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THROMBIN EFFECTS ON OSTEOBLASTIC CELLS II. STRUCTURE-FUNCTION RELATIONSHIPS

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Thrombin has been shown to cause *in vitro* bone resorption and to stimulate osteoblastic cell proliferation, phosphoinositide turnover and cytosolic calcium levels. In the present study, the role of the active site of thrombin in its action on osteoblastic cells was investigated. Either hirudin or (4-amidinophenyl)methanesulfonyl fluoride inhibited, in a dose-dependent manner, the effects of thrombin on human osteoblast-like osteosarcoma cells (G292 and Saos-2 cell lines) and on normal rat calvarial osteoblastic cells. Thrombin-induced stimulation of cell proliferation, cytosolic calcium increases, and stimulation of phosphoinositide metabolism were concomitantly, and to a proportionally similar extent, inhibited. The inhibitors, when present in the absence of thrombin, did not affect the basal levels of cell functions. Both ζ-thrombin and γ-thrombin, forms resulting from proteolytic cleavage of α-thrombin, were capable of stimulating the osteoblastic cells. These data indicate that thrombin's actions on osteoblast-like cells are dependent on the availability of its catalytic site.

Thrombin (coagulation factor IIa, EC 3.4.21.5) is a serine protease with a central regulatory role in hemostasis (1, 2), partly due to its enzymatic activity on fibrinogen and the resulting fibrin formation (3). Thrombin demonstrates several hormone-like actions (4), which include stimulation of cell proliferation (5, 6), prostanoid synthesis by platelets (7) and endothelial cells (8), monocyte chemotaxis (9), and smooth muscle cell contraction (10). There is strong evidence to suggest that some of the hormone-like activities of thrombin on various tissues are related to its catalytic activity. Prostaglandin synthesis (8), platelet-activating factor production (11), phosphoinositide turnover and cytosolic calcium levels (12) stimulated by thrombin in endothelial cells, seem to be catalytic site dependent.

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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; pAPMSF, (4-amidinophenyl)methanesulfonyl fluoride; SA, specific activity; TCA, trichloroacetic acid; U, NIH equivalent clotting units.

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Several studies have shown thrombin effects on bone, including bone resorption (13-16), prostaglandin synthesis by osteoblast-like cells (17, 18), and stimulation of the proliferation of osteoblast-like cells (19, 20). It appears that thrombin's actions on osteoblastic cells are mediated by stimulation of phosphoinositide turnover (19, 20) and increases in cytosolic calcium levels (19-21). A recent study indicated that thrombin-stimulated *in vitro* bone resorption is dependent on the enzymatic activity (22), but there appear to be no studies on the structure-function relationships between the diverse structural regions of thrombin (23) and its effects on osteoblastic cells.

In the present study two human osteoblast-like osteosarcoma cell-lines and normal osteoblastic cells from rat calvaria were used to investigate the relative contribution of the various functional domains of thrombin to its effects on osteoblastic cells.

