Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 119-127

THROMBIN'S EFFECTS ON OSTEOBLASTIC CELLS I. CYTOSOLIC CALCIUM AND PHOSPHOINOSITIDES

Dimitris N. Tatakis*, Calogero Dolce and Rosemary Dziak

Department of Oral Biology, School of Dental Medicine, Foster Hall
State University of New York at Buffalo, Buffalo, New York 14214

Received August 18, 1989

Thrombin, a blood coagulation factor, has been shown to be a very effective *in vitro* bone resorbing agent whose mechanism of action on osteoblastic cells remains to be elucidated. In the present study, the effects of highly purified human thrombin on Saos-2 and G292 cells, two human osteoblast-like osteosarcoma cell lines, were investigated. Thrombin (0.6-16 U/ml) caused a significant, dose-dependent increase in osteoblastic cell proliferation. Thrombin also elicited a dose-dependent increase in cytosolic calcium concentration in both Saos-2 and G292 cells (maximal increases were 38% and 200% over baseline, respectively). Addition of thrombin to the osteoblast-like cells resulted in significant time- and dose-dependent changes in phosphoinositide levels: the percentage of inositol monophosphate levels were decreased, whereas the percentage of inositol bisphosphate, inositol trisphosphate and inositol tetrakisphosphate levels were increased. The relative magnitude of the changes in phosphoinositide levels was similar to the changes in cytosolic calcium concentration. These results suggest that thrombin's mechanism of action on bone cells may involve increases in cytosolic calcium levels and in phosphoinositide metabolism.

Thrombin, a serine protease, has a central role in hemostasis (1, 2). Thrombin participates in the regulation of hemostasis by several mechanisms: it acts on plasma fibrinogen to form fibrin (3), it stimulates platelet serotonin (4) and prostaglandin release (5), endothelial cell secretion of prostacyclin (6), platelet-activating factor (7), and tissue plasminogen activator (8), and smooth muscle cell contraction (9). Thus, thrombin (coagulation factor IIa, EC 3.4.21.5) demonstrates both enzymatic activity and several hormone-like actions (10), including monocyte chemotaxis (11) and stimulation of cell proliferation (12, 13).

Recent studies have indicated that thrombin has profound effects on bone and bone cells. Thrombin can cause *in vitro* bone resorption, in a dose-dependent manner, both in a mouse calvaria (14, 15) and a rat long bone assay (16, 17). Indomethacin and other cyclooxygenase inhibitors are capable of inhibiting the bone resorbing activity of thrombin (14-17), suggesting that this action of thrombin is, at least partly, prostaglandin-mediated. Moreover, thrombin has been shown to stimulate

*Correspondence to: Dr. D.N. Tatakis, Oral Biology Dept., Foster Hall, SUNYAB, 3435 Main Street, Buffalo, NY 14214.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; cAMP, adenosine 3',5'-cyclic phosphate; $[Ca^{2+}]_i$, cytosolic free calcium concentration; DNA, deoxyribonucleic acid; FCS, fetal calf serum; HBSS, Hepes-buffered balanced salt solution; Hepes, N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid; HPLC, high-pressure liquid chromatography; IP, inositol monophosphate(s); IP_2, inositol bisphosphate; IP_3, inositol trisphosphate(s); IP_4, inositol tetrakisphosphate; SA, specific activity; TCA, trichloroacetic acid.

0006-291X/89 $1.50

prostaglandin synthesis by isolated chicken (18) and rat (19) osteoblast-like cells. Feyen *et al* (18) reported that thrombin did not increase cAMP levels in chicken osteoblastic cells. Thus, as much as it can be assessed from the existing literature, the mechanism(s) and the second messenger(s) involved in the action of thrombin on osteoblastic cells are not known.

In the present study we used two human osteoblastic-like osteosarcoma cell-lines to investigate the effects and the mechanism of action of thrombin on osteoblastic cells.

Materials and Methods

Chemicals. Tissue culture media and media supplements were obtained from GIBCO (Grand Island, NY). Tissue culture disposable dishes and flasks were from Corning (Corning, NY). Fura-2AM and fura-2 were from Molecular Probes (Eugene, OR). Highly purified human plasma thrombin (Specific Activity: 4,000 NIH units/mg protein) was obtained from Sigma (St. Louis, MO). *Myo*-[1,2-^{3}H]-inositol and inositol phosphate standards (inositol-[2-^{3}H(N)]-1-phosphate, inositol-[2-^{3}H(N)]-4-phosphate, inositol-[2-^{3}H(N)]-1,4-bisphosphate, inositol-[1-^{3}H(N)]-1,4,5-trisphosphate, and inositol-[1-^{3}H(N)]-1,3,4,5-tetrakisphosphate) were purchased from NEN (Boston, MA). ^{3}H-thymidine was obtained from ICN (Irvine, CA).

