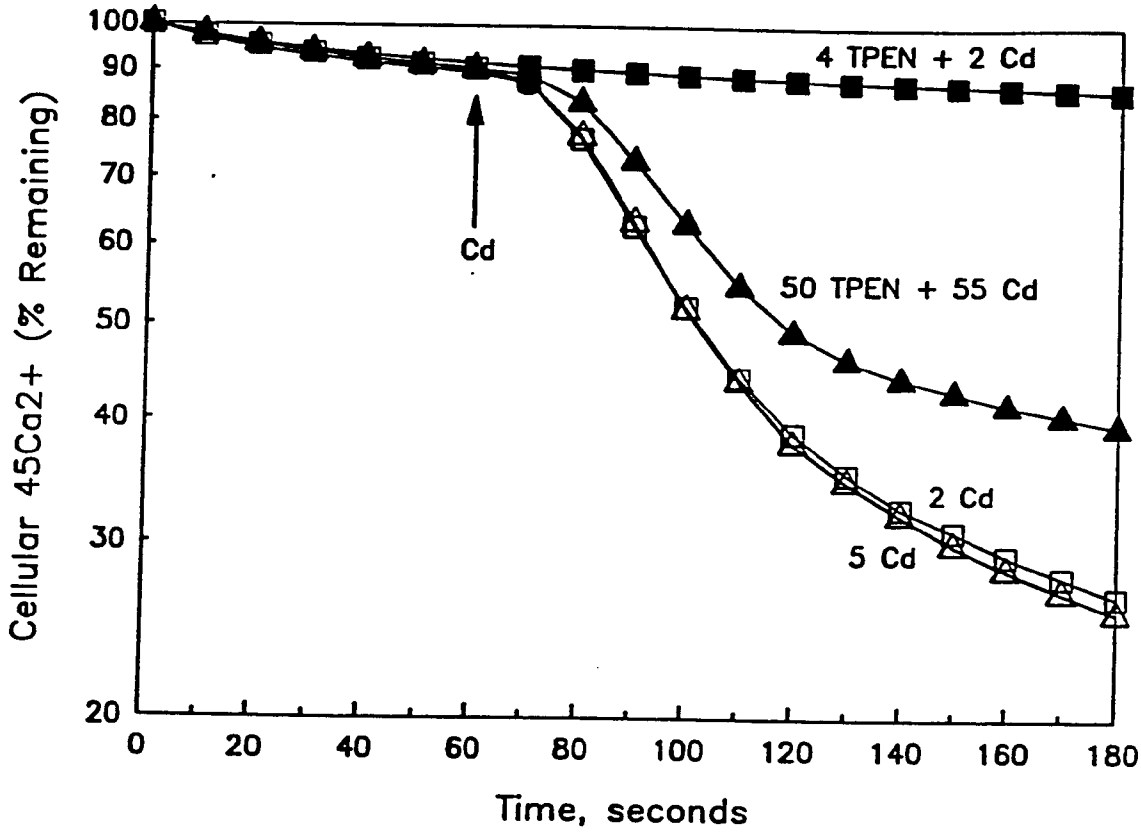

Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
180
Time, seconds
Cd
4 TPEN + 2 Cd
50 TPEN + 55 Cd
2 Cd
5 Cd

Figure 40. Effect of TPEN on the stimulation of $^{45}Ca^{2+}$ efflux by Cd^{2+} or ATP in coronary endothelial cells. Some cultures received TPEN (2 or 50 μM as indicated) during the last 40 min of the $^{45}Ca^{2+}$ labeling. TPEN (2 or 50 μM) was also present in the PSS used to rinse cultures to remove extracellular $^{45}Ca^{2+}$ to start efflux and throughout the efflux assay. Cd^{2+} (1 or 5 μM) or ATP (150 μM) was present from 60 to 180 sec as indicated. Data are representative of three experiments.

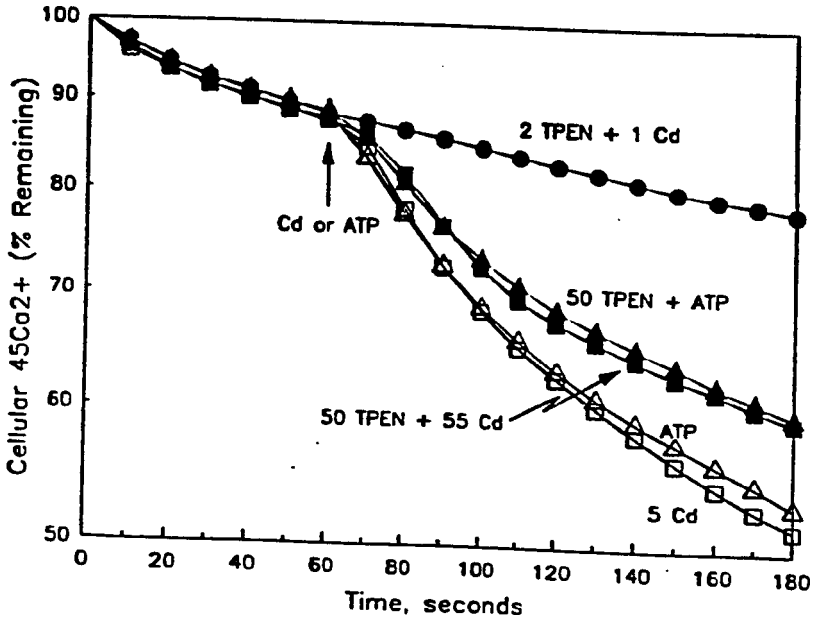

100
90
80
70
60
50
Cellular 45Ca2+ (% Remaining)
0
20
40
60
80
100
120
140
160
180
Time, seconds
2 TPEN + 1 Cd
Cd or ATP
50 TPEN + ATP
50 TPEN + 55 Cd
ATP
5 Cd

Figure 41. Effect of decreasing $[Na^+]_o$ on [^{3}H]inositol phosphate levels in human skin fibroblasts. Control cultures (0.8 mg protein) were incubated with fresh PSS for 2 or 5 min, or PSS was added and immediately removed. The duration of the incubation had no effect on [^{3}H]inositol phosphate levels. Control [^{3}H]inositol phosphate levels (counts/min/culture) were (mean ± SE; n = 6): GPI, 1169 ± 176; IP, 1434 ± 85; IP_2, 251 ± 24; and IP_3 plus IP_4, 248 ± 41. A 15 sec incubation with 100 nM bradykinin in PSS gave the following [^{3}H]inositol phosphate levels (mean ± SE; duplicates): GPI, 1179 ± 118; IP, 4754 ± 60; IP_2, 6864 ± 86; and IP_3 plus IP_4, 3413 ± 102. Values are means of duplicate cultures for one of two independent experiments that gave similar results. The mean value of IP_3 plus IP_4 between 30 and 180 sec (575 ± 30; n = 8) was statistically different from the control value ($p < 0.001$; unpaired Student's t-test). [^{3}H]Inositol phosphate measurements were made by Cindy Smith.