Materials and Methods

Chemicals. Tissue culture media and media supplements were obtained from GIBCO (Grand Island, NY). Tissue culture dishes and flasks were from Corning (Corning, NY). Fura-2AM and fura-2 were from Molecular Probes (Eugene, OR). pAPMSF was obtained from Boehringer Mannheim (Indianapolis, IN). Highly purified human plasma thrombin (SA: 4,000.0 U/mg protein), rat thrombin (SA: 1,118.0 U/mg protein) and recombinant hirudin (SA: 10,300.0 NIH units/mg protein) were purchased 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-tetrakis-phosphate) were obtained from NEN (Boston, MA). ^{3}H-thymidine was from ICN (Irvine, CA).
Alpha thrombin (24), γ-thrombin (25) and ζ-thrombin (26) were prepared and evaluated as described previously. The α-thrombin (lot 318, SA: 2,355.0 U/mg) was 90.6% α, 9.0% β, and 0.4% γ forms; the γ-thrombin (lot 83, SA: 47.66 U/mg) was 0.0% α, 0.0% β, and 100.0% γ forms; the ζ-thrombin preparations consisted of 1.5% α, 0.0% β, and 98.5% ζ (lot 10, SA: 2,399.0 U/mg) or 2.8% α, 1.6% β, and 95.7% ζ (lot 8, SA: 1,672.0 U/mg). pAPMSF-treated thrombin was prepared from the commercially obtained material as follows: serial thrombin dilutions were made, in duplicate, in thrombin carrier (0.15 M NaCl, 0.05 M NaCitrate, pH 6.5) and kept on ice; a 150 μM pAPMSF solution was freshly prepared in thrombin carrier and immediately added (3 μM final concentration) to one set of serial thrombin dilutions, while an equivalent amount of carrier was added to the other set; after a 10 min incubation at 22° C the solutions were used in the various experiments.
Cells and cell culture. The culture method for the two human osteoblastic osteosarcoma cell lines Saos-2 and G292 (19), and the isolation procedure (sequential collagenase treatment) for the normal rat calvarial cells (27) have been described in detail previously.
Cytosolic calcium studies. Cells (both Saos-2 and G292) were loaded with fura-2 and used for cytosolic calcium determinations as previously described in detail (19). Normal rat cells were also used, but the loading period with fura-2AM was 45 min, instead of 30 min. Unless otherwise noted in the figures or tables, all other experimental conditions were as previously described (19).
Cell proliferation. ^{3}H-thymidine incorporation was used to study cell proliferation. The method, a modification of the procedure described by Carney *et al* (28), has been detailed previously (19).
Phosphoinositide metabolism studies. Culture, labelling and phosphoinositide analysis of the human osteosarcoma cells were performed as previously described in detail (19), with two modifications. The first modification was the substitution of 10% FCS with 1mg/ml BSA in the labelling medium (McCoy's 5a without inositol); the elimination of serum from the labelling medium resulted in 1) cells that were quiescent at the time of the experiment and 2) increased incorporation of label into the cells. The second modification was the change of the gradient for the HPLC analysis (from 100% A for 5 min and then linear 100% A to 100% B in 40 min) to 100% A for 10 min and then linear 100% A to 100% B in 40 min. This resulted in better separation of the free inositol peak from the IP peak. The average retention time, in this system, for the various inositol phosphates is: IP-14-15 min, IP_2-21 min, IP_3-31 min, IP_4-41.5 min. The counts corresponding to the IP, IP_2, IP_3 and IP_4 peaks are expressed as percentage (%) of inositol phosphate counts (19). For information purposes, the actual mean counts obtained from the chromatograph are also given in parenthesis in Table 1.
After isolation, populations III and IV of the normal rat cells [populations with the osteoblastic phenotype (27)] were pooled and cultured in 12-well plates (10^6 cells/ml; 1ml/well) in BGJb (Fitton-Jackson modification) medium supplemented with 10% FCS for 48 h. The cells were then washed (2X) with BGJb medium with 1mg/ml BSA, and labelled for 24 h with 30 μCi/ml *myo*-[^{3}H]-inositol (SA: 45-80 Ci/mmol) in 50% BGJb medium with 1mg/ml BSA and 50% McCoy's 5a

without inositol with 1mg/ml BSA. After this labelling period, the normal cells were used for phosphoinositide experiments, in the same manner previously detailed for the G292 cells (19).
Statistical Analysis. Data were analysed using factorial ANOVA, paired and unpaired Student's t test.