Cells and cell culture. The two human osteoblastic osteosarcoma cell lines Saos-2 (ATCC #HTB 85) and G292 (ATCC #CRL 1423) were used. These cells have a characterized osteoblast-like phenotype (20, 21). The cells were maintained under standard conditions. Cells were cultured in McCoy's 5a medium supplemented with 10% FCS, in a humidified, 5% CO_2, 37°C incubator.

Cytosolic calcium measurement. Cells (both Saos-2 and G292) recovered from culture flasks after mild trypsinization were washed three times in a Hepes-buffered pH 7.4 balanced salt solution (HBSS) containing 120 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 3 mM K_2HPO_4, 30 mM mannitol, 30 mM Hepes, 5 mg/ml glucose and 1 mg/ml crystalline endotoxin-free BSA fraction V (Sigma). The cells were then resuspended at $1.5x10^6$ cells/ml in HBSS containing 1 μM fura-2AM and incubated at 37°C for 30 minutes in a shaking water bath. During this incubation period and all subsequent handling the cells were protected from light. At the end of the 30 minute incubation period the cells were again washed thrice and resuspended in HBSS at a concentration of $1.5x10^6$ cells /ml. The cell suspension was then kept at 4°C until it was used in the experiment.

Intracellular calcium levels were measured in the fura-2 loaded cells using a SPEX Fluorolog II cation measurement system (SPEX Industries, Edison, NJ), which is a computer-driven recording spectrofluorometer. The instrument is equipped with a water jacketed cuvette holder (temperature for all experiments was 37°C) and a magnetic stirrer. Two ml of the cell suspension were placed in a quartz cuvette and the cells were allowed to equilibrate (in the dark) for 10 minutes prior to any fluorescence recording. Fura-2 fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm (22, 23). At the end of each experiment, maximum and minimum fluorescence were recorded after the addition of 0.1% Triton-X 100 and 4 mM EGTA - 30 mM Tris (pH 8.5), respectively. Cytosolic calcium concentration was calculated, with the fura-2 dissociation constant for Ca^{2+} assumed to be 224nM at 37°C, using the spectrofluorometer's computer software, based on the equations given by Grynkiewicz *et al* (22).

Phosphoinositide metabolism studies. Isolated cells were suspended in McCoy's 5a with 10% FCS and seeded in 12-well flat bottom polystyrene dishes ($1x10^6$ cells/ml; 1 ml cell suspension/well). After a 24 hour incubation, the media was replaced with McCoy's 5a without inositol plus 10%FCS and 10 μCi/ml *myo*-[^{3}H]-inositol (SA: 45-80 Ci/mmol). Cells were then incubated for an additional 24 hours. Next, the incubating media was removed and the cells were washed 3 times with HBSS. The cells were further incubated for an additional 30 minutes with 1 ml HBSS containing either 10mM (G292) or 100mM (Saos-2) LiCl, to inhibit inositol-1-phosphatase (24). At the end of this period, agents were added to the cells and at the designated time point the reaction was stopped by the addition of 1ml ice cold 15% TCA. The TCA solution contained 2μl/ml phytic acid hydrolysate (mixture of inositol phosphates) prepared according to the method of Wreggett *et al* (25), in order to minimize extraction losses of the labelled cellular inositol phosphates. When ^{3}H-labelled IP_2, IP_3, and IP_4 standards were added (either individually or as a mixture) to unlabelled cells, extracted and assayed as described below, the recovery was over 83% for IP_2, and over 95% for IP_3, and IP_4. After scraping-off the precipitated material and transferring it to test tubes, the wells were washed with 1 ml of the TCA solution which was then combined with the previously collected material. The total reaction mixture (3ml) was centrifuged for 20 min at 2000 x g. The supernatant was collected and extracted 3 times with water saturated ether. The samples were then lyophilized and stored at -20°C, until they were analyzed by strong anion-exchange HPLC, using a modification of the technique by Balla *et al* (26).

