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Thrombin-induced proliferation and expression of platelet-derived growth factor-A chain gene in human vascular smooth muscle cells

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Treatment of human vascular smooth muscle cells (SMC) with human α-thrombin greatly increased DNA synthesis and cell proliferation. Both the integrity of the catalytic site and that of the anion binding exosite were required for expression of this activity. Experiments employing Northerns indicated induction of c-*fos* expression as well as a time-dependent induction of platelet-derived growth factor-A (PDGF-A) gene by thrombin. The thrombin mitogenic activity was potentiated by PDGF-BB, insulin and the vasoconstrictor peptide endothelin-1 suggesting synergism by convergence of intracellular growth-promoting signals. SMC treatment with pertussis toxin and forskolin indicated that the mitogenic activity of thrombin may be induced via signal transduction mechanism(s) involving changes in cAMP levels and activation of a G_i-like protein. These results suggest that thrombin may play a functional role in the regulation of human vascular SMC proliferation.

Platelet-derived growth factor induction, Thrombin; Smooth muscle cell, Proliferation, Pertussis toxin; G protein; Cyclic AMP

1. INTRODUCTION

The properties of thrombin as a serine protease (EC 3.4.21.5) and its role in the blood coagulation–fibrinolysis cascade, have been well characterized [1–4]. Thrombin can also contribute to the thrombogenic properties of the vessel wall. Fibrin-bound thrombin is protected from inactivation by heparin–antithrombin III, and there is a sustained release of α-thrombin after blood clotting during re-endothelialization and enzymatic degradation of the blood clot [5,6]. Furthermore, thrombin can induce gap formation between adjacent endothelial cells [7], and it can bind to subendothelial extracellular matrix (ECM) leaving the enzyme catalytic site intact [8,9]. Thrombin is chemotactic for monocytes [10], it initiates proliferation of a number of cells [11,12], it alters ECM composition and promotes endothelial cell adhesion and spreading [9,13,14]; it induces the expression and release of platelet-derived growth factor (PDGF) from endothelial cells [15,16] and acts as a vasoconstrictor for vascular SMC [17].

The importance of the catalytic activity in thrombin and its role in vascular remodelling have become issues of controversy with regard to vascular SMC proliferation. Although in neonatal rat vascular-SMC, α-thrombin was shown to be mitogenic [18,19] and to induce c-*myc* and PDGF-A chain expression [20], in adult rat-SMC, α-thrombin and its various thrombin forms were shown to induce protein synthesis and growth-related signals including c-*fos* mRNA expression but not DNA synthesis [21,22]. In contrast, immobilized, non-enzymatically active thrombin was shown to induce SMC proliferation [23]. In the present work we demonstrate that human α-thrombin is a potent mitogen for adult human vascular-SMC; that it synergises with PDGF or other mitogens and that it induces the expression of PDGF-A chain gene. The evidence that the potency of thrombin as a mitogen depends on both, the catalytic and anion exosite sites as well as the involvement of a pertussis toxin sensitive G-protein in the signal transduction mechanism(s) of thrombin are discussed.

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2. MATERIALS AND METHODS

2.1 *Materials*

Purified human α-thrombin (specific activity 3,000 U/mg), pertussis toxin, cyclic adenosine monophosphate (cAMP), 3′,5′-cAMP-dependent protein kinase, forskolin, IBMX and anti-α-SMC actin antibodies were from Sigma, D-Phe-Pro-Arg-CH_2Cl (PPACK) from Calbiochem. [^{3}H]Thymidine (5 mCi/mmol), [^{3}H]cAMP (24 Ci/mmol) and [^{32}P]dCTP (3,000 Ci/mmol) were from Amersham; PDGF-BB and fibroblast growth factor (FGF) were from Bachem, insulin was from Boehringer and endothelin-1 was from Peninsula Lab. All other chemicals and culture media were obtained from sources as specified or were of the highest purity available commercially. Purified human α-thrombin was characterized by SDS-PAGE, and its enzymatic activity determined as described [2]. γ-Thrombin prepared by autocatalytic degradation of human α-thrombin [24] retained esterase activity but had only 1.6 U/mg specific clotting activity. PPACK-α-thrombin was prepared as described [25] and dialyzed against serum-free (SF) DMEM prior to use.