Results

Active site inhibition abolishes thrombin-induced cytosolic calcium increases. Addition of thrombin to the G292 human osteoblast-like cells in the presence of hirudin, a specific thrombin inhibitor (29), resulted in inhibition of the thrombin-stimulated increase in $[Ca^{2+}]_i$ (Fig. 1). Similar results were obtained with the Saos cells (data not shown). When pAPMSF-treated thrombin was added to the cells the calcium response was absent (data not shown). Either pAPMSF, a synthetic serine

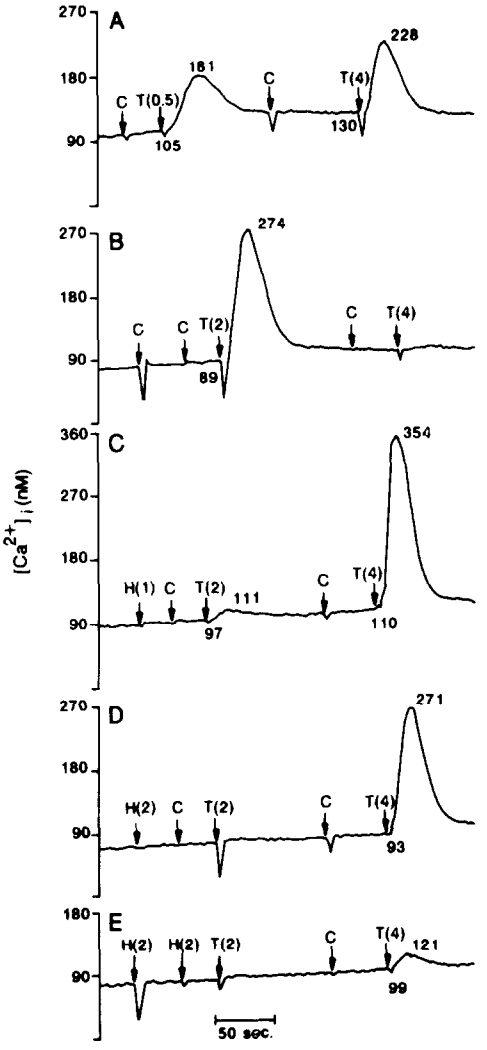

Fig. 1. Hirudin dose-dependently inhibits thrombin-induced $[Ca^{2+}]_i$ increases in G292 cells. A-E: Experiments performed successively on aliquots of the same cell preparation, using the same agonist solutions. The numbers below and above the graph lines represent the $[Ca^{2+}]_i$ peak values (in nM) before and after, respectively, the addition of carrier control (C), hirudin (H) or human thrombin (T) to the cells. Numbers in parentheses indicate the amount of agonist added, in U/ml (final concentration). Occasional downward spikes are instrumental artifacts caused during agent addition (opening of the cuvette chamber).

