Calcium, Lanthanum, Pyrophosphate, and Hydroxyapatite: A Comparative Study in Fibroblast Mitogenicity[1] (42826)

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Abstract. **Calcium-containing crystals and elevated levels of calcium chloride ($CaCl_2$) and lanthanum chloride ($LaCl_3$) have been previously reported to enhance the proliferative activity of cultured fibroblasts. We have investigated the relative mitogenicity of these agents, whether they function via precipitation on the cell surface and whether they interact with one another. Confluent cultures of newborn foreskin fibroblasts provided with fresh medium containing 10% fetal bovine serum (FBS) in the presence of hydroxyapatite (HA), pyrophosphate (PP_i), $LaCl_3$ (La), or additional $CaCl_2$ (Ca) were all stimulated more than control cultures provided with fresh medium and 10% FBS alone as assessed by cell counts 5 days later. Increases in cell yield above the original confluent cell density were 316% for La, 271% for Ca, 189% for HA, 131% for PP_i, and 45% for controls. Addition of fresh medium containing 10% FBS and epidermal growth factor or fresh medium containing 20% FBS as additional points of reference yielded increases of 204 and 107%, respectively, over original confluent density. Stimulation induced by La or Ca was significantly greater ($P < 0.001$) than the stimulations induced by each of the other treatments. The same treatments added to confluent cultures without a change of medium also renewed mitotic activity, with La and Ca again the most mitogenic and approximately doubling the pretreatment cell yields. Cultures incubated in an inverted position to avoid cell contact with precipitates in the medium were also stimulated by La and Ca, but not by HA and PP_i. When added to confluent cultures simultaneously supplemented with optimal additional Ca, La decreased Day 5 cell yields in a dose-dependent manner at low concentrations (0.03–0.2 m*M*) but increased cell yields over those obtained with 0.2 m*M* $LaCl_3$ again in a dose-dependent manner at higher concentrations. Thus, while HA and PP_i act via precipitation on the cell surface, the more mitogenic agents La and Ca function in solution and appear to stimulate cell division by different nonadditive mechanisms. These findings suggest multiple mechanisms of membrane participation in mitogen responsiveness and in density-dependent inhibition of growth.** [P.S.E.B.M. 1989, Vol 190]

Density-dependent inhibition of growth is a characteristic behavior of cultured diploid fibroblasts that is altered by cellular aging *in vitro* (1, 2) but not expressed by cultured malignant or malignantly transformed cells (3, 4). Hence, although its mechanism is poorly understood, density-dependent growth inhibition can be assumed to reflect important aspects of cellular growth regulation.

Recent studies with normal human fibroblasts show that elevating extracellular calcium concentration from 2 to 4–5 m*M* enhances both saturation density and the response of confluent cultures to serum stimulation (5, 6). In fact, serum-stimulated confluent foreskin-derived fibroblasts in the presence of additional calcium chloride ($CaCl_2$) show an increase in cell yield that requires multiple rounds of cell division by a sizable fraction of the cell culture (5). Previous studies by other investigators using a wide variety of experimental approaches have demonstrated similar effects for calcium precipitate formed by the introduction of pyrophosphate (PP_i) (7) or the insoluble calcium crystal hydroxyapatite (HA) (8) or lanthanum chloride ($LaCl_3$), even when presented to cells only briefly under cell culture conditions adjusted so as to prevent precipitation (9). The mechanism by which these agents exert their effects is largely unknown, however. PP_i precipitate has been shown to require cell surface contact for its mitogenicity (10), whereas endocytosis of HA may

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be necessary for its mitogenicity (8). Because La has been shown not to enter a variety of tissues and intact cultured cells [heart cells and fibroblasts (11), heart muscle (12), skeletal muscle, (13), pancreatic islets, (14) red blood cells (15), or chondrocytes, (16)], its mitogenic effect is presumably exerted at the cell surface. Whether the mitogenicity of elevated extracellular Ca is due to formation of a precipitate on the cell surface has not been clarified.