For HPLC analysis, the samples were dissolved in 100µl of HPLC grade water and injected in an HPLC system equipped with a Zorbax Bio series SAX column (DuPont; Wilmington, DE). Elution conditions were as follows: Mobile phases: A: H_2O, and B: 0.7M ammonium dihydrogen phos- phate, pH 3.5; Gradient: 100% A for 5 min and then linear 100% A to 100% B in 40 min; Flow rate: 1ml/min. The column was connected to an inline radioactivity detector. The average retention time, in this system, for the various inositol phosphates is: IP-9-10 min, IP_2-15.5 min, IP_3-24.5 min, IP_4-34.5 min. The counts corresponding to the IP, IP_2, IP_3 and IP_4 peaks were added together and the results for the individual peaks were expressed as percentage (%) of inositol phosphate counts.

Cell proliferation. Cell proliferation was monitored by ^{3}H-thymidine incorporation. The method is a modification of the procedure described by Carney *et al* (27). Cells were seeded in 24-well flat bottom polystyrene dishes ($1x10^6$ cells/ml; 0.5 ml of cell suspension/well). After a 24 hr incubation period in McCoy's 5a medium supplemented with 10% FCS, the cells were washed twice with McCoy's medium supplemented with 1mg/ml crystalline endotoxin-free BSA (McCoy's with BSA). The cells were then cultured in McCoy's with BSA for 24 hrs. At the end of this 24 hr period, the medium was removed and fresh McCoy's with BSA was placed in the wells (0.5 ml/well) with the appropriate agents. The cells were then incubated for another 24-48 hrs. During the last two or six hours of this 24-48 hr incubation period with the agent(s) ^{3}H-thymidine (SA: 10Ci/mmol; 1mCi/ml; 1 µCi/ml final concentration) was added to the medium. After this labeling period the cells were washed once with McCoy's 5a medium and then extracted and rinsed five times with 10% TCA. The acid-precipitable material was then dissolved in 0.5N KOH (0.5 ml, 2 hrs at 23°C). The solution, after neutralization with 1N HCl (0.25 ml), was counted in 10 ml of Liquiscint (National Diagnostics; Manville, NJ) scintillation fluid.

Statistical Analysis. Data were analysed using factorial ANOVA, paired and unpaired Student's t test.

Results

Thrombin stimulates proliferation. Thrombin caused a significant, dose-dependent increase in DNA synthesis, as indicated by the increase in ^{3}H-thymidine incorporation by the Saos-2 cells (Fig 1). Similar effects were observed in the G292 cells (Table 1). This effect appeared to be independent of any prostaglandin synthesis, since it was not altered in the presence of indomethacin (Table 1). The maximum effect was seen at a thrombin concentration of 8U/ml (Fig 1).

Thrombin increases cytosolic calcium. Addition of thrombin to the human osteoblast-like cells resulted in a sharp increase in $[Ca^{2+}]_i$ (Fig 2). Although the data presented are from cells in suspension, the qualitative, quantitative and temporal aspects of the cytosolic calcium response of these cells to thrombin were similar in a few experiments performed with the cells attached on cuvettes (data not shown). Qualitatively, the effect of thrombin was similar in both cell lines. $[Ca^{2+}]_i$ increases were

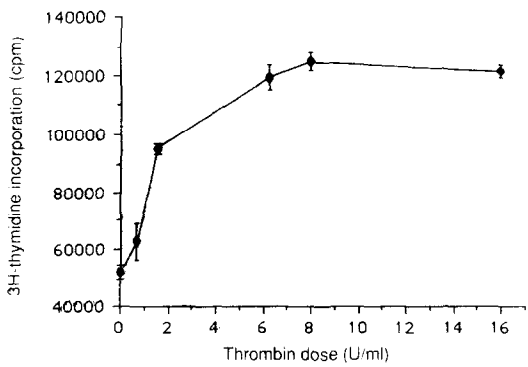

Fig. 1. The effect of thrombin in Saos-2 cell proliferation. The data represent the mean ± standard deviation for quadruplicate determinations, from a representative experiment.