2.2. *Cells and cell culture*

Human SMC were isolated from sections of abdominal normal aorta obtained from kidney transplant donors (10–45 years of age). The aortic tissue was transferred into DMEM supplemented with 100

1U/ml penicillin, 100 μg/ml streptomycin sulphate, 100 μg/ml neomycin and 2.5 μg/ml fungizone (Gibco) Dissected medial tissue was finely minced, rinsed and digested under constant agitation (18–20 h, 37°C) in DMEM 15 mM HEPES (Flow Lab) with 0.1% collagenase Type II (Worthington) and 0.05% elastase Type I (Sigma) supplemented with 10% FCS. Digested cells were cultured in L-glutamine/antibiotics-supplemented DMEM/HEPES and 20% FCS which was reduced to 10% after the second passage Cells were subcultured at a split ratio of 1:4, characterized [26] and used between passages 2–8. Two or three cell cultures were used for each experiment.

2.3. *cAMP measurement*

After treatment of growth-arrested cells (see below) under various conditions, cAMP levels were measured in extracts of cells treated with ethanol (4°C, 30 min). After removal of precipitated protein by centrifugation, the ethanol extracts were dried by evaporation (under vacuum), resuspended in 50 mM Tris-HCl, 4 mM EDTA pH 7.0 and assayed as described previously [27]. Protein was estimated using the Pierce protein-assay reagent kit.

2.4. *DNA synthesis/cell proliferation*

For DNA synthesis, SMC were plated (96-well plates, 1×10^4 cells per well) in DMEM/15 mM HEPES supplemented with 10% FCS and antibiotics. Upon confluency, cells were growth arrested (48 h; 0.5% FCS) Unless otherwise stated, DNA synthesis was measured 40 h post-stimulation by labelling (24 h) with 1 μCi [^{3}H]thymidine prior to harvesting the cells (Brandel harvester; Whatman GF/B filters). For experiments with pertussis toxin cells were pre-incubated for 6 h with the indicated concentration of the toxin in SF medium before stimulation with growth factors. For cell proliferation, SMC were plated (24-well plates; 1×10^5 cells per well) in DMEM/HEPES, 10% FCS and 24 h later growth arrested as above. Cell numbers were measured 4 days later using a Coulter Counter.

2.5. *Northern hybridization*

RNAs were prepared from SMC (≈10^7 cells) according to the method of Wisnievski et al. [28]. For Northern hybridization RNA (10 μg) was electrophoresed through agarose gel (1%), transferred to Hybond membrane filters and hybridized with either the c-*fos* (pf22) DNA probe [29], a c-*myc* probe containing exon 2'–3' coding region [30] or a PDGF-A probe containing the *Hin*dIII–*Sac*I fragment (640 bp) [31]. The DNA probes were labelled with [^{32}P]dCTP by random priming as described [32]. The pGd-T-SB probe of glucose-6-phosphate dehydrogenase (G6PD) [33] was used as a control in all Northerns. Removal of radiolabelled probe for rehybridization of membranes was as described [32]