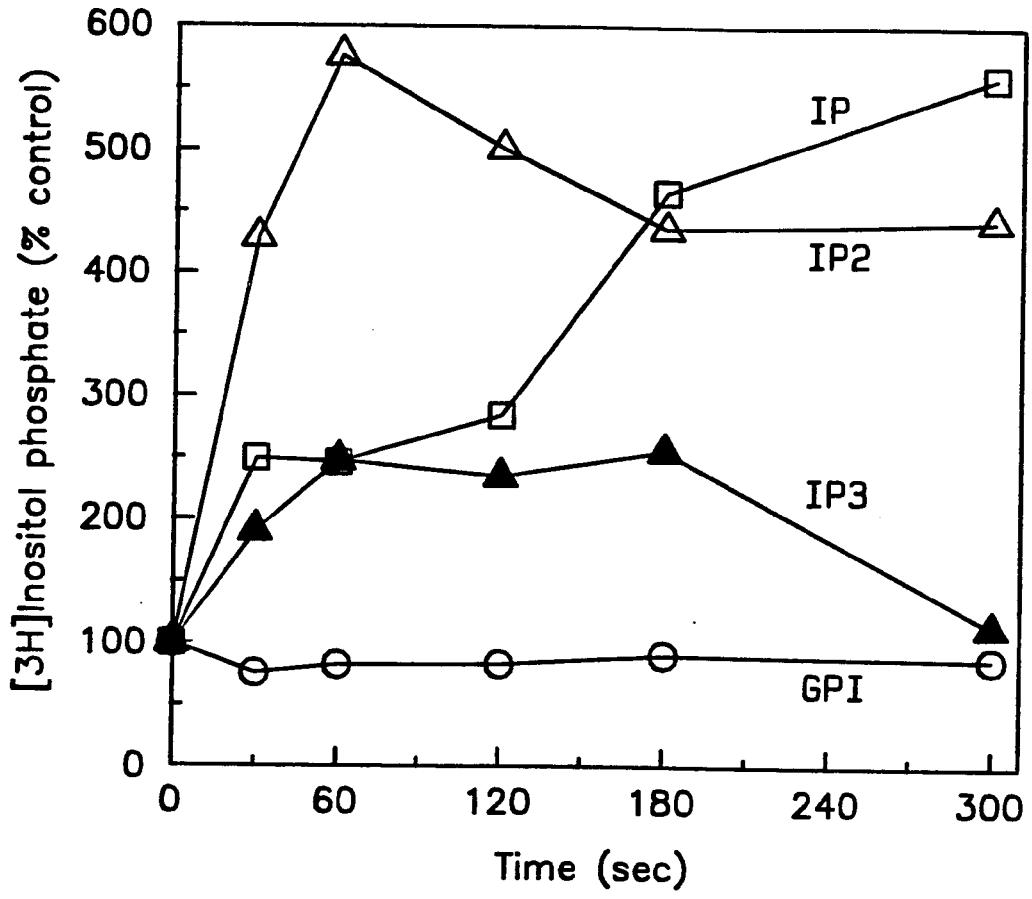

600
500
400
300
200
100
0
[3H]Inositol phosphate (% control)
0
60
120
180
240
300
Time (sec)
IP
IP2
IP3
GPI

Figure 42. Increases in [^{3}H]inositol phosphates produced by Cd^{2+} in human skin fibroblasts. Fibroblast cultures were labeled with [^{3}H]inositol for 48 hrs and incubated with 5 μM $CdCl_2$ in PSS for the indicated interval. Error bars are shown when SE was larger than the marker (n = 3). Control cultures were incubated in PSS without Cd^{2+}. Controls at 15, 30, or 60 sec were no different. Control values (counts/min/culture) were GPI, 2195 ± 125; IP, 1291 ± 44; IP_2, 170 ± 9; IP_3, 166 ± 15; IP_4, 402 ± 37 (mean ± SE, n = 6). [^{3}H]inositol phosphate standards (Du Pont-New England Nuclear) were used to verify the separation of IP, IP_2, IP_3, and IP_4. [^{3}H]Inositol phosphate measurements were made by Cindy Smith.

