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INTRACELLULAR Ca^{2+} STORES AND EXTRACELLULAR Ca^{2+} ARE REQUIRED IN THE REAL-TIME Ca^{2+} RESPONSE OF BONE CELLS EXPERIENCING FLUID FLOW

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Abstract—In this study, we sought to determine if there is a requirement for calcium entry from the extracellular space as well as calcium from intracellular stores to produce real-time intracellular calcium responses in cultured bone cells subjected to fluid flow. Understanding calcium cell signaling may help to elucidate the biophysical transduction mechanism(s) mediating the conversion of fluid flow to a cellular signal. An experimental design which utilized a scheme of pharmacological blockers was employed to distinguish between the biochemical pathways involved in this cell signaling. A parallel-plate flow chamber served as the cell stimulating apparatus and a fluorescence microscopy system using the calcium-sensitive dye fura-2 measured the intracellular calcium changes. In the present study, evidence for a role by the inositol–phospholipid biochemical pathway, specifically inositol trisphosphate (IP_3) was obtained using neomycin which completely inhibited the calcium response to flow. Additionally, a concomitant role of extracellular calcium was demonstrated through experiments performed in calcium-free medium which also eliminated the flow response. Experiments conducted with gadolinium, a stretch-activated channel blocker, partially inhibited ($\sim 30\%$) the flow response while verapamil, a type-L voltage sensitive channel blocker, had no effect on the flow response. These results suggest a requirement of extracellular calcium (or calcium influx) as well as IP_3-induced calcium release from intracellular stores for generating the intracellular calcium response to flow in bone cells. Copyright © 1996 Elsevier Science Ltd.

Keywords: Bone cell; Shear stress; Calcium; Fluid flow; Biochemical pathway.

INTRODUCTION

Bone cells are exposed to a fluid environment *in vitro* and *in vivo*. Fluid flow in the microporous spaces and channels of cortical bone have been hypothesized to produce signals or forces which may act on bone cells, influencing cell function and subsequently bone maintenance. *In vitro* studies have demonstrated that bone cells exhibit responses to fluid flow with increases in real-time intracellular calcium concentration ($[Ca^{2+}]_i$), cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP_3), prostaglandins, and bone proteins (Hung *et al.*, 1995; Reich *et al.*, 1990, 1991; Shin *et al.*, 1993; Williams *et al.*, 1994). These types of cellular level investigations intend to elucidate the fundamental mechanism(s) of the signal transduction that occurs via the biophysical interaction of the bone cell and its dynamic aqueous environment.

It was previously reported that the flow-induced $[Ca^{2+}]_i$ response of bone cells was extinguished in the presence of an inhibitor of calcium mobilization from cytoplasmic stores (Hung *et al.*, 1995). To gain further insight to the events mediating the fluid-induced calcium signaling of bone cells, we posed the following question; Is there a requirement for calcium entry from the extracellular space as well as calcium release from intracellular stores to produce the $[Ca^{2+}]_i$ response of cultured bone cells to fluid flow? This investigation explicitly examined the contributions of IP_3-induced calcium release, extracellular calcium, stretch-activated ion channels, and L-type voltage-sensitive ion channels to further establish the role of intracellular calcium stores as well as determine the importance of extracellular calcium in the fluid flow induced bone cell $[Ca^{2+}]_i$ response.

MATERIALS AND METHODS

Primary bone cells were harvested from 1- to 3-day old neonatal Sprague-Dawley rat calvaria (IACUC protocol #E-1109) using a serial enzymatic digestion as described in Hung *et al.* (1995). Isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM/NCTC-135, Gibco, Grand Island, NY) plus 10% newborn bovine serum. Cells obtained in this manner have been shown to express the osteoblastic phenotype when mature (Brand and Hefley, 1984; Brighton *et al.*, 1991; Luben *et al.*, 1976) and were observed to express alkaline phosphatase synthesis (Lowry *et al.*, 1954) in the course of this study. Isolated cells were seeded at 40,000 cells cm^{-2} in a 1 cm^2 central portion of custom quartz slides (Friedrich and Dimmock, Millville, NJ) and were examined preconfluent, 3 days after plating.