3. RESULTS AND DISCUSSION

Media SMC migration and proliferation into the intima in response to chemoattractants and growth factors released at the site of vascular injury, have been considered as major contributors to the proliferative nature and progression of atherosclerotic lesions [34]. Such lesions are often occluded by a thrombus [35] which during lysis can become the source of enzymatically active thrombin [24]. This potential availability of thrombin to VSMC has placed particular emphasis on its post-clotting role in promoting SMC proliferation. Fig. 1 shows that in SF medium, enzymatically active, human α-thrombin induces DNA synthesis in human aortic SMC (Fig. 1a) in a dose-dependent manner with maximal stimulation obtained between 2 and 4 U/ml (20–40 nM). The γ-thrombin concentrations used were based on the original concentration of α-thrombin; this preparation retained little clotting activity as a result of autocatalytic cleavage that lowers the affinity of the fibrinogen binding exosite [1–4,24]. γ-Thrombin as compared to α-thrombin and when used at equimolar concentrations was found to be less potent in stimulating [^{3}H]thymidine uptake (Fig. 1a). α-Thrombin inactivated at the catalytic site by the D-Phe-Pro-Arg-chloromethyl ketone inhibitor [25], PPACK-thrombin, failed to induce DNA synthesis at the same range of concentrations (Fig. 1a) suggesting that an intact catalytic site and a high-affinity anion-exosite are important for the mitogenic activity of thrombin in agreement with data obtained in fibroblasts [24]. As expected from the [^{3}H]thymidine uptake data, there was a significant increase (36 ± 2%) in cell numbers upon 4-day exposure

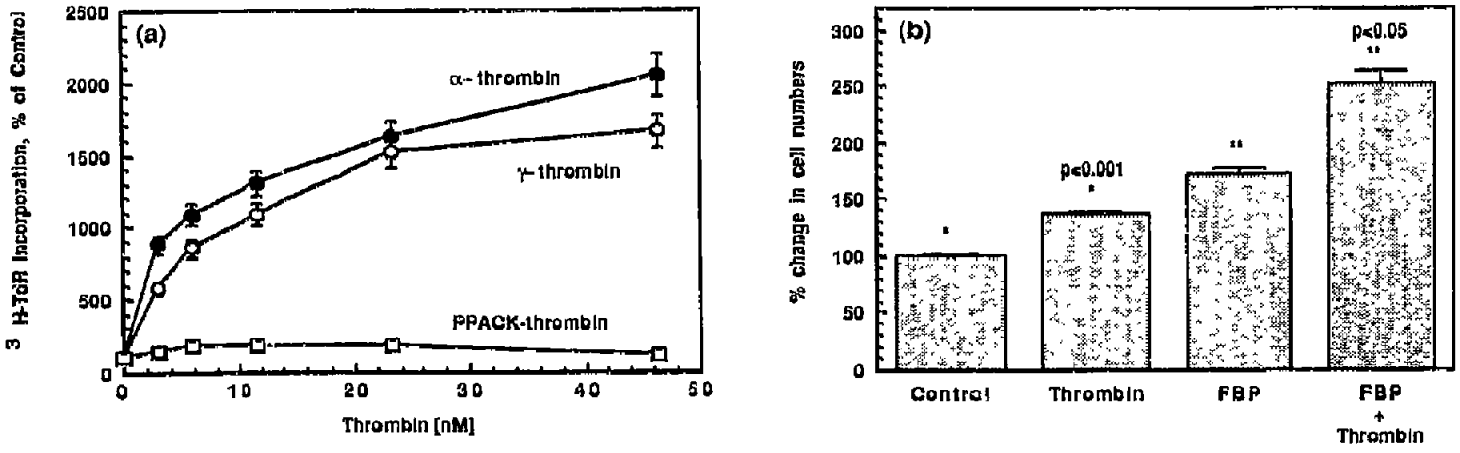

Fig 1. Thrombin induces DNA synthesis and proliferation of human aortic smooth muscle cells. (a) Induction of DNA synthesis in SF medium by α-thrombin (●), γ-thrombin (○) or PPACK-α-thrombin (□). (b) Cell proliferation induced by 2 U/ml thrombin in SF medium supplemented with or without 2% FBP Data are expressed as a percentage of control cells incubated for the same time period in SF medium alone, and represent the mean ± S.E.M. (n = 4) of one of three or four representative experiments. P values were calculated using Student's t-test analysis.