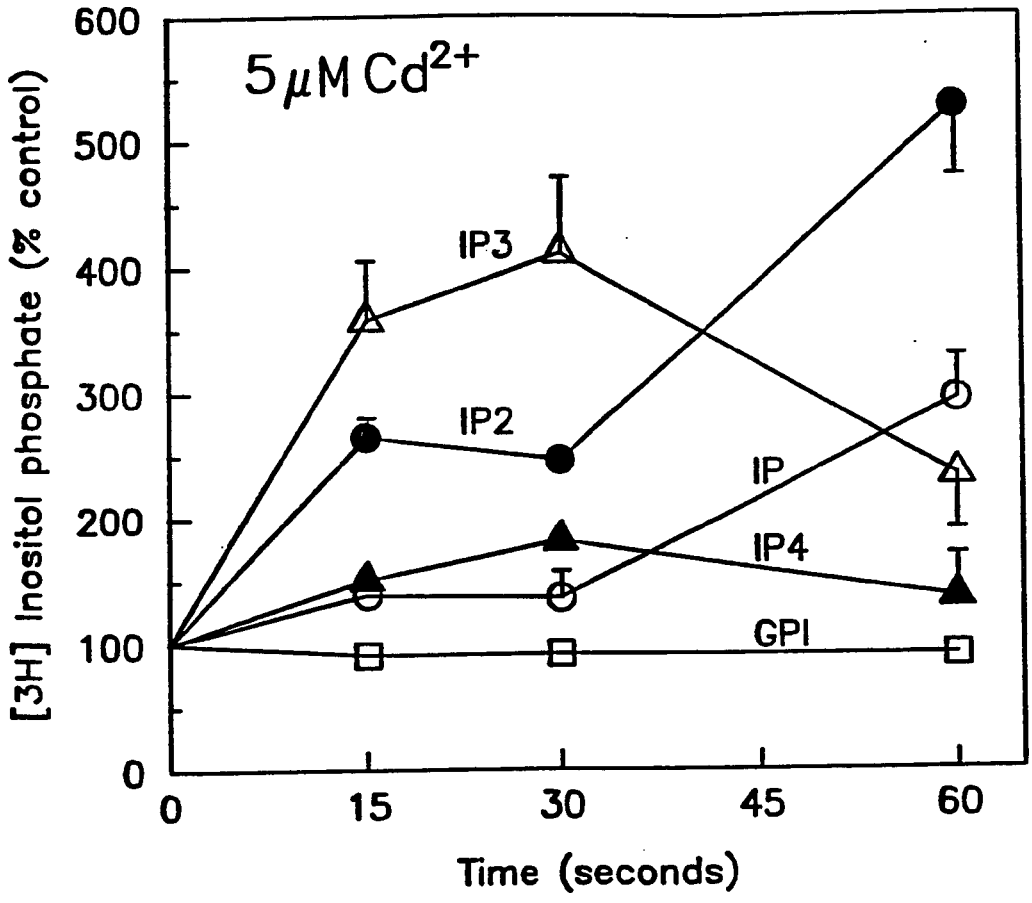

5 μM Cd2+
IP3
IP2
IP
IP4
GPI
[3H] Inositol phosphate (% control)
600
500
400
300
200
100
0
0
15
30
45
60
Time (seconds)

Figure 43. Effects of bradykinin or decreasing pH_o on the production of [^{3}H]inositol phosphates in human skin fibroblasts. The cultures were labeled for 48 hrs with 20 μCi of [^{3}H]inositol. The cultures were rinsed 5 times with PSS and incubated for 2 hr in 10 ml of PSS containing 10 mM glucose before aspirating the medium and adding PIPES-PSS (pH 6.1) or adding 20 nM bradykinin. Controls at 15, 30, and 60 sec did not differ significantly in [^{3}H]inositol phosphates. Control values (counts/min/culture) were: GPI, 2195 ± 125; IP, 1291 ± 44; IP_2, 170 ± 9; IP_3, 166 ± 15; IP_4, 402 ± 37 (mean ± SE, n = 6). [^{3}H]Inositol phosphate measurements were made by Cindy Smith.

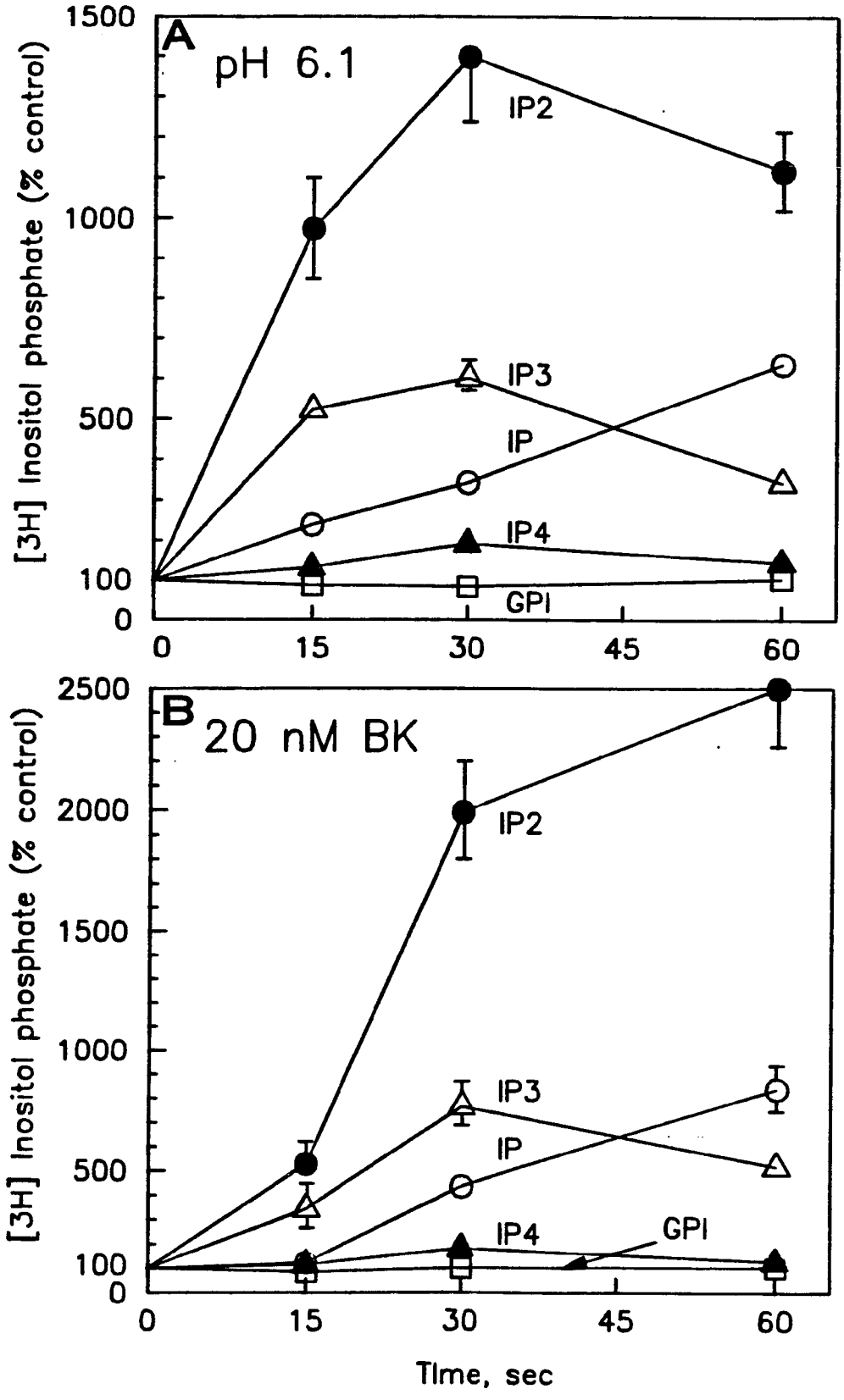

A
pH 6.1
IP2
IP3
IP
IP4
GPI
[3H] Inositol phosphate (% control)
1500
1000
500
100
0
0
15
30
45
60
B
20 nM BK
IP2
IP3
IP
IP4
GPI
[3H] Inositol phosphate (% control)
2500
2000
1500
1000
500
100
0
0
15
30
45
60
Time, sec

Figure 44. Effect of pertussis toxin on the increase in $[Ca^{2+}]_i$ evoked by decreasing $[Na^+]_o$ in human skin fibroblasts. Fibroblasts grown on cover glasses were incubated with 30 µg/ml pertussis toxin or pertussis toxin vehicle for 19 or 25 hr. Data are from three independent experiments that gave similar results. $[Ca^{2+}]_i$ measurements were made by Tao Zheng.