In order to clarify the mode of action of additional $CaCl_2$, and specifically to determine whether it is similar to $LaCl_3$ or the more extensively studied HA and PP_i, we conducted studies comparing the mitogenic effects of these agents. Herein, we report differences in maximal amount of stimulation, the extent to which cell contact with precipitate is necessary for stimulation, and evidence for a complex interaction between the proliferative responses induced by La and additional Ca.

Materials and Methods

Cell Culture. Fibroblast explant cultures were established from foreskin specimens of term infants as previously described (5, 17). Explants were provided fresh medium weekly and harvested at confluence, approximately 4 weeks after plating. Fibroblasts were then serially passaged one to five times prior to experimental use.

Fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DME) (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD, and Hyclone Laboratories, Inc., Logan, UT) at 37°C in a humidified 95% air-5% CO_2 atmosphere. This serum-supplemented medium contained approximately 2 m*M* extracellular calcium, based on the proportional amounts of Ca supplied by DME, as specified by the manufacturer (0.9 of 1.8 m*M*, or 1.62 m*M*), and serum, as determined by atomic absorption spectroscopy of several serum lots (10% of 2.9–3.9 m*M*, or 0.29–0.39 m*M*). At each passage, cells were detached from culture vessels with a Ca^{2+}-Mg^{2+}-free 0.25% trypsin solution; during experiments, cells were detached and counted in a solution containing 0.25% trypsin and 0.1% EDTA.

Experimental Design. Fibroblasts were seeded at 10^4 cells/cm^2, equivalent to 90,000 cells per 35-mm dish (Falcon, Oxnard, CA), in 2 ml of serum-supplemented DME and grown to confluence without a change of medium. Confluent monolayer cultures were then provided with 2 ml of fresh DME supplemented with 10% FBS (v:v). With the change in medium, the monolayers received 20-μl aliquots from stocks of $CaCl_2$ (ACS grade; J. T. Baker, Phillipsbury, NJ), $LaCl_3$ (ACS grade; Baker), $Na_4P_2O_7$ (ACS grade; Aldrich, Milwaukee, WI), or HA (Aldrich) maintained in deionized distilled water at 100 times the desired concentration. HA, an insoluble crystal, was vortexed to form a suspension immediately before aliquoting to each dish.

Epidermal growth factor (EGF; Collaborative Research, Waltham, MA) or 20% FBS was added at confluence to some cultures as a mitogenic stimulus. For acidification of the medium, appropriate aliquots of 1 *M* HCl were added to the medium and the medium was kept in a sealed 50-ml plastic centrifuge tube until cultures were refed. Acidified medium was quickly placed on cultures, appropriate treatments added, and cultures promptly placed in a 5% CO_2 incubator. Additional $CaCl_2$ was aliquoted before $LaCl_3$ so as to minimize the effect La^{3+} might have on inhibition of the formation of Ca^{2+}-induced precipitation by acidified medium. The pH of the medium ranged from 7.37 to 7.60 for basal medium (DME + 10% FBS) and 6.13 to 6.47 for medium to which 16 m*M* HCl had been added. Within 2 hr after being placed in the 5% CO_2 incubator, medium acidified with 16 m*M* HCl was approximately 0.2 pH units lower than basal medium. Typically, after 24 hr, the pH of acidified medium was 7.3, while the pH of basal medium was 7.5.

For experiments involving cell growth in 25-cm^2 flasks (Falcon), 2.5×10^5 cells were seeded per flask into 10 ml of medium. On reaching confluence 7–12 days later, flasks were totally filled with medium (approximately 64 ml) and 0.64-ml aliquots of treatment were added while the flasks were upright. The flasks were then placed either in an upright position so that the cell sheet was on the bottom below the medium, or in an inverted position so that the cell sheet was at the top facing down into the medium. Flasks were placed in a 5% CO_2 incubator at 37°C and cell yields determined 7 days later. Cell number of duplicate cultures was determined electronically with a Coulter counter Model ZM (Coulter Electronics, Inc., Hialeah, FL).

Statistical Analysis. Statistical significance was determined by the Newman-Keuls test for pairwise comparisons and by the *t* test for differences between paired means. For evaluating levels of significance from several experiments, a technique proposed by Sokal and Rohlf (18) was used.