A parallel-plate flow chamber (Levesque and Nerem, 1985) was used to introduce fluid flow over the cells and an Olympus fluorescence microscopy system (Tokyo, Japan) with the software capability of cell-by-cell analysis

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was used to measure $[Ca^{2+}]_i$. Both the flow chamber and fluorescence microscopy system have been described previously (Hung *et al.*, 1995). In brief, the flow chamber consists of a plexiglass block in which a uniform depth flow channel has been milled and fitted with a borosilicate glass window, a uniform thickness silastic 250 μm spacer (h), and a custom quartz slide with cultured bone cells. The chamber design permits the shear stress acting at the chamber wall (where the cells are located) to be determined as a function of the applied fluid flow rate by assuming laminar flow between two infinite plates (White, 1986). Chamber performance was verified by strong agreement between pressure transducer measurements of the pressure drop across the chamber and theoretical pressure values derived from the expression $\Delta P = -12Q\mu\Delta x/(h^3 b)$ where ΔP is the pressure gradient, Q the flow rate, Δx the distance between the pressure reading ports, μ the fluid visocity, h the gap distance between the plates or height of the flow channel, and b the width of the flow channel (White, 1986). A wall shear stress of 35 dynes cm^{-2} (flow rate of 0.47 ml s^{-1}) was chosen for study because it is in the physiologic range estimated from poroelastic continuum models of fluid flow in bone (Spirt and Pollack, 1993; Weinbaum *et al.*, 1994). The actual shear stress acting on the cells is unknown but is likely to be non-uniform and to be characterized by shear gradients (Hyman, 1972; Satcher Jr *et al.*, 1992).

The bone cells were loaded with the calcium sensitive dye Fura-2 AM (2.5 μM) and 20% w/v pluronic-DMSO (Molecular Probes, Eugene, OR) in Liebovitz medium (L-15, Sigma Chemical, St. Louis, MO) for 45 min at 25–27°C (Malgaroli *et al.*, 1987). $[Ca^{2+}]_i$ measurements were obtained by recording the intensities of the dye's single wavelength (510 nm) fluorescent emissions from two excitation wavelengths (340 and 360 nm) every 4 s (integration of 32 images to reduce noise). The ratio of the two fluorescent emission intensities (340 nm/360 nm) was calculated and converted to $[Ca^{2+}]_i$ using an *in vitro* calibration curve performed with the salt form of fura-2 (Grynkiewicz *et al.*, 1985; Roe *et al.*, 1990). Experiments were 4 min long, consisting of an initial 2 min no-flow basal period followed by a 1.5 min flow period and ending with a 0.5 min no flow period. All experiments were performed at room temperature (25–27°C) with solutions at a pH of 7.4. Cells were chosen for study from the central region of the 1 cm^2 rectangular patch of cells on the quartz slide to avoid edge effects. The preconfluent nature of the cells facilitated outlining individual cells in a field of view (typically 20–30 cells) with rectangular boxes using *Casals* (Olympus, Japan) image processing software.

All drug studies were performed with four experimental groups: a no-flow control, no-flow + blocker group, a flow group, and a flow + blocker group, where the blocker was either calcium-free HBS (CFHBS), neomycin, gadolinium (Gd^{3+}), or verapamil. To assess the role of extracellular Ca^{2+}, a calcium-free perfusate consisting of HBS without the normally present 1.25 mM $CaCl_2$ was used. In some instances, 0.1 mM EGTA was added in place of 1.25 mM $CaCl_2$ in the normal HBS. The role of plasma membrane stretch-activated channels was assessed by experiments incorporating Gd^{3+} (50–100 μM) (Yang and Sachs, 1989) while the role of L-type voltage-gated channels (Bean, 1989) was examined using verapamil (10 μM). Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) was used as a positive agonist control (Sigma Chemical, St. Louis, MO). This agent has been observed to elicit $[Ca^{2+}]_i$ responses in a number of osteoblastic cells, including rat calvarial bone cells, via a pathway mediated by phosphoinositide metabolism (Tatakis *et al.*, 1992).