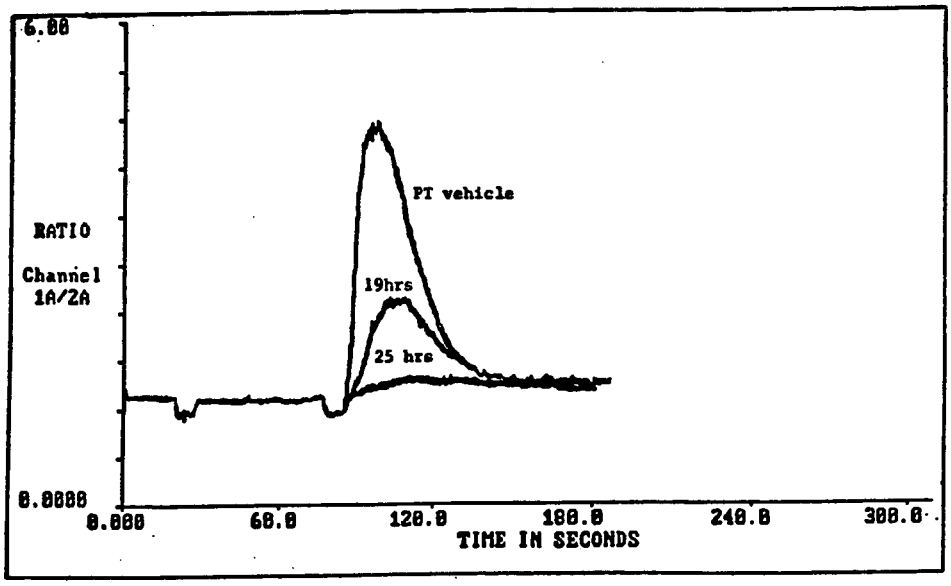
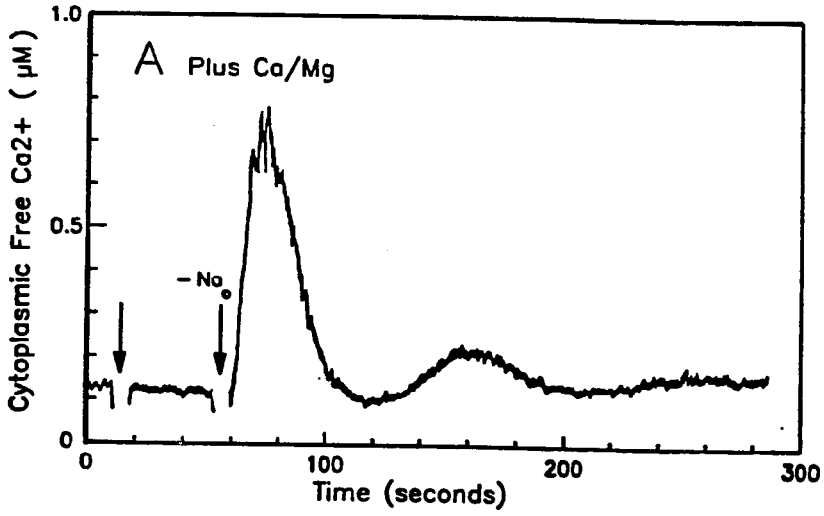

6.000
RATIO
Channel
1A/2A
0.0000
PT vehicle
19hrs
25 hrs
0.000
60.0
120.0
180.0
240.0
300.0
TIME IN SECONDS

Figure 45. Effect of extracellular Ca^{2+} and Mg^{2+} on the increase in $[Ca^{2+}]_i$ evoked by decreasing $[Na^+]_o$ in human skin fibroblasts. A, PSS was removed and replaced at the first arrow. At the second arrow ($-Na^+_o$), PSS was removed and replaced with choline PSS. Data are from one of four similar experiments with similar results. B, PSS was removed and replaced at the first arrow. At the second arrow and each subsequent arrow, Ca^{2+}/Mg^{2+}-free solutions containing 0.5 mM EDTA were used in the order: -Ca/Mg arrow, Ca^{2+}/Mg^{2+}-free PSS; $-Na^+_o$ arrow, Ca^{2+}/Mg^{2+}-free choline PSS; $+Na^+_o$ arrow, Ca^{2+}/Mg^{2+}-free PSS; A23187 arrow, 3 μM A23187 in Ca^{2+}/Mg^{2+}-free PSS. Data are from one of five experiments with similar results. $[Ca^{2+}]_i$ measurements were made by Dr. Jeff Smith.


A Plus Ca/Mg
$-Na_o$
Cytoplasmic Free Ca2+ (μM)
1.0
0.5
0
0
100
200
300
Time (seconds)