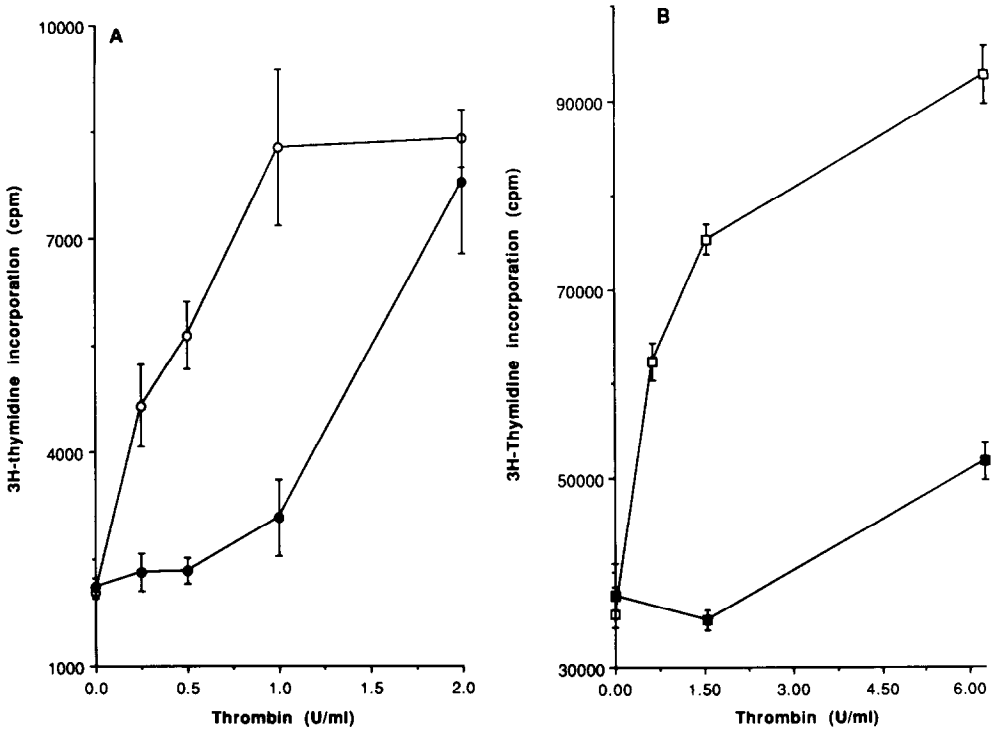

Fig. 2. Thrombin inhibition abolishes the thrombin-induced osteoblastic cell proliferation. A. G292 cells received serial thrombin dilutions either pretreated with 3 μM pAPMSF (filled circles) or left untreated (open circles). B. Saos-2 cells were treated with serial thrombin dilutions either in the presence (filled squares) or absence (open squares) of 2 U/ml hirudin. Similar findings were obtained with G292 cells (data not shown). Results are the mean ± standard deviation for quadruplicate (A) or triplicate (B) determinations.

proteinase inhibitor (30), alone (data not shown) or hirudin alone (Fig. 1) did not affect basal cytosolic calcium levels in the osteoblast-like cells. Hirudin inhibited the thrombin effect in a dose-dependent manner (Fig. 1C-E).

The functional catalytic site is required for stimulation of proliferation. Thrombin's proliferative effect on the osteoblast-like cells was inhibited when the enzyme was pretreated with pAPMSF (Fig. 2A). When thrombin was added to the cells in the presence of hirudin the dose-response curve was shifted to the right (Fig. 2B). Neither hirudin alone (Fig. 2B) nor pAPMSF alone (Fig. 2A) had an effect on the basal proliferation of the cells. Both ζ-thrombin and γ-thrombin [two forms of the enzyme that are derived from proteolytic cleavage of the alpha form (see discussion)] were capable of stimulating osteoblastic cell proliferation, but γ-thrombin was less potent in this effect (data not shown).

Catalytic site inhibition abolishes thrombin-stimulated phosphoinositide turnover. As with cytosolic calcium and proliferation, the stimulation of osteoblastic cell phosphoinositide turnover by thrombin was inhibited in the presence of hirudin (Fig. 3A). Similarly, pAPMSF-treated thrombin was not capable of stimulating the inositol phosphate metabolism of the osteoblast-like cells (Fig. 3B).

All three thrombin forms, α-thrombin, ζ-thrombin, and γ-thrombin were capable of stimulating the phosphoinositide turnover of osteoblastic cells (Table 1). Since γ-thrombin was less potent than the other forms in stimulating proliferation, the potency of this form in eliciting a phosphoinositide response was evaluated further. At 0.2 μM γ-thrombin was invariably capable of stimulating the phosphoinositide turnover of human osteoblast-like osteosarcoma cells (Fig. 4A) and of rat calvarial