To more directly assess the role of inositol-containing phospholipids (Fig. 1) particularly, IP_3 which binds to receptors on the membrane of internal calcium stores and triggers the release of calcium (Berridge, 1993; Williamson and Hansen, 1988), experiments incorporating neomycin (10 mM) were performed. Neomycin disrupts the inositol–phospholipid pathway by blocking phospholipase C (PLC) from hydrolyzing the polyphosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP_2) to two intracellular messengers: IP_3 and diacylglycerol (DG) (Prentki *et al.*, 1986), see Fig. 2. Prentki *et al.* (1986) have also suggested that neomycin inhibits the release of calcium from intracellular stores directly through selective binding to IP_3 rather than indirectly via disruption of PIP_2 phosphorylation.

Neomycin, Gd^{3+}, and verapamil were introduced to the cells for the final 15 min of the Fura-2 AM incubation period. After incubation, cells were rinsed with HBS or HBS + blocker and placed into the flow chamber which was filled with the same solution. The cells were allowed to equilibrate for approximately 10–15 min before experimentation. All blocker agents were obtained from Sigma Chemical (St. Louis, MO) except for Gd^{3+} (Aldrich Chemical, St. Louis, MO). The concentration of neomycin was identical to that used previously by Brighton *et al.* (1992) in substrate-stretch, bone cell

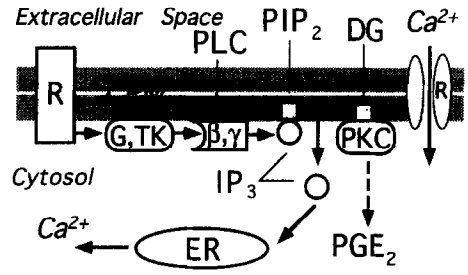

Fig. 1. A schematic of the inositol–phospholipid cell-signaling pathway. (CPM) refers to the cell plasma membrane, (R) to a GTP-binding protein (G)-linked receptor or tyrosine kinase (TK)-linked receptor which when activated produces phospholipase C (beta and gamma types, respectively) which phosphorylates phosphatidylinositol 4,5-biphosphate (PIP_2) to give both diacylglycerol (DG) and inositol trisphosphate (IP_3). IP_3 binds to receptors on intracellular calcium (Ca^{2+}) stores such as the endoplasmic reticulum (ER) resulting in the release of Ca^{2+} into the cytosol of the cell. Also illustrated is the influx of Ca^{2+} from the extracellular space via receptor-linked channels, (R) and inward arrow.

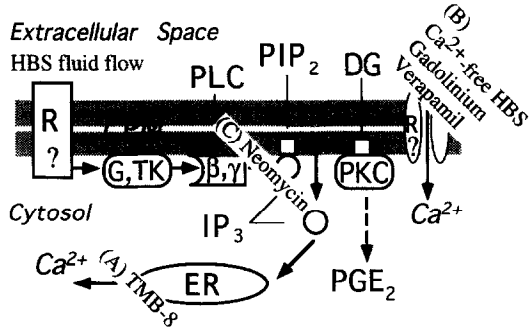

Fig. 2. Schematic from Fig. 1 displaying the blocking agents used to first elucidate the role of intracellular calcium: (A) TMB-8, then extracellular calcium: (B) Ca^{2+}-free HBS, gadolinium (Gd^{3+}), and verapamil, and lastly the role of inositol–phospholipids in the cell response to flow: (C) neomycin. (?) indicates unknown flow receptors (R) linked to tyrosine kinases or G-proteins and to plasma membrane ion channels.