Results

Microscopic Observation of Precipitation. HA, an insoluble crystal, rapidly settled onto the monolayer surface. Clumps of precipitated material appeared to vary markedly in size, as observed under phase microscopy at 100×, although no attempt was made to formally measure the clumps. PP_i and La are known to form precipitates upon being introduced to the medium, but most of the PP_i-induced precipitate and, to a lesser extent, the La-induced precipitate appeared to remain suspended in the medium. Additions of up to 5 m*M* $CaCl_2$ did not result in an immediately visible precipitate, but after several days there was a scattering of precipitate on the monolayer surface without any

observable floating precipitate. Additions of 1 m*M* $CaCl_2$, although stimulatory, rarely formed a visible precipitate. In general, the amount of precipitate formed by the addition of 3 m*M* $CaCl_2$ appeared to be less than that formed by 10 μg/ml HA, a minimally mitogenic supplement.

Proliferative Response of the Cultures. The optimal dose for each treatment was determined by preliminary studies (data not shown) and resulted in significant ($P < 0.001$) increases in growth relative to control cultures in either the presence or absence of serum stimulation (Fig. 1). Concentrations of $PP_i < 1$ m*M* occasionally stimulated less well than did 1 m*M*, whereas 2 m*M* PP_i resulted in a lower cell yield; HA was equally mitogenic over the dose range 100–500 μg/ml. $LaCl_3$ and additional $CaCl_2$ resulted in the greatest and second greatest increase in cell yield, respectively, in each of the five experiments in both the presence and absence of serum stimulation. The well-characterized mitogen EGF and additional serum (20% FBS with change of medium or 0.2 ml of FBS added to cultures without a change of medium), used for comparative purposes, resulted in increased growth, but cell yields were less than those of cultures stimulated by $LaCl_3$ or additional $CaCl_2$. Increases in growth for refed cultures were 316% for La, 271% for Ca, 204% for EGF, 189% for HA, 131% for PP_i, 107% for 20% FBS, and 45% for controls. In particular, cultures treated with La or Ca showed highly significant ($P < 0.001$) increases in cell yield relative to each of the other treatments. The differences in cell yields for the several treatments with refed cultures are greater than with non-refed cultures, probably because depleted medium cannot support further growth as well as fresh medium.

Growth of Cells in Inverted Cultures. The presence of obvious amounts of La-induced precipitate and, to a lesser extent, precipitate induced by additional $CaCl_2$ raises the question of whether the stimulation observed by these agents is due to the accompanying precipitate or to the ions in the solution. To address that question, confluent cultures in both upright and inverted positions were exposed to additional $CaCl_2$, $LaCl_3$, HA, and PP_i. Loss of stimulation in the inverted position would indicate a need for cell contact by precipitates for the agents to be mitogenic, whereas a stimulatory effect in the inverted position would indi-