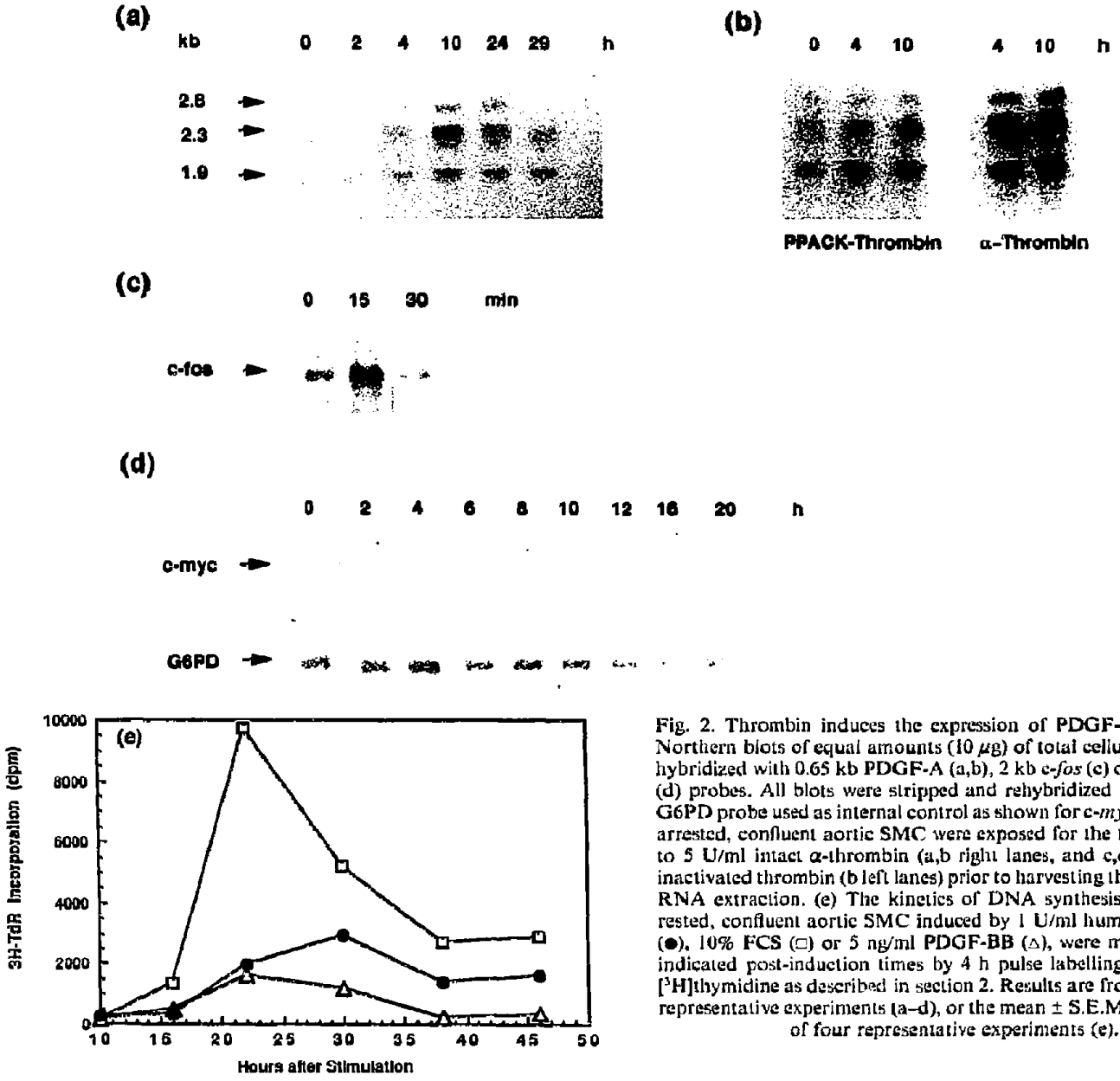

Fig. 2. Thrombin induces the expression of PDGF-A chain gene. Northern blots of equal amounts (10 μg) of total cellular RNA were hybridized with 0.65 kb PDGF-A (a,b), 2 kb c-*fos* (c) or 1.3 kb c-*myc* (d) probes. All blots were stripped and rehybridized with a 0.53 kb G6PD probe used as internal control as shown for c-*myc* (d). Growth-arrested, confluent aortic SMC were exposed for the times indicated to 5 U/ml intact α-thrombin (a,b right lanes, and c,d) or PPACK-inactivated thrombin (b left lanes) prior to harvesting the cells for total RNA extraction. (e) The kinetics of DNA synthesis in growth-arrested, confluent aortic SMC induced by 1 U/ml human α-thrombin (●), 10% FCS (□) or 5 ng/ml PDGF-BB (△), were measured at the indicated post-induction times by 4 h pulse labelling the cells with [^{3}H]thymidine as described in section 2. Results are from one of three representative experiments (a–d), or the mean ± S.E.M. (n = 4) of one of four representative experiments (e).

of aortic SMC to thrombin as compared to quiescent control cells (Fig. 1b). Exposure to fetal bovine plasma (FBP) (platelet poor plasma; with no esterase activity) increased cell numbers to 72 ± 1% and co-exposure with thrombin to 151 ± 12% (Fig. 1b), indicating a synergistic effect between thrombin and the growth promoting activity of plasma.

Thrombin induced the expression of PDGF-A chain mRNA which, as indicated by the time course of expression in Fig. 2a, peaked and fell significantly much later than previously reported for thrombin or other mitogens [20]. PDGF-A expression levels normalised for G6PD mRNA levels used as control, showed that PPACK-inactivated thrombin stimulated mRNA expression by 2-fold (Fig. 2b, left lanes) as compared to active α-thrombin which elevated mRNA levels by 5-fold after 10 h of stimulation (Fig. 2b, right lanes). The low stimulation of PDGF-A mRNA expression by this preparation of PPACK-inactivated thrombin was attributed to a residual mitogenic activity (data not shown) invariably seen in some preparations as a result of incomplete inactivation of thrombin due to the rapid hydrolysis of the chloromethyl ketone inhibitor. Although we cannot exclude the induction of expression of other growth factors, the kinetics of PDGF-A gene induction by thrombin and the delay for thrombin-induced maximal DNA synthesis as compared to FCS or PDGF-BB (Fig. 2a vs. 2e) are similar to those of the autocrine