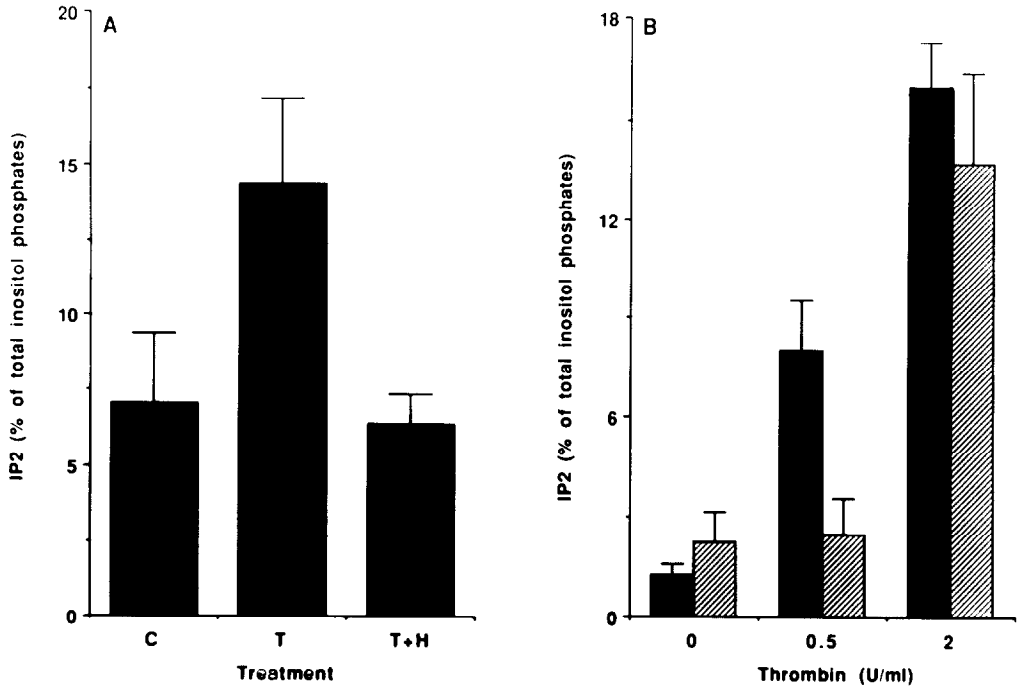

Fig. 3. Thrombin inhibition abolishes the thrombin-induced osteoblastic cell inositol phosphate turnover. A. normal rat calvarial osteoblastic cells were treated with thrombin carrier (C) or 2 U/ml rat thrombin (T) or T in the presence of 2 U/ml hirudin (T+H). *p<0.05 from other groups (ANOVA). B. G292 cells received serial thrombin dilutions either treated with 3 μM pAPMSF (hatched bars) or left untreated (black bars) [Compare with Fig. 2A]. Similar findings were obtained with Saos-2 cells (data not shown). Cells were assayed 45 s after thrombin addition. Results are the mean + standard deviation from triplicate (A) or quadruplicate (B) determinations.

Table 1. Effect of human thrombins on inositol phosphate levels in G292 cells

Treatment	IP	IP_2	IP_3	IP_4
Control	91.30 ± 0.99 (3068.0)	5.06 ± 1.35 (168.5)	2.03 ± 0.49 (68.5)	1.61 ± 0.83 (54.8)
Thrombin [0.04μM, Sigma]	45.79 ± 1.88 (4233.7)	42.39 ± 1.92 (3928.3)	9.76 ± 0.70 (908.0)	2.06 ± 0.74 (186.7)
α-thrombin [0.05μM]	42.99 ± 3.18 (3618.3)	45.05 ± 3.31 (3792.2)	9.29 ± 0.46 (780.8)	2.68 ± 0.56 (225.5)
γ-thrombin [0.05μM]	62.15 ± 2.89 (3648.8)	28.18 ± 1.76 (1647.0)	8.25 ± 1.22 (493.5)	1.80 ± 0.91 (109.5)
ζ-thrombin [0.04μM, Lot 10]	46.40 ± 2.64 (3879.0)	41.05 ± 2.57 (3402.2)	9.75 ± 0.66 (817.0)	2.86 ± 0.51 (235.5)
ζ-thrombin [0.06μM, Lot 8]	44.87 ± 2.21 (3973.5)	43.08 ± 1.91 (3814.0)	8.75 ± 1.09 (775.2)	3.30 ± 0.38 (293.2)

Values are the percentage of the total inositol phosphate counts. Results are the mean ± standard deviation for quadruplicate determinations (triplicates for Sigma thrombin). Values in parenthesis are the mean counts (above background) for the corresponding peaks, from the quadruplicates. Controls received an appropriate dilution of thrombin carrier. Reactions were terminated 45 s after the addition of the agents. All IP, IP_2 and IP_3 values are significantly different from control ($p<0.05$, ANOVA). The γ-thrombin IP and IP_2 values are significantly different from other forms ($p<0.05$, ANOVA). Similar findings were observed with the Saos-2 cells (data not shown), although γ-thrombin was much less effective in this cell-line, which is less responding to thrombin when compared to G292 cells (19).