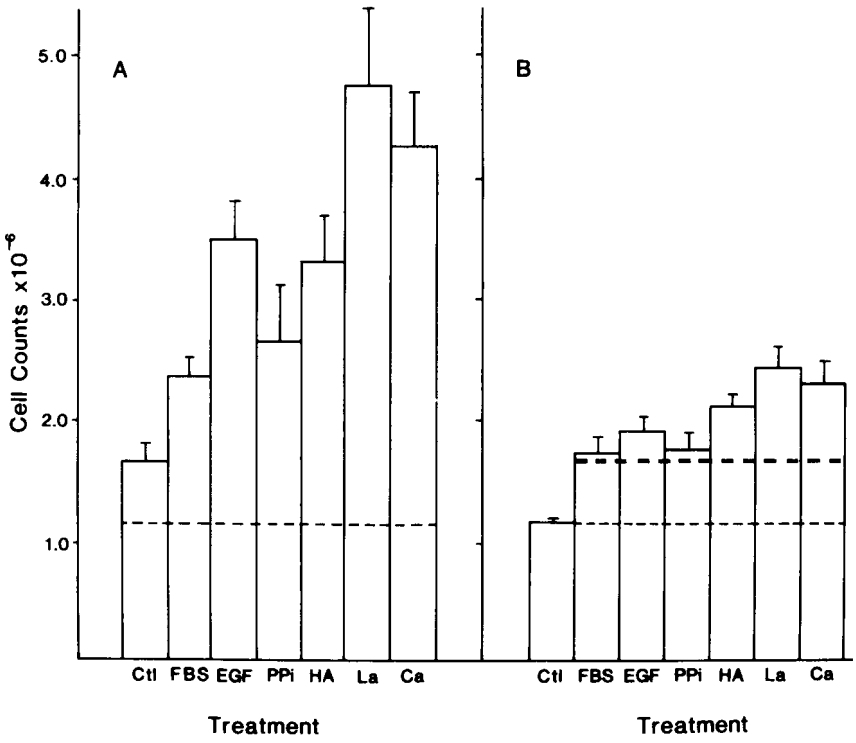

Figure 1. Response of confluent cultures to $CaCl_2$, $LaCl_3$, $Na_4P_2O_7$, and hydroxyapatite in the presence or absence of fresh serum-containing medium. Control cultures (ctl) received fresh DME plus 10% FBS (A) or no medium change (B). Treatments provided with the fresh DME and 10% FBS (A) or alone (B) were 3 m*M* additional $CaCl_2$ (Ca), 0.75 m*M* $LaCl_3$ (La), 100 μg/ml HA, 1 m*M* $Na_4P_2O_7$ (PP_i), 50 ng/ml EGF, and an additional 10% FBS in the medium change in A or 0.2 ml of FBS alone in B, both equivalent to 20% FBS. Cell yields were determined in paired dishes at refeeding when treatments were introduced (Day 7) and 5 days later at the end of the experiment. Bar heights are mean ± SEM of duplicate dishes in five experiments. EGF and additional FBS were tested in only four of the five experiments. Average cell yield at the time of refeeding is indicated by the dashed line. The heavy dashed line in B represents the cell yield obtained by the refed control cultures. Highly significant differences ($P < 0.001$) in cell yields exist between La or Ca and HA, EGF, PP_i and 20% FBS; between HA and 20% FBS or PP_i; and between EGF and 20% FBS. Significant differences ($P < 0.01$) also exist between La and Ca and between EGF and PP_i. No differences exist between EGF and HA or between 20% FBS and PP_i ($P > 0.05$). All treatments give highly significant increases in cell yield relative to controls ($P < 0.001$).

cate that the agents function in solution, regardless of how much precipitation is formed.

Ca- and La-treated cultures showed significant increases ($P < 0.001$) in cell yields compared with controls in both an upright and inverted position (Fig. 2). Percentage of stimulations relative to control cultures for cultures treated with Ca and La were 119 and 113% for upright cultures and 106 and 116% for inverted cultures, respectively. In contrast, cultures treated with HA and PP_i were not stimulated in an inverted position, although they did show stimulation in the upright position. In the case of PP_i, the loss of stimulation occurred in the presence of a visible floating precipitate, implying that floating precipitate is not mitogenic and/or it does not adhere to the surface of the inverted monolayer. The control cultures grew equally well upright or inverted. The different volumes of growth medium per cm^2 of available culture surface area for the filled T-25 flasks vs the 35-mm petri dishes, 2.6 ml vs 0.22 ml, probably accounts for the absolute differences in stimulation of control cultures: 210 and 198% for upright and inverted control cultures in filled flasks (Fig. 2) vs 45 and 55% in control cultures in petri dishes (Figs. 1, 3).