Table 1.
Effect of thrombin on osteoblastic cell proliferation

Cell Type	Exp.	Treatment	^{3}H-thymidine incorporation (cpm)
Saos-2	1	Control	60,693.2 ± 3,334.7
		Control + Indomethacin 1μM	58,466.0 ± 3,580.2
		Thrombin 6.25 U/ml	116,811.5 ± 8,151.7[a]
		Thrombin 6.25 U/ml + Indomethacin	116,934.7 ± 4,548.7[a]
	2	Control	41,769.2 ± 1,385.0
		Control + Indomethacin 1μM	45,538.0 ± 1,594.2
		Thrombin 6.25 U/ml	66,865.7 ± 3,405.4[a]
		Thrombin 6.25 U/ml + Indomethacin	60,140.2 ± 2,280.9[a]
G292		Control	28,281.7 ± 3,107.2
		Control + Indomethacin 1μM	25,197.5 ± 1,223.6
		Thrombin 6.25 U/ml	46,510.5 ± 4,181.7[a]
		Thrombin 6.25 U/ml + Indomethacin	45,707.7 ± 1,558.3[a]

Results are the mean ± standard deviation for triplicate or quadruplicate determinations. a: $p<0.01$ from corresponding control (two-tail, unpaired Student's t-test). Controls received an appropriate dilution of the thrombin carrier. Cultures without indomethacin received the appropriate amount of indomethacin carrier.

immediate (with peaks occurring within 15-25 seconds after thrombin addition) and transient (levels returned at or close to baseline values within 2.0 minutes). Quantitatively, the dose-dependent, thrombin-induced increase in $[Ca^{2+}]_i$ was different for the Saos and the G292 cells. The maximal response in Saos-2 cells represented a 38% increase over baseline, whereas G292 cells responded with a 200% increase over baseline (Table 2). Thrombin was capable of stimulating $[Ca^{2+}]_i$ increases, in both cell lines, even in the absence of extracellular calcium or in the presence of 50 μM of verapamil (data not shown). Addition of equivalent amounts of thrombin carrier (control) did not affect $[Ca^{2+}]_i$ levels (Fig 2).

The dose-response curve for proliferation appeared to be almost superimposable to the $[Ca^{2+}]_i$ curve, when both were on a similar scale. When the mean values for Saos-2 proliferation from Fig 1

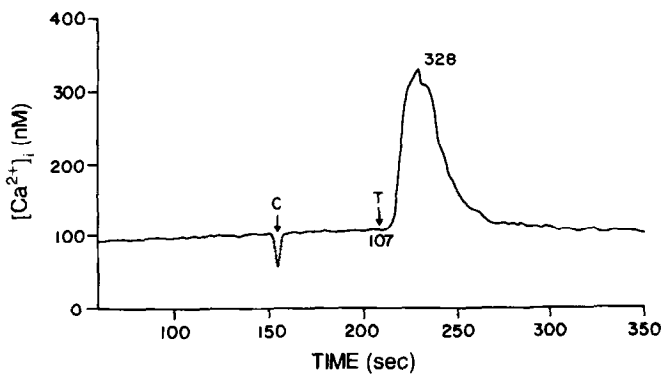

Fig. 2. The effect of thrombin in G292 cell $[Ca^{2+}]_i$. The numbers below and above the graph line represent the $[Ca^{2+}]_i$ peak values (in nM) before and after, respectively, the addition of 6.25U/ml of thrombin (T) to the cells. Addition of an equivalent amount of thrombin carrier (C) did not affect baseline $[Ca^{2+}]_i$. The dose responses (Table 2) were calculated from such independent determinations.

Table 2.
Effect of thrombin on cytosolic calcium concentration

Thrombin dose (U/ml)	Saos-2 cells $[Ca^{2+}]_i$ (nM)	G292 cells $[Ca^{2+}]_i$ (nM)
0.00 (Baseline)	117.7 ± 16.5 (n=20)	111.7 ± 12.6 (n=22)
0.62	128.5 ± 23.4 (n=6)	208.5 ± 52.7 (n=6)
1.56	153.6 ± 14.4 (n=5)	179.1 ± 15.2 (n=8)
6.25	164.7 ± 20.5 (n=3)	294.8 ± 64.3 (n=5)
15.60	151.2 ± 14.8 (n=6)	331.0 ± 95.5 (n=3)

Results are the mean ± standard deviation of cytosolic calcium concentration. In parentheses is the number of determinations. All thrombin doses were statistically significant ($p<0.05$; two-tail t-test) when compared to their respective baselines.

were plotted against, and correlated with the mean values for $[Ca^{2+}]_i$ from Table 2 there was a linear relationship (r=0.992) between $[Ca^{2+}]_i$ and ^{3}H-thymidine incorporation values.