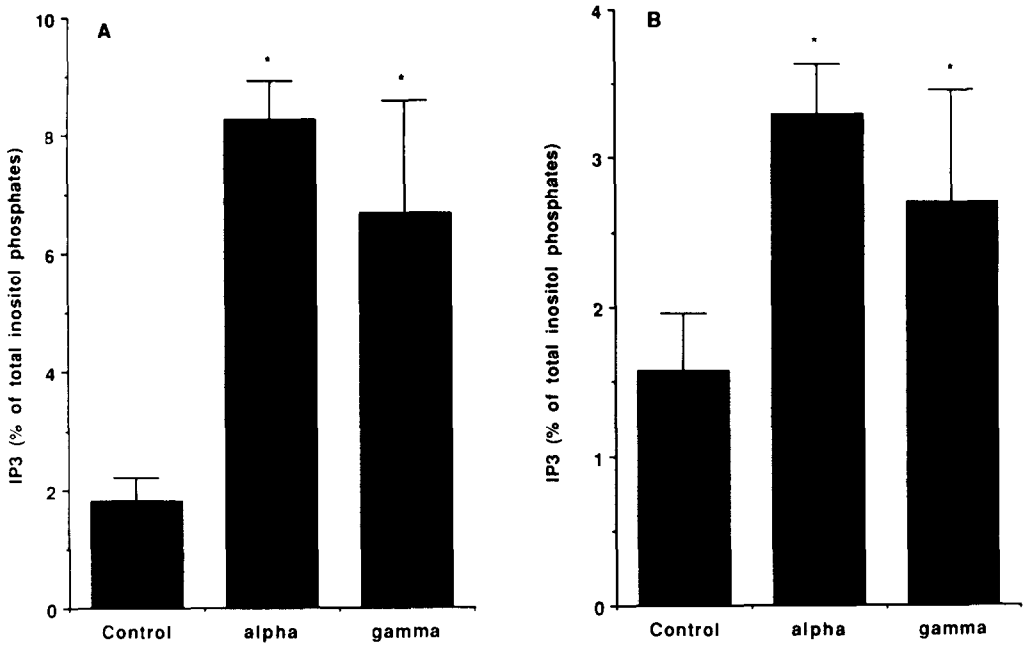

Fig. 4. Comparison between α-thrombin and γ-thrombin. A. G292 cells, and B. normal rat calvarial osteoblastic cells received 0.2 μM of either human thrombin or an appropriate amount of thrombin carrier (control) and were assayed 45 s later. Results are the mean + standard deviation from quadruplicate determinations. *p<0.05 vs. control (ANOVA).

normal osteoblastic cells (Fig. 4B), although the cellular response was not of the same magnitude as to α-thrombin. Gamma-thrombin did stimulate the inositol phosphate metabolism of the osteoblastic cells at doses below 0.1 μM (Table 1), but the effect, unlike that of α-thrombin or ζ-thrombin at the same dose, was not consistent; it appeared to depend on the overall state of cell responsiveness (e.g. cell passage, data not shown). The rat osteoblastic cells responded to both rat (Fig. 3A) and human (Fig. 4B) thrombins.

Discussion

In a previous study we have shown, using the human osteoblast-like osteosarcoma cell-lines G292 and Saos-2, that thrombin is a potent agonist for osteoblastic cell cytosolic calcium increases and phosphoinositide turnover (19). The results of the present study extend these findings in normal rat calvarial osteoblastic cells and indicate that thrombin's activities on osteoblastic cells depend on the availability of its catalytic site.