PDGF-AA loop induced in VSMC by interleukin-1 [36] or transforming growth factor-β [37]. Catalytically active thrombin induced the expression of c-*fos* (Fig. 2c) in accordance with the action of other known mitogens and in agreement with other studies on thrombin [21]. c-*myc* expression appeared to peak at 8 h (Fig. 2d) which is much later than has been observed for thrombin in fibroblast cells (1 h) or in neonatal VSMC (4 h), or for other mitogens [20,39,40]. When it is compared with the time course of PDGF-A mRNA expression, our data support that the mediation of c-*myc* expression is possibly via autocrine growth factor activity. Therefore, in human VSMC thrombin may affect cell growth by direct induction of early growth-related signals as well as via induction of expression of growth factor activity. Variations in experimental conditions, the conformation of thrombin or variations in growth factor receptor types and numbers may account for variations on thrombin mitogenicity in the various cell types observed previously [18–24]. Similarly, species specific differences may account for the earlier induction of c-*myc* and PDGF-A expression (4–7 h) observed in neonatal rat VSMC [20].

The DNA synthesis induced by thrombin was potentiated by insulin, which by itself failed to induce DNA synthesis in human VSMC, or by PDGF-BB and the vasoconstrictor peptide endothelin [41] which were not mitogenic at the low concentrations tested here (Fig. 3a–c). This potentiation was more apparent at higher concentrations of thrombin for insulin and at lower concentrations for the other ligands (Fig. 3) suggesting synergism via several mechanisms of signal transduction, and/or that potentiation may depend on the level of concentration of second messenger(s) induced by thrombin. These observations are in agreement with the evidence in support of cooperative effects on cell proliferation of G protein-dependent signal pathways and growth factor–receptor tyrosine kinases [42] and the fact that thrombin and endothelin have similar mitogenic and vasoconstrictor activities in SMC [17,43].