Thrombin stimulates phosphoinositide metabolism. Thrombin caused a significant, time-dependent increase in the percentage of IP_2, IP_3, and IP_4, along with a decrease in the percentage of IP in both cell lines (Table 3 and 4). For information purposes, the actual counts obtained from the chromatograph are also given in parenthesis in Table 3. For both Saos-2 and G292 cells, IP levels continued to decrease up to 45 seconds after thrombin addition, IP_2 levels appeared to peak at 45 seconds and stabilize thereafter, whereas IP_3 levels appeared to peak at 45 seconds and decrease thereafter (Table 3 and 4). IP_4 levels exhibited the least change with time. Basal (0 sec) levels of inositol phosphates

Table 3.
Effect of thrombin on inositol phosphate levels in Saos-2 cells

Time	IP	IP_2	IP_3	IP_4
Experiment 1				
Thrombin				
0 sec	95.65 ± 1.52	1.55 ± 1.04	0.97 ± 0.40	1.83 ± 0.13
45 sec	87.78 ± 4.88	6.18 ± 2.46	2.73 ± 1.20	3.30 ± 1.58
Control				
45 sec	94.61 ± 1.17	2.33 ± 1.54	1.01 ± 0.26	2.05 ± 1.75
Experiment 2				
0 sec	97.03 ± 1.49 (5008.2)	2.10 ± 1.02 (109.5)	0.24 ± 0.09 (12.2)	0.63 ± 0.44 (32.5)
15 sec	93.54 ± 1.03 (4382.0)	3.70 ± 0.44 (171.2)	0.87 ± 0.73 (45.2)	1.89 ± 1.14 (80.7)
30 sec	90.36 ± 2.16 (5103.7)	6.21 ± 1.67 (343.5)	1.87 ± 0.28 (103.7)	1.56 ± 0.71 (90.0)
45 sec	85.86 ± 1.85 (4503.5)	8.33 ± 2.29 (421.5)	2.74 ± 0.99 (147.5)	3.06 ± 0.66 (155.2)
60 sec	87.12 ± 0.77 (5616.0)	8.23 ± 1.41 (523.0)	2.46 ± 0.36 (161.7)	2.19 ± 1.14 (120.8)
75 sec	85.96 ± 0.79 (5265.7)	8.78 ± 1.93 (550.7)	2.34 ± 0.58 (142.7)	2.98 ± 1.81 (173.2)

The values are the percentage of the total inositol phosphate counts. Results are the mean ± standard deviation for quadruplicate determinations. Values in parentheses are the mean counts (above background) for the corresponding peaks, from the quadruplicates. Cells were treated with 8 U/ml thrombin. Controls received an appropriate dilution of the thrombin carrier.

Table 4.
Effect of thrombin on inositol phosphate levels in G292 cells

Time	IP	IP_2	IP_3	IP_4
Thrombin				
0 sec	92.83 ± 0.85	3.43 ± 0.79	1.72 ± 0.49	2.00 ± 0.85
15 sec	86.50 ± 2.63	7.10 ± 1.92	4.32 ± 1.20	2.07 ± 0.46
30 sec	69.64 ± 3.06	20.61 ± 2.69	6.25 ± 0.57	3.51 ± 1.09
45 sec	56.52 ± 2.79	33.79 ± 1.48	7.07 ± 1.71	2.61 ± 0.34
60 sec	54.39 ± 1.52	34.82 ± 2.01	6.42 ± 1.07	4.37 ± 1.29
75 sec	57.35 ± 2.57	34.17 ± 1.98	6.02 ± 1.27	2.31 ± 1.54
90 sec	57.62 ± 2.40	35.05 ± 3.60	5.43 ± 1.02	1.89 ± 0.67
Control				
45 sec	91.02 ± 2.39	5.87 ± 1.68	1.68 ± 0.78	1.43 ± 0.46

The values are the percentage of the total inositol phosphate counts. Results are the mean ± standard deviation for quadruplicate determinations, from a single experiment. Cells were treated with 8 U/ml thrombin. Controls received an appropriate dilution of the thrombin carrier.

remained unaltered 45 sec after the addition of equivalent amounts of thrombin carrier (control) (Table 3 and 4).