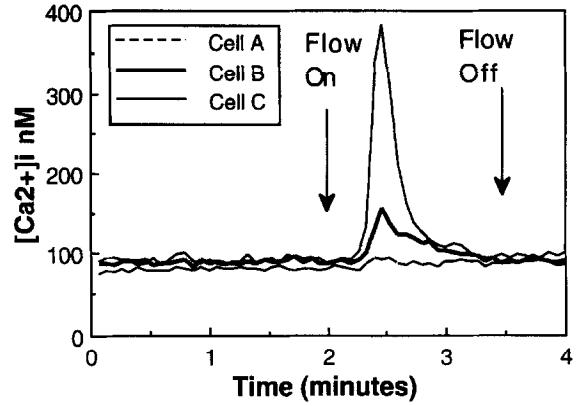

Fig. 3. A representative graph of the $[Ca^{2+}]_i$ versus time for 3 cells from the same slide preparation exposed to 35 dynes cm^{-2} of wall shear stress with a HBS perfusate. Notice the heterogeneity in the magnitude of response as well as the latency to peak response which has been reported previously (Hung *et al.*, 1995). The 1.5 min flow period is indicated by the arrows (flow on and flow off).

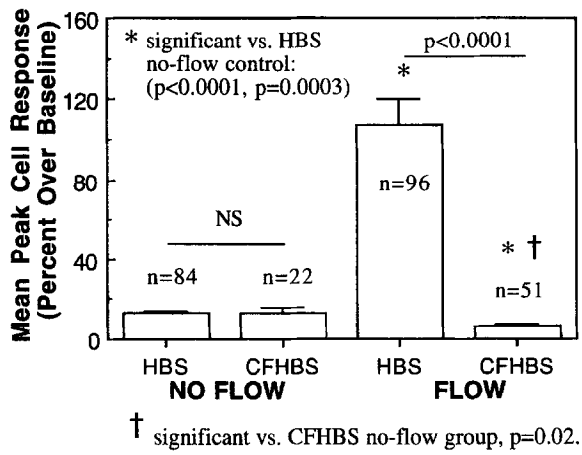

Fig. 4. A graph depicting the inhibitive effect of calcium-free HBS (CFHBS) on the mean peak $[Ca^{2+}]_i$ response ($p < 0.0001$). Cells were exposed to 35 dynes cm^{-2} of wall shear stress of 1.5 min. There was no significant difference between the no-flow HBS control and calcium-free HBS no-flow group, $p > 0.99$. The HBS flow group was significantly greater than the no-flow HBS control, $p < 0.0001$, while the calcium-free HBS flow group was significantly suppressed, $p = 0.0003$. Results from experiments performed with calcium-free HBS supplemented with EGTA were similar. Error bars represent the standard error for n cells. NS: not significant.

investigations measuring IP_3 and DNA synthesis (Brighton *et al.*, 1992) and is comparable to that used by others (Prasad *et al.*, 1993). The EGTA concentration was obtained from the literature and was chosen because it did not cause the cells to become detached during flow exposure (Shen *et al.*, 1992). Gadolinium and verapamil concentrations were also obtained from the literature (Corsetti *et al.*, 1992; Lorich *et al.*, 1993; Sadoshima *et al.*, 1992; Xia and Ferrier, 1992).

The amplitude of the peak $[Ca^{2+}]_i$ response during flow was expressed as a percentage over the initial no-flow baseline for each cell. Analysis of variance (ANOVA) using the Scheffe Test for multiple comparisons was used to analyze mean peak $[Ca^{2+}]_i$ response data. In some cases, the ANOVA was performed on the logarithmic transformation of the data in order to satisfy the requirement for homogeneous variances. Differences were considered significant at $\alpha = 0.05$. The mean $\pm$ standard deviation for n cells from a specified number of slide preparations is presented in the text.