Pertussis toxin treatment attenuated the thrombin-induced DNA synthesis in the range of thrombin concentrations shown, and this attenuation was dependent on the concentration of the toxin (Table I) in agreement with the findings reported previously for Chinese hamster lung fibroblasts [44]. Pertussis toxin (10 ng/ml) treatment of cells did not affect their mitogenic response to PDGF-BB or FGF as compared to untreated cells (data not shown) suggesting that the effect of pertussis toxin on the thrombin mitogenic activity is specific. Since pretreatment with forskolin which is believed to stimulate adenylate cyclase directly [45] and was shown to inhibit endothelial PDGF-induction by thrombin [46], also inhibited DNA synthesis induced by thrombin (Table I), our data indicates the involvement of a G_i-like protein which regulates negatively adenylate cyclase as also reported for platelets [47]. The involvement of a

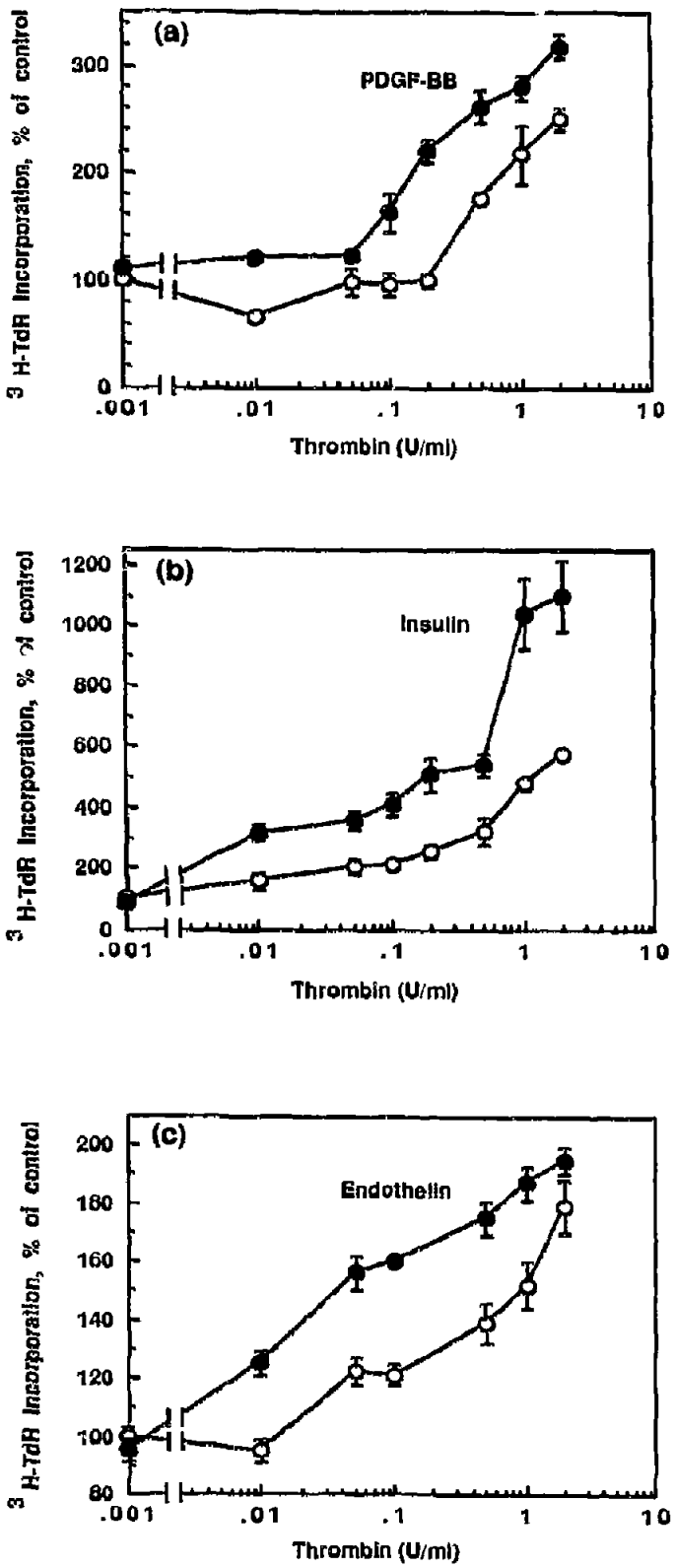

Fig. 3. Thrombin synergizes with other growth promoting factors Dose-dependent induction of DNA synthesis in aortic SMC by human α-thrombin, was measured in the absence (○) or presence (●) of (a) 0.5 ng/ml PDGF-BB, (b) 1 μg/ml insulin, or (c) 2.5 ng/ml endothelin-1 as described in section 2. Values represent the mean ± S.E.M (n = 4) of one of 3 or 4 representative experiments.

Table I

Thrombin effect on cAMP levels, and the effect of pertussis toxin and forskolin on thrombin-induced DNA synthesis in human aortic smooth muscle cells

Thrombin (U/ml)	cAMP[a] (pmol/mg protein)	DNA synthesis[b] (dpm)				
		Pertussis toxin (ng/ml)			Forskolin (μM)	
		0	1	10	0	10
0.00	94.4 ± 2.4	766 ± 80	571 ± 45	474 ± 28	700 ± 80	635 ± 45
0.10	nd[c]	2,358 ± 259	1,274 ± 130	939 ± 162	2,860 ± 245	815 ± 75
0.5	85.3 ± 3.7	2,922 ± 80	1,617 ± 178	1,149 ± 235	nd	nd
1	nd	4,439 ± 600	1,995 ± 235	1,579 ± 69	4,645 ± 80	1,255 ± 305
2	75.4 ± 2.1	5,439 ± 1,679	2,874 ± 287	1,981 ± 28	nd	nd
5	69.2 ± 2.6	nd	nd	nd	nd	nd

[a]cAMP levels in confluent, growth-arrested SMC were measured 60 min post-stimulation with human α-thrombin in SF medium in the presence of 100 μM IBMX. cAMP levels in control cells were measured after 60 min incubation with 100 μM IBMX in SF medium.