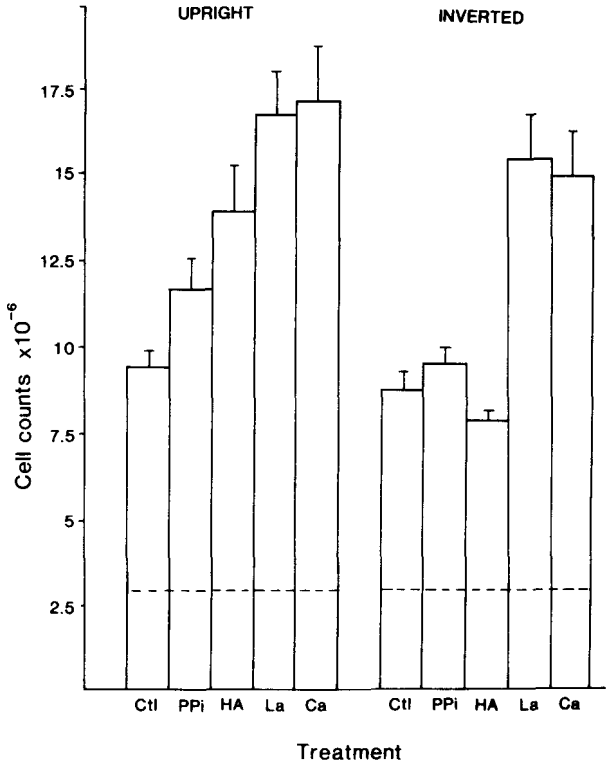

Figure 2. Response of fibroblast cultures in upright or inverted position to $CaCl_2$, $LaCl_3$, PP_i, and HA. Flasks containing confluent cultures were filled with medium and placed in the upright (A) or inverted position (B). Untreated controls (Ctl) were compared with cultures receiving 3 m*M* additional $CaCl_2$ (Ca), 0.75 m*M* $LaCl_3$ (La), 1 m*M* $Na_4P_2O_7$ (PP_i), or 100 μg/ml HA. Cell yields were determined in paired flasks at refeeding and 7 days later. Bar heights are mean cell yield ± SEM per treatment group for five experiments. Average cell yield at the time of refeeding is indicated by a dashed line. All treatments gave significant increases in cell yield relative to controls when cultures were in an upright position ($P < 0.001$ for Ca, La, and HA: $P < 0.005$ for PP_i) but only La- and Ca-treated cultures were significantly stimulated in an inverted position ($P < 0.001$).

Interaction between Ca and La. A biphasic pattern of stimulation is observed with combined doses of $CaCl_2$ and $LaCl_3$ (Fig. 3). Doses of $LaCl_3$ that provide suboptimal stimulation if added alone to confluent cultures, when combined with 3 m*M* or 5 m*M* $CaCl_2$ result in La-dose-dependent decreases in growth relative to that obtained with $CaCl_2$ alone. In the case of 3 m*M* $CaCl_2$ plus 0.2 m*M* $LaCl_3$, there is a significant ($P < 0.01$) decrease in growth relative to the growth observed with either 3 m*M* $CaCl_2$ or 0.2 m*M* $LaCl_3$ alone. However, higher concentrations of $LaCl_3$ in the presence of an additional 3 m*M* $CaCl_2$ reverse the pattern of response and result in a La-dose-dependent increase in growth. The growth obtained with 3 m*M* $CaCl_2$ plus 0.75 m*M* $LaCl_3$ or 1.0 m*M* $LaCl_3$ is significantly greater ($P < 0.01$) than the growth obtained with 3 m*M* $CaCl_2$ plus 0.2 m*M* $LaCl_3$. Hence, the proliferative processes induced by $LaCl_3$ and additional $CaCl_2$ are at least in part mutually antagonistic, but La does not serve simply as an inhibitor of the Ca effect.