When assayed at a single time point (45 sec after thrombin addition), thrombin caused a dose-dependent stimulation of phosphoinositide metabolism (Fig 3 and 4). IP_4 levels showed the least change (data not shown). Analogous to the above reported $[Ca^{2+}]_i$ increases, the results on phosphoinositide metabolism were qualitatively similar in both lines, but quantitatively there were differences. In the Saos-2 cells the observed significant changes were much smaller than the very pronounced changes seen in the G292 cells (Table 3 and 4, Fig 3 and 4). When the dose-response experiment was performed at a later time point (60 sec, data not shown) the response for IP and IP_2

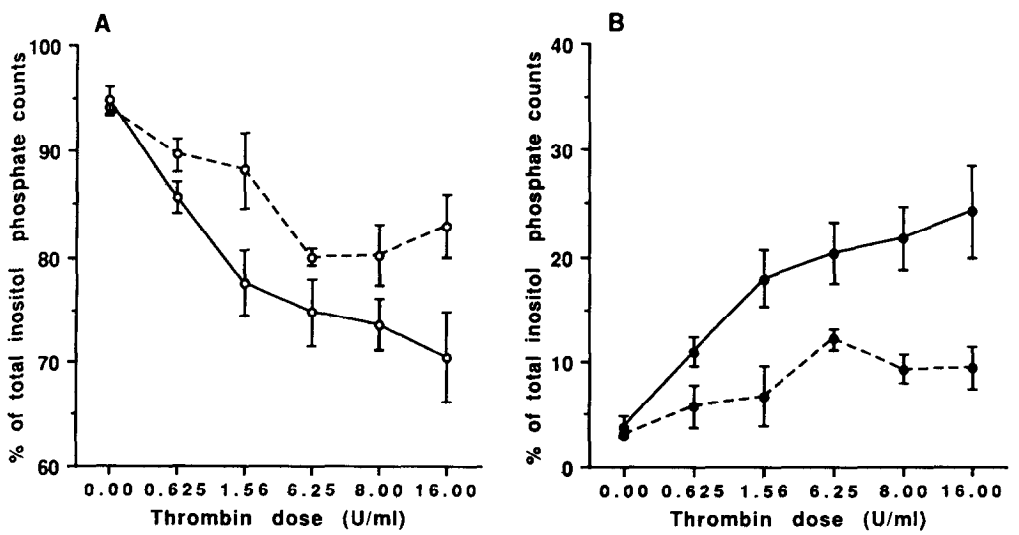

Fig. 3. The effect of thrombin on IP (panel A) and IP_2 (panel B) levels in Saos-2 and G292 cells. The data represent the mean ± standard deviation for quadruplicate determinations. IP (open circles) and IP_2 (closed circles) levels in Saos-2 (dashed line) and G292 (solid line) cells were assayed 45 seconds after the addition of thrombin to the cells.

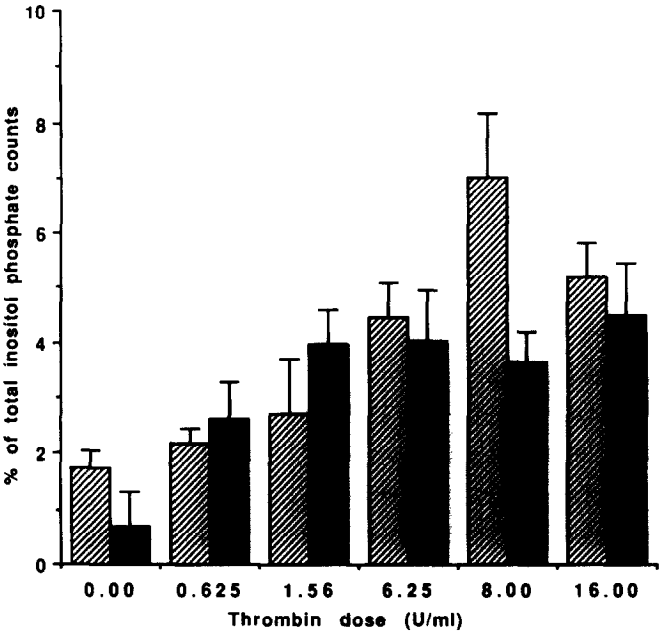

Fig. 4. The effect of thrombin on IP_3 levels in Saos-2 and G292 cells. The data represent the mean (plus standard deviation) for quadruplicate determinations, from the same experiments shown in Fig 3. Saos-2 (hatched bars) and G292 (solid bars) cells were assayed 45 seconds after the addition of thrombin to the cells.

was still evidently dose-dependent, whereas the IP_3 and IP_4 response had become less dose-dependent, thus confirming the time-dependence (Table 3 and 4) of the thrombin effect.