RESULTS

As expected, exposure to 35 dynes cm^{-2} of steady wall shear stress (1.5 min) elicited a transient and heterogeneous calcium increase in terms of the peak $[Ca^{2+}]_i$ response in the cultured bone cells (Fig. 3). The requirement of extracellular calcium in this response was demonstrated by a complete inhibition of the flow response in the presence of a calcium-free perfusate, $p < 0.0001$ (Fig. 4) (ANOVA), F-value of 120.01, DF = 3 Residual = 249, $p < 0.0001$). There was no difference between the no-flow HBS control (12.9 ± 8.8, $n = 84$ cells, 3 slides) and calcium-free HBS no-flow group (13.3 ± 10.3, $n = 22$ cells, 1 slide), $p > 0.99$. The HBS flow group (106.8 ± 134.2, $n = 96$ cells, 4 slides) was significantly greater than the no-flow HBS control, $p < 0.0001$, while the calcium-free HBS flow group (6.5 ± 3.4, $n = 51$ cells, 2 slides) was significantly decreased, $p = 0.0003$. The calcium-free HBS flow group was also significantly decreased from the calcium-free HBS no-flow group, $p = 0.02$. Results from experiments performed with calcium-free HBS supplemented with EGTA were similar but with a more dramatic decrease in the mean peak $[Ca^{2+}]_i$ response relative to the no-flow HBS control.

Gadolinium partially ($\sim 30\%$) inhibited the flow response in both day 3 and day 6 preconfluent cells ($p < 0.0001$), suggesting stretch-sensitive channels as a potential source of calcium entry into the cell (Fig. 5) (ANOVA (DF = 3, $F = 62.86$, Residual = 465,

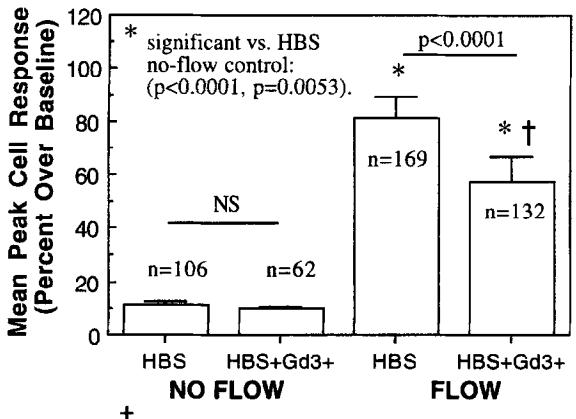

Fig. 5. A graph depicting the partial inhibition of the mean peak $[Ca^{2+}]_i$ response by gadolinium (Gd^{3+}), $p < 0.0001$. The HBS flow group and Gd^{3+} flow groups were significantly increased from the no-flow HBS group, $p < 0.0001$ and $p = 0.0053$, respectively. Furthermore, the response of the Gd^{3+} flow group was significantly decreased from that of the HBS flow group, $p < 0.0001$. The no-flow HBS and no-flow Gd^{3+} groups were not different, $p = 0.50$. Error bars represent the standard error for n cells. NS: not significant.

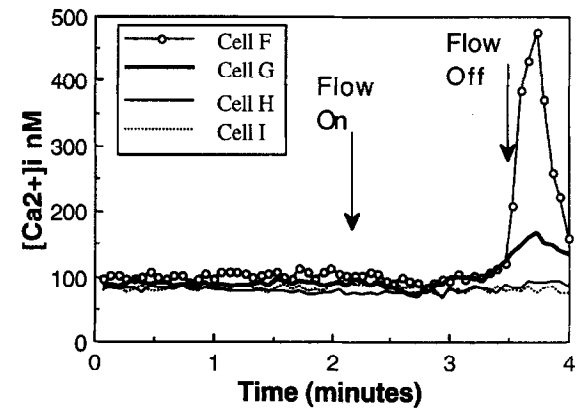

Fig. 6. A graph of the $[Ca^{2+}]_i$ vs time for 4 cells from the same slide exposed to 35 dynes cm^{-2} of wall shear stress with a HBS supplemented with Gd^{3+} perfusate. Note the flow responses initiated at flow termination, flow off. A greater frequency of such postflow responses when compared to the HBS flow group may indicate an increased cell sensitivity to alterations in the steady flow induced by the Gd^{3+}.