The structure (2) and the functional domains (23) of thrombin have been extensively reviewed. Besides the active site, responsible for the proteolytic activity, there are several exosites with various functions attributed to them. Such domain(s) have been shown to mediate fibrinogen binding (31), monocyte chemotaxis (9), and macrophage proliferation (32), which are thrombin functions that do not depend on a functional active site. In contrast, thrombin-induced smooth muscle contraction depends entirely on the catalytic site (33). Finally, the effects of thrombin on fibroblasts (34) and platelets (35) appear to depend on more than one domain. Therefore, a certain cellular response to thrombin can be mediated either entirely by the active site of the molecule, or entirely by an exosite, or by a combined action of the catalytic site and an exosite.

The relative contribution of the various functional domains of thrombin to its effects on bone cells had not been previously investigated. The present study is the first report that the catalytic site of

thrombin is required for osteoblastic cell response. Hirudin, a specific thrombin inhibitor (29), completely inhibited the effects of thrombin on the osteoblastic cells. Hirudin is a small (~7 kd) peptide that binds to thrombin over an extended contact region (36-38) that includes thrombin's exosite involved in chemotaxis (9, 37). Therefore, a possible contribution of this functional domain to the action of thrombin on osteoblastic cells could not be ruled out on the basis of hirudin inhibition studies. However, inhibition with pAPMSF, an agent that acts by irreversibly modifying the active site serine (30), also resulted in complete inhibition of thrombin's effects on osteoblastic cell proliferation, cytosolic calcium and phosphoinositide metabolism. These results strongly suggest that a free catalytic site is required for thrombin to have an effect on osteoblast-like cells. This does not mitigate the proposed (19) role of thrombin in the fibrin sealant system, since studies have shown that: i) the fibrin clot can be a reserve of releasable, enzymatically active thrombin (39, 40) and ii) thrombin can retain its functional properties even after it binds to extracellular matrix (41).

The present data reaffirm the previously reported (19) relation between thrombin-stimulated phosphoinositide metabolism, cytosolic calcium, and proliferation in osteoblastic cells, since all three responses were concomitantly inhibited to the same extent (e.g., compare Fig. 2A and Fig. 3B). The results of the present study, in conjunction with the results of a recent study by Stern *et al* (22) which indicated that thrombin-stimulated *in vitro* bone resorption is dependent on the catalytic site, support further the role of the osteoblast as the cell regulating and/or mediating the response(s) of bone to thrombin.

Proteolytic or autoproteolytic cleavage of thrombin results in the formation of various thrombin forms with altered structures (23-26). A tryptic digestion form is γ-thrombin (25), which lacks clotting activity [because it lacks the fibrin(ogen)/anion-binding exosite] but retains proteolytic activity. Another form of proteolytically altered thrombin is ζ-thrombin (26), which retains its clotting ability and appears to be physiologically more relevant than γ-thrombin. Contrary to its inability to stimulate fibroblastic cells (34) γ-thrombin could stimulate the osteoblastic cells, even though it was a less potent agonist than α- or ζ-thrombins. In the study by Stern *et al* (22) γ-thrombin was consistently less potent than α-thrombin in stimulating *in vitro* bone resorption. These results suggest that the anion-binding exosite may play a secondary role in the action of thrombin on osteoblastic cells, possibly by providing an additional contact region between the cell surface and the enzyme. The comparable potency of α- and ζ-thrombin in stimulating osteoblastic cells reported herein is the first report on the ability of the ζ-form to stimulate any cell type, and corroborates the recently reported similarities in activity (when tested for their enzymatic properties) between α- and ζ-thrombin (26).

Studies with cells that respond to enzymatically active thrombin have indicated that on the cell surface there are protein substrates for the protease (42, 43). The results of the present study strongly suggest that osteoblastic cells should have an analogous thrombin substrate on their surface, although the nature of such a membrane protein is not presently known.

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