When the mean IP_3 levels (Fig 4) obtained in Saos-2 and G292 were correlated with the corresponding mean $[Ca^{2+}]_i$ values (Table 2), there was a strong correlation between them (r=0.905 and r=0.829, respectively). Similarly strong correlations were observed between mean $[Ca^{2+}]_i$ values and IP or IP_2 levels (data not shown).

Discussion

It is becoming progressively evident in recent years that many resorptive agents mediate their effects through activation of the osteoblast. Among them, parathyroid hormone (28) and prostaglandin E_2 (29) have been shown to activate the phosphoinositide metabolism and to raise the free cytosolic calcium levels of osteoblastic osteosarcoma cells. The present study is the first report on the capability of thrombin to stimulate osteoblastic cell proliferation, $[Ca^{2+}]_i$ increases and phosphoinositide metabolism. The dose-response of the effects of thrombin on osteoblastic osteosarcoma cell proliferation, $[Ca^{2+}]_i$, and phosphoinositide metabolism is comparable to the dose-response reported for the effect of thrombin on bone resorption (15, 17).

The biological significance of the reported stimulation of osteoblastic cell proliferation by thrombin is not clear, since there appear to be no direct studies on a possible osteogenic effect of thrombin. Other resorptive agents, like interleukin-1 and prostaglandin E_2, have been shown to stimulate (under certain conditions) bone formation and/or osteoblastic cell proliferation (30, 31).

Thrombin, as an integral component of a fibrin sealant system, may be partly responsible for the osteogenic effect obtained with this agent. Recent studies have pointed out the applicability and the clinical potential of this fibrin sealant, which has been used as a bone healing agent in trauma, orthopedic and periodontal surgery (32-34). The presently reported significant increases in osteoblastic proliferation may be of relevance to the aforementioned clinical observation.

Thrombin has significant effects on bone tissue (14-19). These studies have led to the implication of thrombin in inflammation-induced bone resorption, particularly in conditions such as arthritis and periodontal disease (17). The previously reported, by Feyen *et al* (18), lack of thrombin effect on cAMP levels, along with the presently reported increases in $[Ca^{2+}]_i$ and the almost perfect correlation between $[Ca^{2+}]_i$ levels and proliferation, strongly suggest that thrombin mediates its action on osteoblast-like cells via changes in $[Ca^{2+}]_i$. This is consistent with the effects of thrombin on other cells. Thrombin stimulates platelet functions via $[Ca^{2+}]_i$ increases (35, 36), whereas $[Ca^{2+}]_i$ increases are involved in the action of thrombin on endothelial (37) and smooth muscle (38) cells.

Many agents stimulating $[Ca^{2+}]_i$ increases have been shown to act via the phosphoinositide cycle (39, 40). Thrombin is reported to stimulate the phosphoinositide pathway in platelets (41), fibroblasts (27) and endothelial cells (37). The results of the present study indicate that thrombin mobilizes, in a time- and dose-dependent manner, the inositol phosphate pathway in two different osteoblast-like osteosarcoma cells. The relative extent of the inositol phosphate changes observed in the two cell-lines parallel the magnitude of $[Ca^{2+}]_i$ increases. This fact, together with the observation that thrombin increases $[Ca^{2+}]_i$ even in the absence of extracellular calcium, provides strong evidence that thrombin acts via the phosphoinositide cycle and the release of calcium from intracellular stores in the osteoblast-like cells.

Acknowledgments: We thank Paul Dressel for his art work on the figures. This work was partly supported by USPHS grants DE07034, DE08240, DE00158 and AR25271 from the National Institutes of Health, Bethesda, MD.