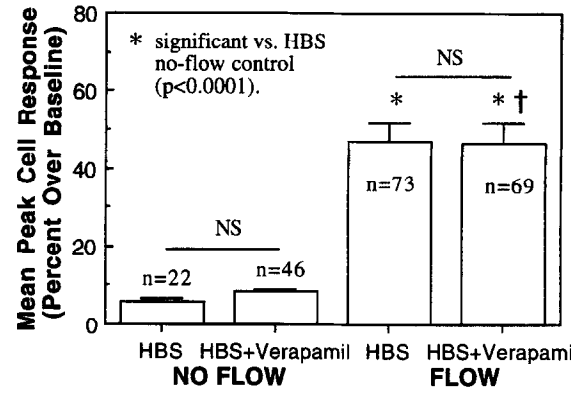

Fig. 7. A graph depicting the lack of an effect of verapamil on the mean peak $[Ca^{2+}]_i$ response ($p = 0.95$) . Both flow groups were significantly greater than the no-flow HBS control, $p < 0.0001$. The verapamil no-flow group was not different from the no-flow HBS control, $p = 0.22$. Error bars represent the standard error for n cells. NS: not significant.

$p < 0.0001$). There was no difference between the no-flow HBS control (11.5 ± 8.5, $n = 106$ cells, 4 slides) and no-flow Gd^{3+} group (9.6 ± 9, $n = 62$ cells, 3 slides), $p = 0.50$. The Gd^{3+} flow group was significantly different from the Gd^{3+} no-flow control $p = 0.0001$. Both the HBS flow group (81 ± 109, $n = 169$ cells, 7 slides) and the Gd^{3+} flow group (57.5 ± 102.2, $n = 132$ cells, 6 slides) were significantly different from the no-flow HBS control, $p < 0.0001$ and $p = 0.0053$, respectively. In addition to the disparity in the mean peak $[Ca^{2+}]_i$ response, the latency to peak response during flow was increased in the Gd^{3+} treated flow group when compared to the HBS flow group ($p = 0.012$ from unpaired t-test). Data from a single slide of each flow group, having robust cell responses, revealed a nearly two-fold increase in the latency to peak response in Gd^{3+} exposed cells (47.8 ± 31.6 s vs 27 ± 20.1 s. Furthermore, there were an increased number of responses initiated after the flow period in the Gd^{3+} treated cultures (Fig. 6). Such post-flow responses were rare in the HBS flow group.

Calcium influx from verapamil sensitive voltage-gated channels does not appear to play a contributing role in the fluid flow response. No difference was observed between the response of the HBS flow group (47 ± 40.6, $n = 73$, 3 slides) and verapamil flow group (46.2 ± 44.7, $n = 69$ cells, 3 slides), $p = 0.95$ (ANOVA, F-value of 71.53, DF = 3, Residual = 206, $p < 0.0001$). Both flow groups were significantly greater than the no-flow HBS control (6 ± 3.4, $n = 22$ cells, 1 slide), $p < 0.0001$. The verapamil flow group was significantly greater than the no-flow verapamil group was not different from the HBS no-flow control, $p = 0.22$ (Fig. 7).

Neomycin completely inhibited the flow response ($p < 0.0001$), demonstrating a role by the inositol–phospholipid pathway and intracellular calcium stores in the flow response (Fig. 8) (ANOVA, F-value of 140.33, DF = 3, Residual = 224, $p < 0.0001$). The no-flow HBS control (8.3 ± 3.4, $n = 67$ cells, 4 slides) was not different from that of the no-flow neomycin group (9.2 ± 9.7, $n = 47$, 3 slides), $p = 0.97$, nor the neomycin flow group (10.4 ± 16.4, $n = 47$ cells, 3 slides), $p = 0.83$. The neomycin flow group was not different from the neomycin no-flow group, $p = 0.98$. The HBS flow group (116.1 ± 127.2, $n = 67$, 4 slides) responded significantly over the no-flow HBS control, $p < 0.0001$.