Effect of Acidified Medium. The stimulatory effect of 0.01 m*M* $LaCl_3$ is not affected by the acidification of the medium and the stimulation induced by an additional 3 m*M* $CaCl_2$ is drastically reduced (Fig. 4). In contrast, the stimulatory effect of 3 m*M* $CaCl_2$ in the presence of 0.01 m*M* $LaCl_3$ is largely preserved in acidified medium: 365% in acidified medium vs 472% in basal medium. Indeed, the stimulation obtained with 3 m*M* $CaCl_2$ plus 0.01 m*M* $LaCl_3$ in acidified medium is more than twice the sum of the stimulations obtained by the separate treatments (365% vs 41% + 129% = 170%). These results suggest that the inhibitory effect of increased hydrogen ion concentration on Ca-induced stimulation is to a large extent blocked by the presence of a minute amount of $LaCl_3$. A 0.2 m*M* concentration of $LaCl_3$, which normally results in precipitate, does not precipitate in acidified medium, but nevertheless induces on average an increase in stimulation in acidified medium relative to that observed with 0.2 m*M* $LaCl_3$ in basal medium (data not shown). Considered in combination with the data presented in Figure 2, these results strongly suggest that neither $LaCl_3$ nor $CaCl_2$ need form a precipitate in order to be mitogenic. Rather, La and Ca appear to interact in a complex fashion with H^+ and possibly other cations at the cell surface.

Discussion

We have demonstrated that additional $CaCl_2$, $LaCl_3$, HA, and PP_i markedly enhance the proliferative response of confluent human fibroblast cultures to serum stimulation. Cultures treated with La or Ca attain significantly higher densities than do cultures