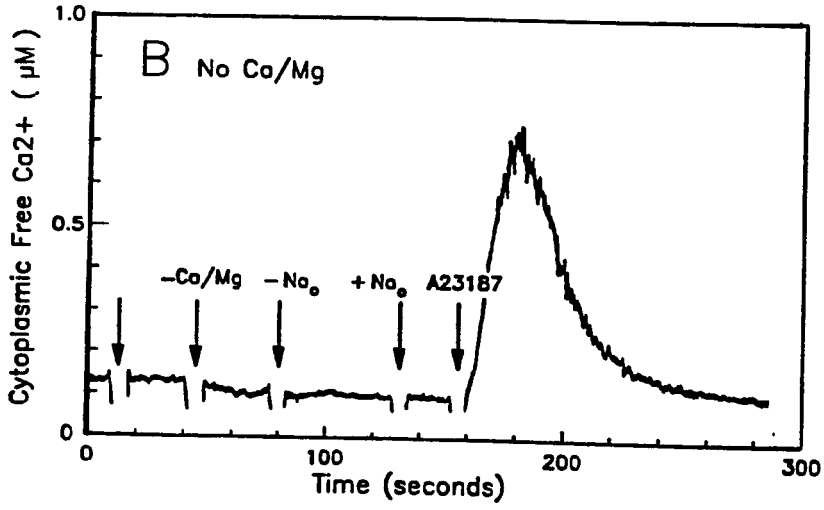
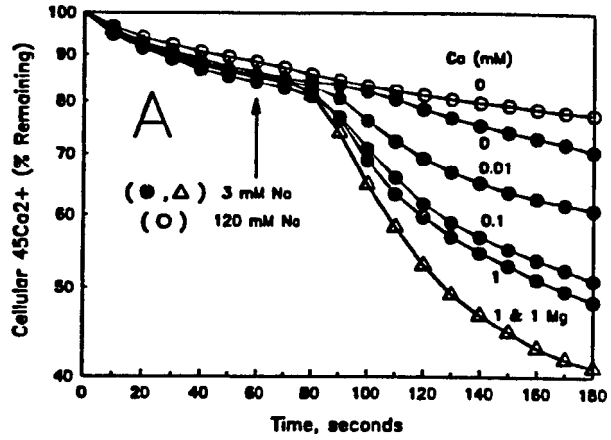
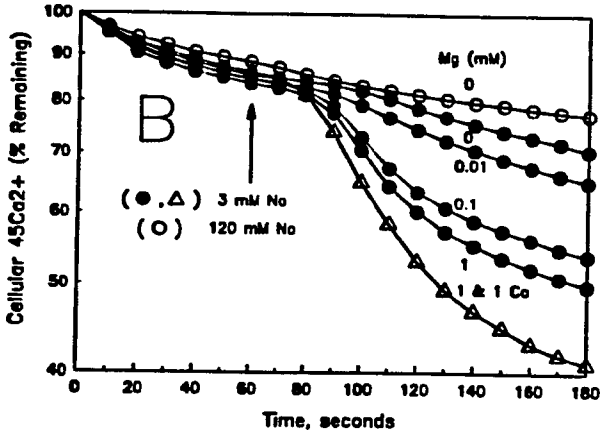

B No Ca/Mg
-Ca/Mg
$-Na_o$
$+Na_o$
A23187
Cytoplasmic Free Ca2+ (μM)
1.0
0.5
0
0
100
200
300
Time (seconds)

Figure 46. Effect of extracellular Ca^{2+} and Mg^{2+} on the stimulation of $^{45}Ca^{2+}$ efflux evoked by decreasing $[Na^+]_o$ in human skin fibroblasts. A, from 0 to 60 sec, $^{45}Ca^{2+}$ efflux was assayed in Ca^{2+}/Mg^{2+}-free PSS (unfilled circles), Ca^{2+}/Mg^{2+}-free PSS with the indicated concentration of Ca^{2+} (filled circles), or PSS (unfilled triangles). At 60 sec (arrow), the buffer was changed to Ca^{2+}/Mg^{2+}-free choline PSS the indicated concentration of Ca^{2+} (filled circles), choline PSS (unfilled triangles), or maintained in Ca^{2+}/Mg^{2+}-free PSS (unfilled circles) through 180 sec. B, from 0 to 60 sec, efflux was assayed in Ca^{2+}/Mg^{2+}-free PSS (unfilled circles), Ca^{2+}/Mg^{2+}-free PSS with the indicated concentrations of Mg^{2+} (filled circles), or PSS (unfilled triangles). At 60 sec (arrow), the buffer was changed to Ca^{2+}/Mg^{2+}-free choline PSS with the indicated concentration of Mg^{2+} (filled circles), choline PSS (unfilled triangles), or maintained in Ca^{2+}/Mg^{2+}-free PSS (unfilled circles). C, first-order rate constants were determined as described in Methods. The basal rate of $^{45}Ca^{2+}$ efflux (dashed line) was the same in the presence or absence of Ca^{2+} and Mg^{2+}. Statistical analysis by Student's t-test for unpaired observations showed: "a," significantly different from 0.01 mM Mg^{2+}, $p < 0.015$; "b," not different from 0 Mg^{2+}, $p < 0.09$; "c," not different from 0.1 mM Mg^{2+}, $p < 0.543$; "d," not different from 1 mM Mg^{2+}, $p < 0.345$. Values are the mean ± SE, n = 7-19.


A
Ca (mM)
0
0
0.01
0.1
1
1 & 1 Mg
(●,△) 3 mM Na
(○) 120 mM Na
Cellular 45Ca2+ (% Remaining)
Time, seconds


B
Mg (mM)
0
0
0.01
0.1
1
1 & 1 Ca
(●,△) 3 mM Na
(○) 120 mM Na
Cellular 45Ca2+ (% Remaining)
Time, seconds

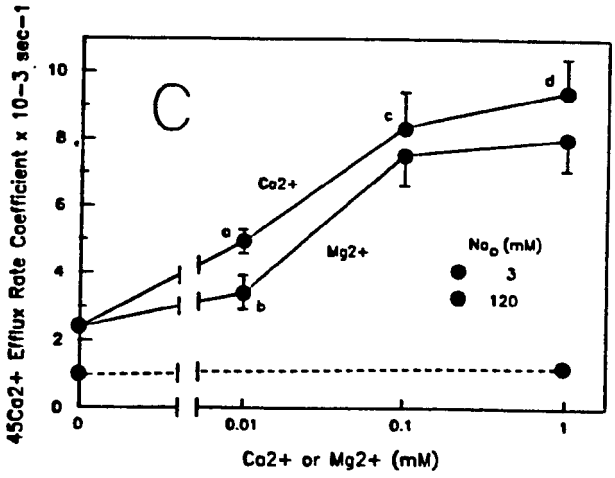

C
Ca2+
Mg2+
Nao (mM)
3
120
45Ca2+ Efflux Rate Coefficient x 10-3 sec-1
Ca2+ or Mg2+ (mM)

Figure 47. Effect of Ca^{2+} and Mg^{2+} on Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux in human skin fibroblasts. First-order rate constants were determined as described in Methods. Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux was measured in the presence (filled circles) and absence (unfilled circles) of 1 mM each Ca^{2+} and Mg^{2+}. Ca^{2+}/Mg^{2+}-free PSS was prepared by passing PSS without added Ca^{2+} or Mg^{2+} through a Chelex 100 column. Values are the mean ± SE, n = 3-6 from five different experiments. Statistical analysis was by Student's t-test for unpaired observations using STAT-PLAN III (The Futures Group, Glastonbury, CT). * = $p < 0.05$, ** = $p < 0.01$.