Experiments with bradykinin (500 nM) indicated that the pharmacological blockers were performing accordingly. Consistent with an inositol–phospholipid pathway mediated response, neomycin was observed to inhibit or reduce the $[Ca^{2+}]_i$ response to bradykinin (data not

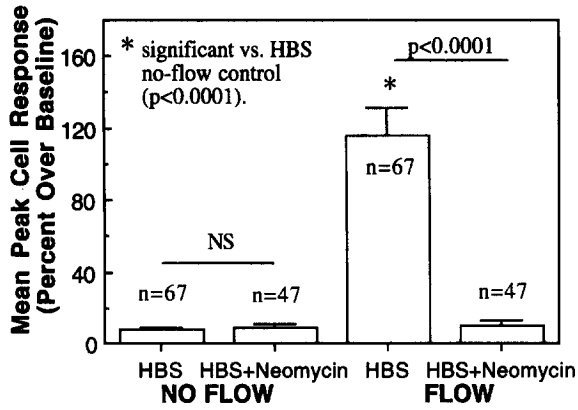

Fig. 8. A graph depicting the complete inhibition of the mean peak $[Ca^{2+}]_i$ response by neomycin. Cells were exposed to 35 dynes cm^{-2} of wall shear stress for 1.5 min. Only the HBS flow group was found to be significantly different from the no-flow HBS control, $p < 0.0001$. The flow groups were significantly different, $p < 0.0001$. Error bars represent the standard error for n cells. NS: not significant.

reported). In further support of neomycin, the PLC specific blocker U73122 (10 μM, Calbiochem, La Jolla, CA) also inhibited the $[Ca^{2+}]_i$ response to fluid flow (data not shown). Bradykinin was also able to elicit large $[Ca^{2+}]_i$ responses in cells exposed to calcium-free HBS or calcium-free HBS + EGTA, and in separate experiments, to Gd^{3+} (data not reported). This observation is particularly important in that it suggests that despite being exposed to the non-physiologic calcium-free environment, the calcium within the intracellular stores remained viable and was not entirely depleted.

DISCUSSION

In this investigation, we addressed the question of whether calcium entry from the extracellular space and calcium release from intracellular stores are required in generating the real-time $[Ca^{2+}]_i$ response to fluid flow in cultured bone cells. The biochemical pathways mediating this response were distinguished using a scheme of pharmacological blockers. The results presented in this paper stem from our previous work (Hung *et al.*, 1995) and relate to the experimental conditions germane to the present study—preconfluent primary cultured bone cells, 35 dynes cm^{-2} of wall shear stress, HBS (serum-free) perfusate, and a 1.5 min steady flow exposure. The relevance of these findings to experiments performed under different conditions is not yet known.

The participation of both intracellular and extracellular calcium in the transient calcium response to fluid flow suggests that IP_3 and extracellular Ca^{2+} may be coagonists which together mediate this response. Such a coagonist relationship has been demonstrated in *in vitro* findings wherein the IP_3-induced calcium release from the endoplasmic reticulum of certain cells was potentiated by the presence of extracellular calcium in a bell-shaped dose-response relationship (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). It has been suggested that the structure of the IP_3 receptors bound to the membrane of the endoplasmic reticulum is important in triggering the release of calcium. These receptors may process not only binding sites for IP_3 but also calcium ions (Finch *et al.*, 1991). The role for IP_3–Ca^{2+} "cofactors" has been suggested by Xia and Ferrier (1992) to mediate the propagation of a calcium pulse between osteoblastic cells initiated by the mechanical perturbation of one cell in a monolayer. Berridge (1993) has proposed a conformational coupling hypothesis in which the IP_3 receptor controls the mobilization of internal and external calcium.