[b]Thrombin-induced DNA synthesis was measured as in section 2 without or with pre-treatment (6 h) of cells with pertussis toxin or in the absence or presence of forskolin added immediately before thrombin. Results represent the mean ± S.E.M. (n = 4) of one of 2 or 3 representative experiments.

[c]nd = not determined.

G-like protein was also suggested by the thrombin inhibition of cAMP levels which we were able to observe after inclusion of the phosphodiesterase inhibitor IBMX (Table I). However, since the direct coupling of the thrombin receptor to a G_i-like protein has yet to be demonstrated, we cannot exclude the possibility of an indirect effect on the activity of adenylate cyclase via thrombin receptor coupling to additional pertussis toxin-sensitive or -insensitive G proteins and/or induction of other pathways which may co-operate in the mediation of DNA synthesis as suggested previously for other cell types [18–24,44,46]. Recent findings support the possibility of receptor coupling with more than one G protein [48]. Alternatively, there may be more than one type of thrombin receptor the expression of which as well as coupling to signal transducers may differ between cell types. The requirement for an intact catalytic site of thrombin and the involvement of the anion exosite for the induction of DNA synthesis, are in agreement with the novel thrombin-receptor activation mechanism proposed recently [49–51]. Similarly, the signal transduction mechanism of thrombin receptor activation indicated by our data, are in agreement with receptor coupling to a G protein proposed from molecular cloning expression and transfection studies of the human thrombin receptor [49–51].

The induction of growth factor expression like PDGF in human vascular SMC by thrombin, and the thrombin potentiation of growth factor- or vasoconstrictor-mitogenic activity emphasize the growth factor-like properties of this serine proteinase enzyme. It also provides a new perspective from which to consider (a) the mitogenic and proliferative effects of thrombin as a link between blood clotting and wound healing, and (b) the post-clotting contributions of thrombin to atherogenesis.

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