References

1. Colman, R.W., Marder, V.J., Salzman, E.W., and Hirsh, J. (1987) In "Hemostasis and Thrombosis" (R.W. Colman, J. Hirsh, V.J. Marder, and E.W. Salzman, Eds) pp 3-17. J.B. Lippincott, Philadelphia, PA.
2. Fenton II, J.W. (1986) Ann. NY Acad. Sci. 485, 5-15.
3. Doolittle, R.F. (1984) Ann. Rev. Biochem. 53, 195-229.
4. Grette, K. (1962) Acta Physiol. Scand. 56(suppl 195), 1-93.
5. Smith, J.B., Willis, A.L. (1970) Br. J. Pharmacol. 40, 545P-546P.
6. Weksler, B.B., Ley, C.W., and Jaffe, E.A. (1978) J. Clin. Invest. 62, 923-930.
7. Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. (1984) Proc. Natl. Acad. Sci. (USA) 81, 3534-3538.
8. Levin, E.G., Marzec, U., Anderson J., and Harker, L.A. (1984) J. Clin. Invest. 74, 1988-1995.
9. White, R.P., Chapleau, C.E., Dugdale, M., and Robertson J.T. (1980) Stroke 11, 363-368.
10. Shuman, M.A. (1986) Ann. NY Acad. Sci. 485, 228-239.
11. Bar-Shavit, R., Kahn, A., Wilner, G.D., and Fenton II, J.W. (1983) Science 220, 728-731.
12. Chen, L.B., and Buchanan, J.M. (1975) Proc. Natl. Acad. Sci. (USA) 72, 131-135.
13. Carney, D.H., and Cunningham, D.D. (1978) Cell 14, 811-823.
14. Gustafson, G.T., and Lerner, U. (1983) Biosci. Rep. 3, 255-261.
15. Hoffmann, O., Klaushofer, K., Koller, K., and Peterlik, M. (1985) Prostaglandins 30, 857-866.

16. Hoffmann, O., Klaushofer, K., Koller, K., Peterlik, M., Mavreas, T., and Stern, P. (1986) Prostaglandins 31, 601-608.
17. Lerner, U.H., and Gustafson, G.T. (1988) Biochim. Biophys. Acta 964, 309-318.
18. Feyen, J.H.M., Van der Wilt, G., Moonen, P., Di Bon, A., and Nijweide, P.J. (1984) Prostaglandins 28, 769-781.
19. Partridge, N.C., Hillyard, C.J., Nolan, R.D., and Martin, T.J. (1985) Prostaglandins 30, 527-539.
20. Rodan, S.B., Imai, Y., Thied, M.A., Wesolowoski, G., Thompson, D., Bar-Shavit, Z., Shull, S., Mann, K., and Rodan, G.A. (1987) Cancer Res. 47, 4961-4966.
21. Shupnik, M.A., and Tashjian Jr, A.H. (1982) J. Biol. Chem. 257, 12161-12164.
22. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
23. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) J. Cell Biol. 105, 2145-2155.
24. Berridge, M.J., Downes, C.P., and Hanley, M.R. (1982) Biochem. J. 206, 587-595.
25. Wreggett, K.A., Howe, L.R., Moore, J.P., and Irvine, R.F. (1987) Biochem. J. 245, 933-934.
26. Balla, T., Baukal, A.J., Guillemette, G., Morgan, R.O., and Catt, K.J. (1986) Proc. Natl. Acad. Sci. (USA) 83, 9323-9327.
27. Carney, D.H., Scott, D.L., Gordon, E.A., and LaBelle, E.F. (1985) Cell 42, 479-488.
28. Reid, I.R., Civitelli, R., Halstead, L.R., Avioli, L.V., and Hruska, K.A. (1987) Am. J. Physiol. 252, E45-E51.
29. Yamaguchi, D.T., Hahn, T.J., Beeker, T.G., Kleeman, C.R., and Muallem, S. (1988) J. Biol. Chem. 263, 10745-10753.
30. Canalis, E. (1986) Endocrinology 118, 74-81.
31. Chyun, Y.S., and Raisz, L.G. (1984) Prostaglandins 27, 97-103.
32. Lack, W., Bösch, P., and Arbes, H. (1987) J. Bone Joint Surg. [Br] 69-B, 335-337.
33. Pini Prato, G.P., Cortellini, P., and Clauser, C. (1988) J. Periodontol. 59, 679-683.
34. Schlag, G., and Redl, H. (1988) Clin. Orthop. 227, 269-285.
35. Rink, T.J., Smith, S.W., and Tsien, R.Y. (1982) FEBS Lett 148, 21-26.
36. Johnson, P.C., Ware, J.A., Cliveden, P.B., Smith, M., Dvorak, A.M., and Salzman, E.W. (1985) J. Biol. Chem. 260, 2069-2076.
37. Jaffe, E.A., Grulich, J., Weksler, B.B., Hempel, G., Watanabe, K. (1987) J. Biol. Chem. 262, 8557-8565.
38. Huang, C.-L., and Ives, H.E. (1989) J. Biol. Chem. 264, 4391-4397.
39. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.
40. Berridge, M.J., and Irvine, R.F. (1984) Nature 312, 315-321.
41. Rittenhouse-Simmons, S. (1981) J. Biol. Chem. 256, 4153-4155.