The findings of this study are consistent with those in the literature which have examined the biological response of primary bone cells to applied mechanical stimuli. The inositol–phospholipid pathway has been demonstrated to mediate the proliferative response of bone cells to substratum stretch (Brighton *et al.*, 1992) and hydrostatic pressure (Brighton *et al.*, 1996). Additionally, increases in IP_3 production and protein kinase C mediated prostaglandin synthesis, the latter associated with the DG branch of the pathway, have been reported in bone cells subjected to fluid flow (Reich *et al.*, 1991; Reich and Frangos, 1993).

Several mechanosensitive and voltage-gated channels have been reported to exist in osteoblast-like cells (Davidson *et al.*, 1990; Duncan and Misler, 1989). Alterations of ion channel activity in the bone cell membrane may be important in the transduction of physical forces by these cells. The participation of Gd^{3+}-sensitive stretch-activated ion channels in the flow response provides evidence for a role by mechanical forces rather than electrokinetic forces. Also consistent with a mechanically mediated response is the lack of an observed role by verapamil-sensitive voltage-gated channels which have been demonstrated to mediate electric field responses of cultured primary bone cells (Corsetti *et al.*, 1992; Lorich *et al.*, 1993). The role of mechanical stimuli is further supported by the findings that the streaming potential does not contribute to the $[Ca^{2+}]_i$ response of cultured primary bone cells to fluid flow under the conditions of this study (Hung *et al.*, 1996) nor to the cAMP response as measured by Reich *et al.* (1990).

Both the increased latency to peak response and increased frequency of cells experiencing $[Ca^{2+}]_i$ transients upon flow cessation suggest that the inhibitory effect of Gd^{3+} was only temporary in some instances. The post-flow responses suggest an increased sensitivity in these cells to changes in the established steady fluid flow. This heightened responsiveness may be brought on directly or indirectly by Gd^{3+}. Relaxation of the cell membrane and/or stretch receptors resulting from stimulus removal (flow cessation) may be involved in this phenomena (He and Grinnell, 1994). Future work will further examine these findings.

Variation between the response magnitude of cells from the different experiments may reflect variability in the cell population (different isolations) and/or experimental conditions (Hung *et al.*, 1995). This variability is apparent in the response of the no-flow HBS control

cells, representing the noise due to spontaneous calcium fluctuations or transients, associated with the different experiments. It is also apparent in the heterogeneous display of the calcium response to fluid flow within a given experimental trial. Similar observations have been reported in endothelial cells exposed to fluid flow (Geiger *et al.*, 1992). To account for this variation in the present study, no-flow controls were performed on each day of experiment. We direct interested readers to our earlier manuscript in which potential sources of this heterogeneous response (cell, stimulus, and response heterogeneity) were addressed (Hung *et al.*, 1995). We emphasize that the sources of heterogeneity (associated with the cells and/or stimulus) would be expected to be pervasive in all cell preparations (slides), independent of the presence of a blocking agent in the perfusate, and would therefore not jeopardize the pharmacological blocker approach of the present study.

As in any blocker study, the extent to which the pharmacological agents interfered with other normal cell functions (in addition to those intended) as well as the impact of these additional perturbations on the final results are unknown. The lack of calcium in the perfusate, for example, may have affected the cell adhesion properties (such as the binding proteins) of the bone cells. This was apparent in earlier aborted experiments using EGTA (0.5 –1 mM) which caused cell detachment. It is noted that under the experimental conditions of this study and with respect to intracellular calcium, the basal $[Ca^{2+}]_i$ level was unaltered by blockers in the absence of fluid flow.

In conclusion, this work strongly suggests that both calcium from internal calcium stores and extracellular calcium are required in the $[Ca^{2+}]_i$ response of bone cells experiencing fluid flow.

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