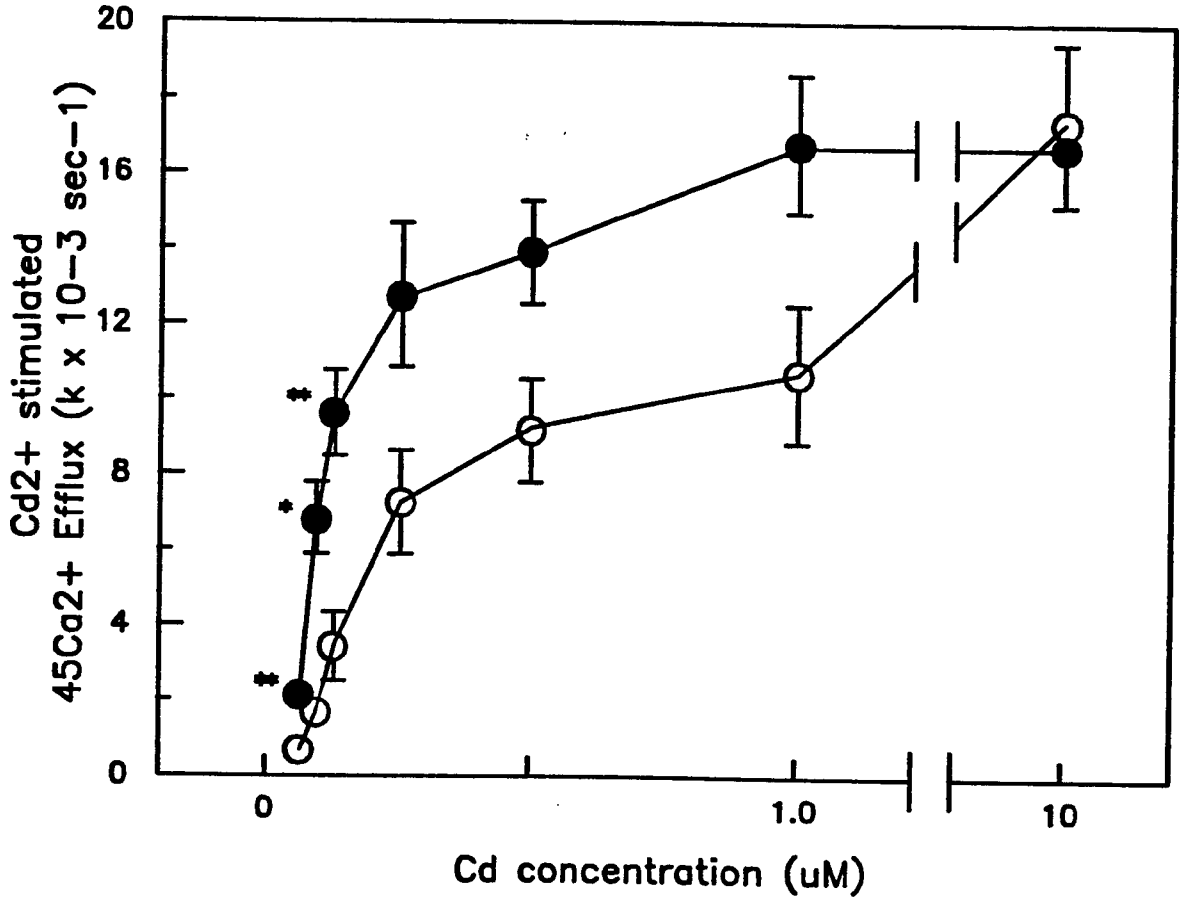

20
16
12
8
4
0
Cd2+ stimulated
45Ca2+ Efflux (k x 10-3 sec-1)
*
*
*
0
1.0
10
Cd concentration (uM)

Figure 48. Effect of Ca^{2+} and Mg^{2+} on bradykinin-stimulated $^{45}Ca^{2+}$ efflux in human skin fibroblasts. First-order rate constants were determined as described in Methods. Bradykinin-stimulated $^{45}Ca^{2+}$ efflux was measured in the presence (filled circles) and absence (unfilled circles) of 1 mM each Ca^{2+} and Mg^{2+}. Values are the mean ± SE, n = 4, from four experiments that gave similar results.