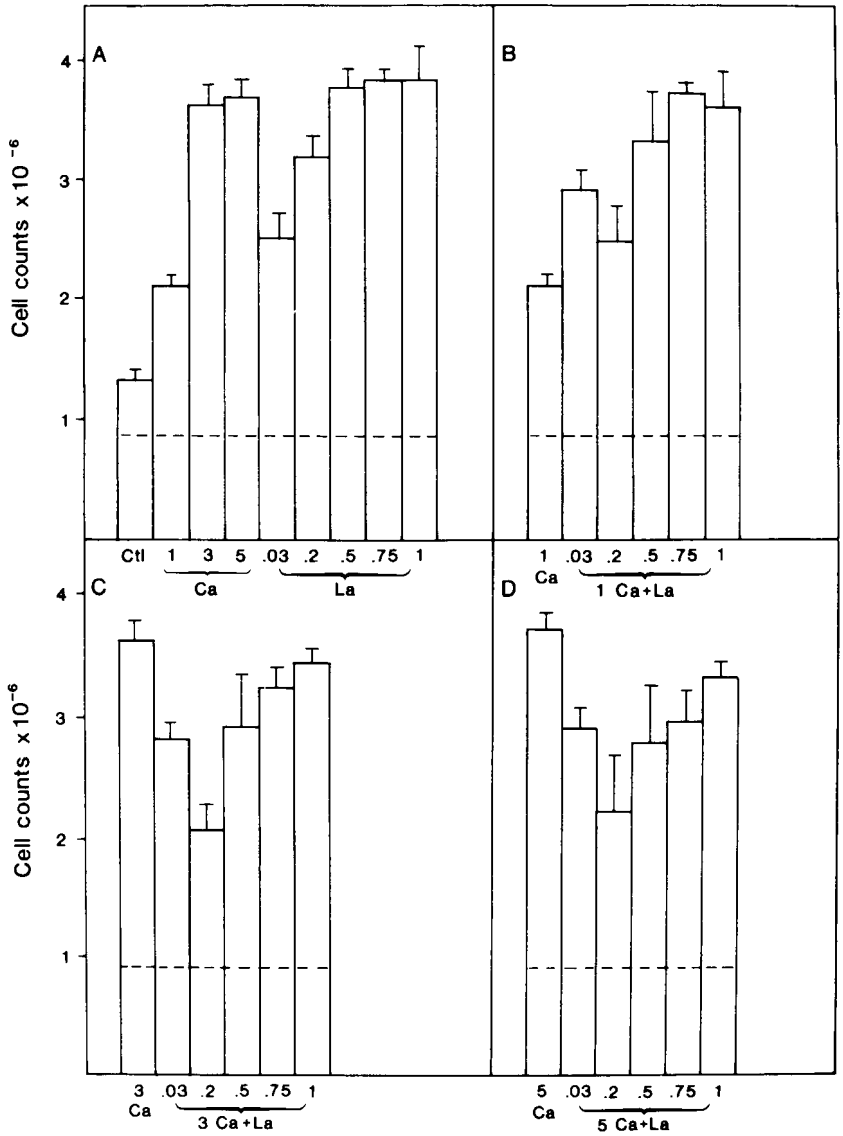

Figure 3. Effect of combined $LaCl_3$ and additional $CaCl_2$ on serum- stimulated confluent fibroblast cultures. Confluent cultures received a fresh medium change at Day 7 without additional treatment (Ctl) or with an additional 1, 3, or 5 m*M* $CaCl_2$ (1 Ca, 3 Ca, 5 Ca), or 0.03, 0.2, 0.5, 0.75, or 1 m*M* $LaCl_3$ (0.03 La, 0.2 La, 0.5 La, 0.75 La, 1 La), or combinations of the $CaCl_2$ and $LaCl_3$ doses: *A*, Ctl plus each separate treatment; *B*, 1 Ca alone and combined with each La dose; *C*, 3 Ca alone and combined with each La dose; *D*, 5 Ca alone and combined with each La dose. Cell yields were determined at refeeding and 5 days after refeeding. Bar heights are mean cell yields ± SEM for duplicate dishes in six experiments. Additions of 5 m*M* CaCl and 0.5 and 1.0 m*M* $LaCl_3$ were tested in only four of the six experiments. Average cell yield at refeeding is indicated by a dashed line.

treated with HA or PP_i, or with EGF or additional FBS, suggesting a different mechanism of action. Addition of these agents to cultures without a change of medium results in renewed mitotic activity that is as great in the case of HA and PP_i, or greater in the case of La and Ca, than the mitotic activity observed with the addition of 50 ng/ml EGF or 10% additional fresh serum. Those agents that produce more than a doubling of the original confluent density logically stimulate more than a single round of cell division. Furthermore, while HA and PP_i act as precipitates on the cell surface, Ca and La function in solution. These findings are consistent with a major role for the cell membrane in mitogen responsiveness and in density-dependent growth inhibition and suggest multiple mechanisms of membrane participation in these processes.

Rubin and Sanui (7) discovered the mitogenic effect of PP_i using an established 3T3 mouse cell line. Smith and Smith (9) subsequently reported that $LaCl_3$ was mitogenic for 3T3 cells in the presence of insulin and found a degree of stimulation relative to quiescent controls similar to that observed in the most comparable assays in the present experiments. Cheung and McCarty (8) used confluent human fibroblasts to demonstrate the mitogenicity of HA crystals in the presence of 1% calf serum, and Cheung *et al.* (19) reported comparable stimulation of quiescent cultures by 10% calf serum and by 1% calf serum plus 100 μg/ml HA.