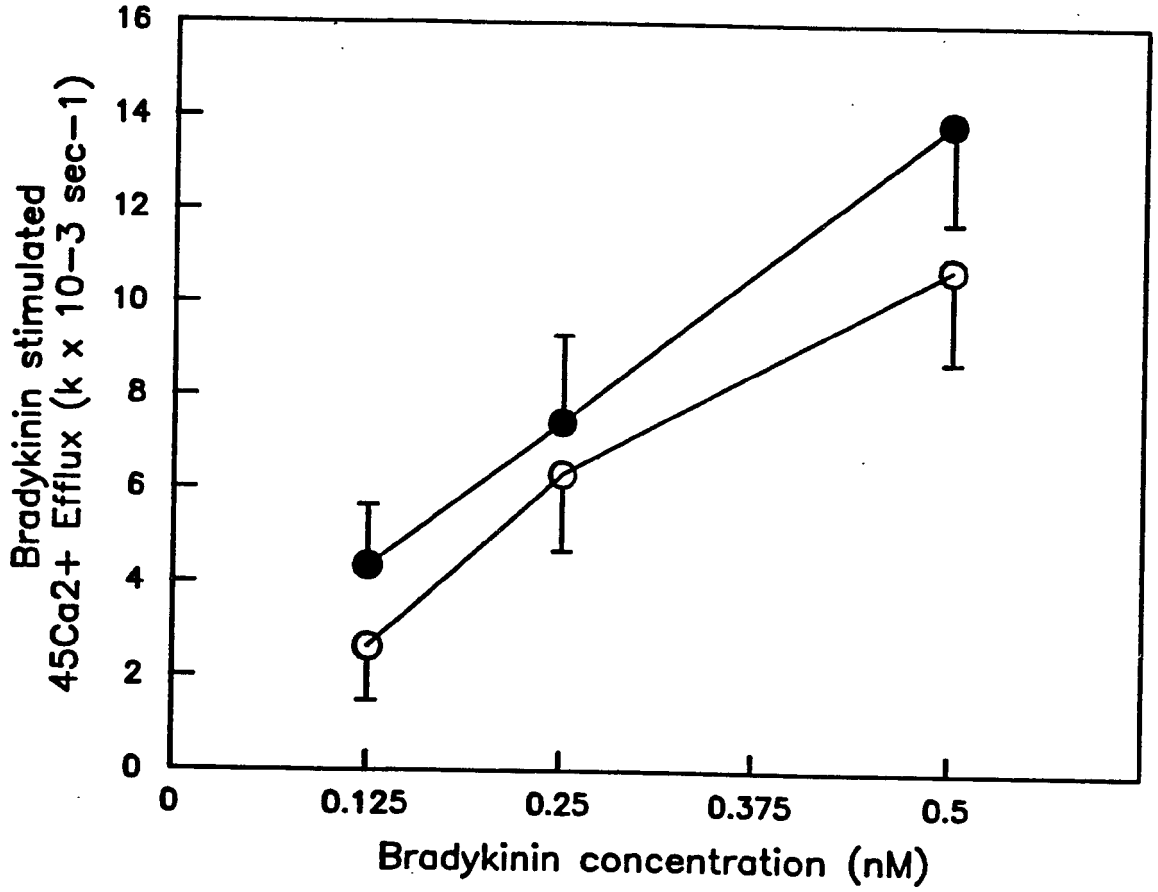

Bradykinin stimulated
45Ca2+ Efflux (k x 10−3 sec−1)
16
14
12
10
8
6
4
2
0
0
0.125
0.25
0.375
0.5
Bradykinin concentration (nM)

Figure 49. Effect of La^{3+} on the increase in $[Ca^{2+}]_i$ evoked by bradykinin or decreasing $[Na^+]_o$ in human skin fibroblasts. A, at the first arrow, PSS was removed and replaced. The stirring bar was started at the second arrow. At the third arrow, 200 nM bradykinin was added. Data are from one of three similar experiments, n = 6. B, at the first arrow, PSS was removed and replaced. At the second arrow, PSS was removed and replaced with choline PSS containin 1 mM La^{3+}. At the third arrow, 200 nM bradykinin was added. Similar results were observed when bradykinin was added to PSS containing 1 mM La^{3+}. Data represent five experiments with choline PSS, n = 18, and three experiments with bradykinin, n = 9. $[Ca^{2+}]_i$ measurements were made by Dr. Jeff Smith.

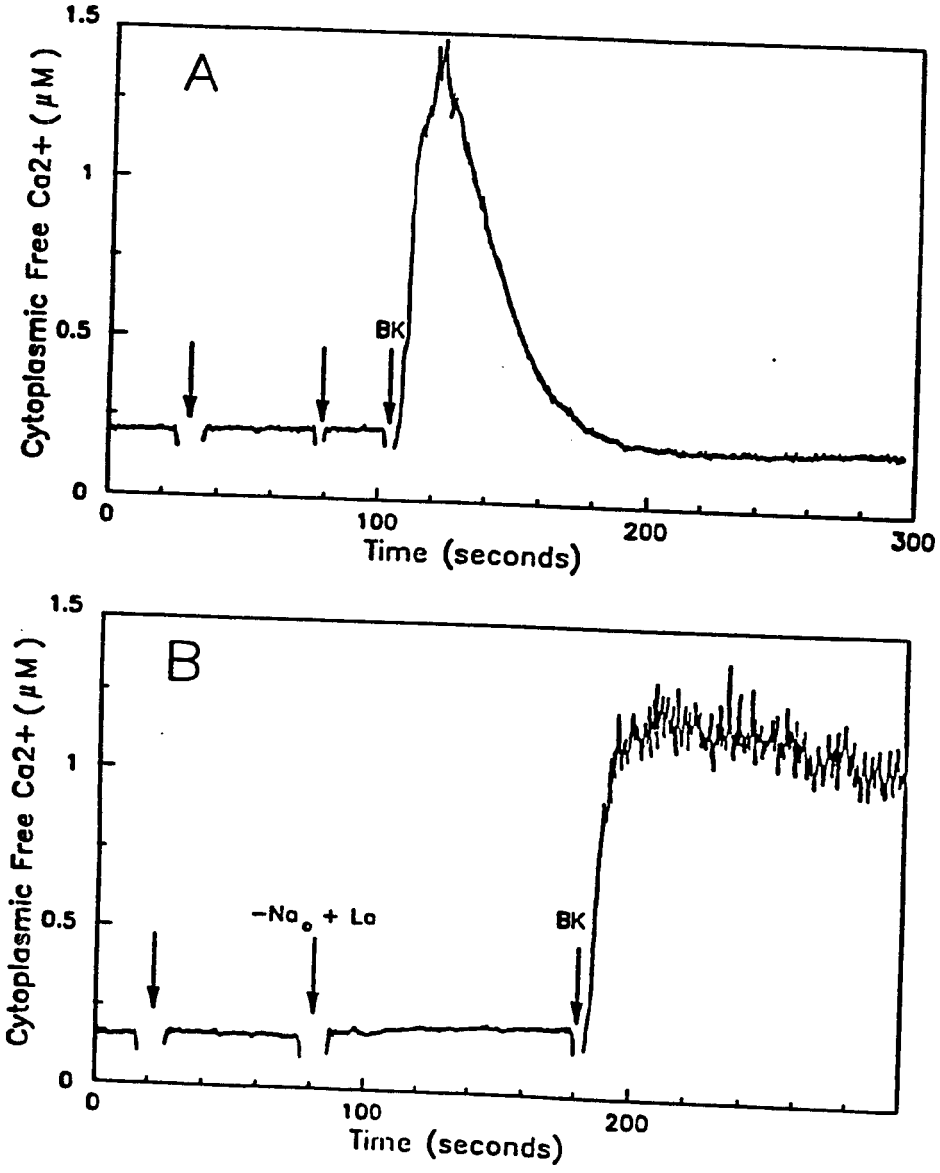

A
Cytoplasmic Free Ca2+ (μM)
1.5
1
0.5
0
BK
0
100
200
300
Time (seconds)
B
Cytoplasmic Free Ca2+ (μM)
1.5
1
0.5
0
−Na$_o$ + La
BK
0
100
200
Time (seconds)

Figure 50. Concentration dependence of the effect of La^{3+} on the increase in $[Ca^{2+}]_i$ produced by decreasing $[Na^+]_o$ in human skin fibroblasts. At the first arrow, (La), PSS was removed and replaced with PSS containing 0 to 100 μM La^{3+}, as indicated. At the second arrow, ($-Na^+_o$), the buffer was changed to choline PSS containing the indicated concentration of La^{3+}. Data are from one experiment, n = 5, no La^{3+}; n = 3, 5 μM La^{3+}; n = 3, 10 μM; n = 2, 20 μM; n = 2, 100 μM. $[Ca^{2+}]_i$ measurements were made by Dr. Jeff Smith.