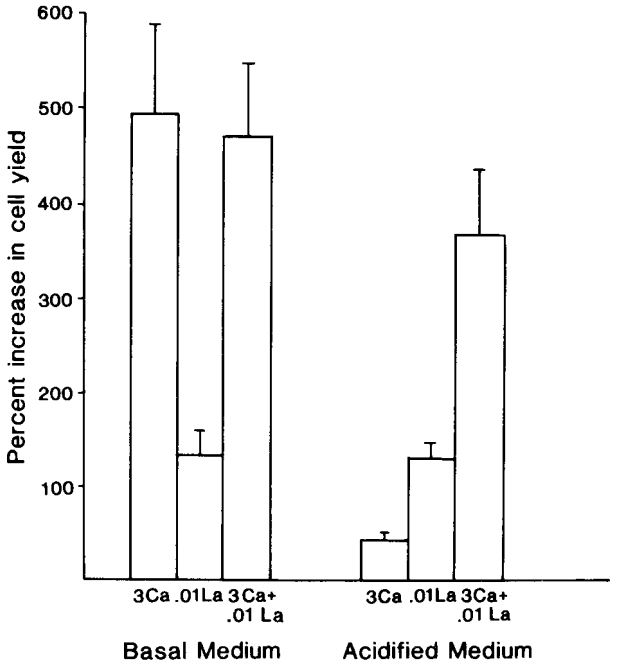

Figure 4. Recovery of $CaCl_2$ induced stimulation in acidified medium. Confluent fibroblast cultures received a fresh medium change at Day 7 in the presence or absence of 16 m*M* HCl without additional treatment (control) or with an additional 3 m*M* $CaCl_2$ (3 Ca), 0.01 m*M* $LaCl_3$ (0.01 La), or a combination of the two treatments (3 Ca + 0.01 La). Cell yields were determined at refeeding and 5 days after refeeding. The percentage of increase in cell yield was determined as difference in cell yield between the cultures refed with and without additional treatments divided by the difference in cell yield before (Day 7) and after (Day 12) refeeding of the control cultures, 100×: [(treatment − control)/(control − Day 7)] × 100. Bar heights are the mean percentage of increase in cell yield ± SEM for duplicate dishes in six experiments. Day 7 cell yields were $8.79 \pm 0.62 \times 10^5$ (mean ± SEM): average cell yields of controls in fresh medium in the presence and absence of 16 m*M* HCl were $13.11 \pm 0.86 \times 10^5$ and $13.63 \pm 0.88 \times 10^5$, respectively. The percentage of increases in cell yield of the combined treatments (3 Ca + 0.01 La) in acidified medium was significantly greater than the sum of the percentage of increases in cell yield of the separate treatments ($P < 0.01$).

Despite different cell culture conditions and measurements of proliferation, the optimal dose of 100 μg/ml HA in the present report is similar to that observed previously (19). As well, we found that confluent fibroblasts respond equally well to EGF and HA, meanwhile Cheung *et al.* (20) found 15 ng/ml platelet-derived growth factor (PDGF) and 100 μg/ml HA were equally stimulatory. The comparability of these findings is further supported by the independent observation that EGF and PDGF are equally mitogenic for confluent human fibroblasts (21).

Hydroxyapapatite and pyrophosphate crystals in the present study and PP_i previously (10) have been shown by comparison of inverted and upright cultures to act via a surface precipitate. The ability of $LaCl_3$ and additional $CaCl_2$ to stimulate inverted cultures, whereas HA and PP_i do not, indicates that La and Ca do not function via the settling of precipitate on the cell surface. Lanthanum has previously been shown with mouse Swiss 3T3 cells to function mitogenically in solution (9). Our results extend this effect to human fibroblasts and suggest that Ca also functions mitogenically in solution. The possibility that La-induced precipitate may also be mitogenic is not examined.

The results of the experiments combining additional $CaCl_2$ and $LaCl_3$ were not anticipated. Added separately to the cultures, both additional $CaCl_2$ and $LaCl_3$ increased cell yields compared with controls. Hence, the $LaCl_3$ effect appears additive to the $CaCl_2$ effect in the basal medium. However, in the presence of higher $CaCl_2$ concentrations (5 and 7 m*M*) that are themselves maximally stimulatory, $LaCl_3$ supplementation initially appears inhibitory, in that cell yields are lower than in the $CaCl_2$-treated controls; while at higher $LaCl_3$ concentrations, cell yields again increase in a dose-dependent fashion. To our knowledge, this type of dose-dependent interaction between mitogenic agents has not previously been reported. This observation suggests that calcium stimulates cell division by different mechanisms at high vs low (basal) concentration and that the La- and Ca-induced proliferative processes differ. Earlier work (6) also suggested that different proliferative processes were operative at low and high calcium concentrations, in that fibroblast cell yields increased in a stepwise fashion rather than continuously with increasing calcium supplementation.

The observation that the calcium-induced stimulation is substantially diminished in acidified medium suggests that hydrogen ions also affect the mechanism operative at the higher Ca concentration; of interest, this effect is partially reversed by minute additions of lanthanum. At least in the case of lanthanum, the interaction is probably but not necessarily at the cell surface, since La does not enter the cell but is known to readily displace Ca from the membrane (13, 22–24).

We find statistically significant differences in stimulation of fibroblast confluent density by optimal doses of $CaCl_2$, $LaCl_3$, HA, and PP_i, as well as evidence that these agents differ in the character of their interaction with the cell membrane. These data suggest that cell surface events strongly influence mitogen responsiveness and density-dependent growth inhibition and do so through multiple mechanisms.

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