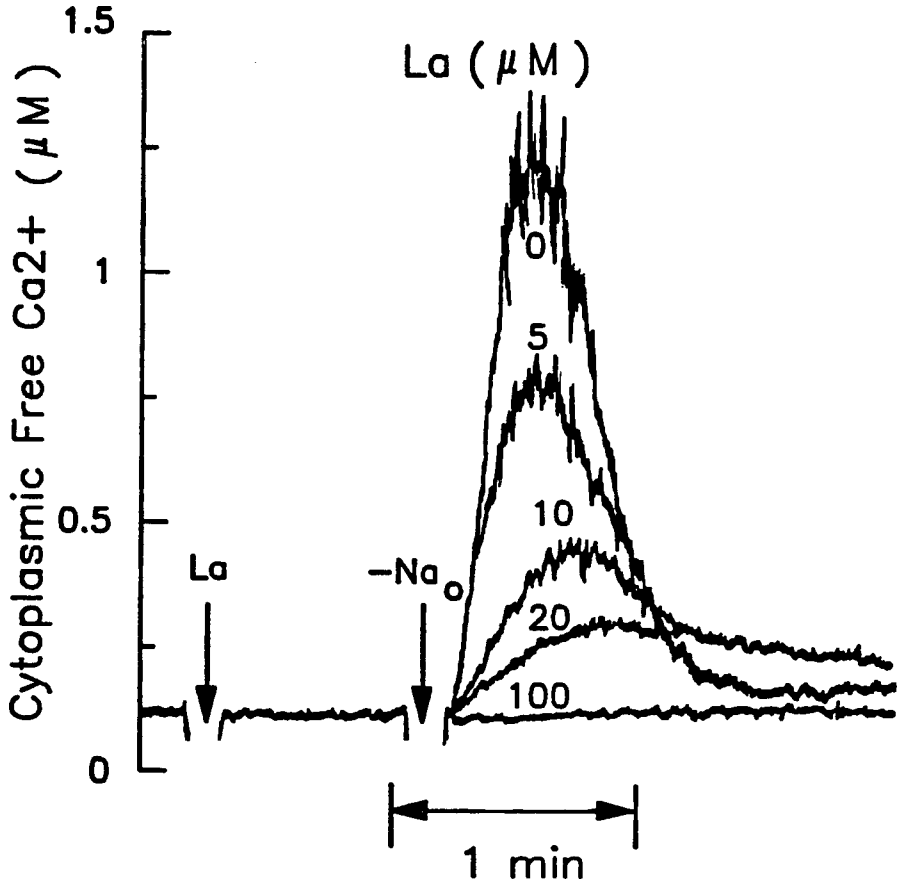
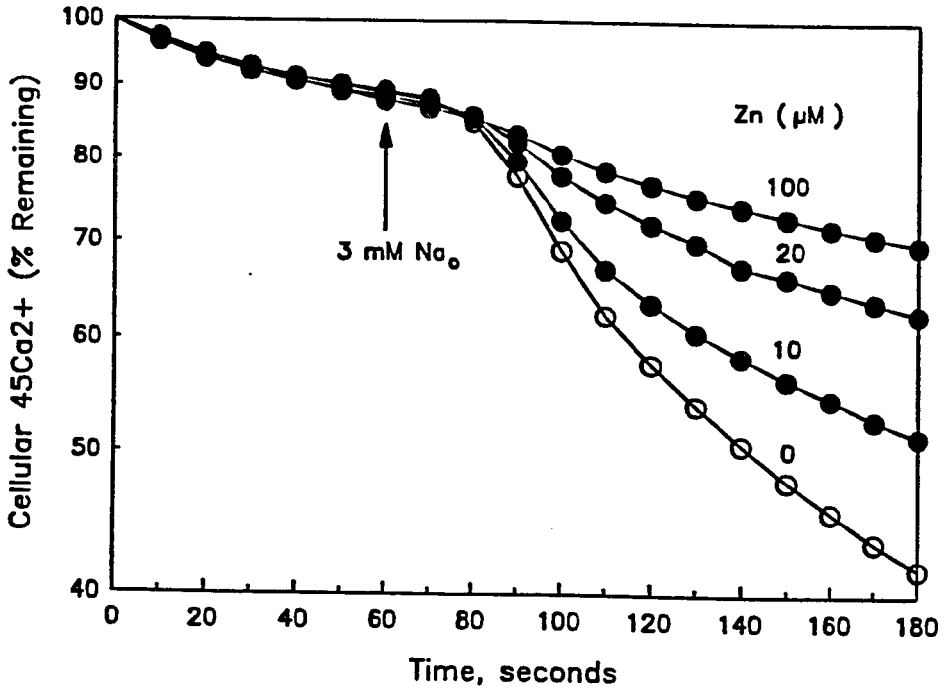

1.5
1
0.5
0
Cytoplasmic Free Ca2+ (μM)
La (μM)
0
5
10
20
100
La
−Na o
1 min

Figure 51. Effect of Zn^{2+} on the stimulation of $^{45}Ca^{2+}$ efflux by decreasing $[Na^+]_o$ in human skin fibroblasts. From 0 to 60 sec, $^{45}Ca^{2+}$ efflux was assayed in PSS with (filled circles) or without (unfilled circles) the indicated concentration of Zn^{2+}. At 60 sec, (arrow), the efflux buffer was changed to choline PSS and the indicated concentration of Zn^{2+}. Values are the mean ± SE from four experiments, n = 6, no Zn^{2+}; n = 3, 10 μM Zn^{2+}; n = 3, 20 μM; n = 5, 100 μM. Error bars are absent because SE did not exceed the size of the symbols.


Cellular 45Ca2+ (% Remaining)
100
90
80
70
60
50
40
0
20
40
60
80
100
120
140
160
180
Time, seconds
3 mM Na$_o$
Zn (μM)
100
20
10
0

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