Thrombin-stimulated events in cultured vascular smooth-muscle cells

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Thrombin is present in high concentrations at sites of clots and may have important post-clotting effects on adjacent vascular tissue. This may be particularly important for vascular smooth-muscle cells (VSMC), whose growth and contractility are altered following atherosclerotic-associated thromboses. To study the cellular signal events by which thrombin exerts its actions, the effects of purified human α -thrombin were examined in cultured rat aortic VSMC. α -Thrombin stimulated a biphasic change in intracellular pH (pH_i), causing an early rapid acidification, followed by a sustained alkalinization. The increase in pH_i was dependent on extracellular Na^+ and inhibited by 5'-(*NN*-dimethyl)amiloride, consistent with mediation by Na^+/H^+ exchange. α -Thrombin rapidly increased free intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). The increase in $[Ca^{2+}]_i$ was secondary to activation of phospholipase C, as demonstrated by increases in $InsP_3$ (226 %) and $InsP_2$ (387 %) and decreases in polyphosphoinositides at 15 s. Expression of the mRNA for the proto-oncogene *c-fos* was induced by α -thrombin. Stimulation of *c-fos* mRNA was not dependent on alterations in pH_i , but required a rise in $[Ca^{2+}]_i$. Despite many growth-related signals shared by α -thrombin with platelet-derived growth factor, α -thrombin failed to stimulate $[^3H]$ thymidine incorporation or cell division, although there was a maximal increase of 52 % in protein synthesis. The data suggest that there are cellular signal events not activated by α -thrombin which are required for proliferation of these aortic VSMC.

INTRODUCTION

The procoagulant α -thrombin has central bioregulatory functions in haemostasis (Fenton, 1987) and is generated at concentrations up to 130 nM during blood clotting (Walz *et al.*, 1985). It is actively incorporated into clots and released gradually during clot retraction and fibrinolysis (Wilner *et al.*, 1981). As such, thrombin and its degradative products may participate in post-clotting vascular events. For example, human coronary atherosclerotic lesions that become clinically unstable are characterized by platelet thrombi and enhanced growth and vasoreactivity (Ku, 1982; Falk, 1985). This enhanced reactivity may be attributable in part to the consequences of proliferation of vascular smooth-muscle cells (VSMC).

In previous studies, we found that substances released by platelets, including epidermal growth factor and platelet-derived growth factor (PDGF), are VSMC mitogens and vasoconstrictors (Berk *et al.*, 1985, 1986). These platelet factors are stored in α -granules and are released only during platelet degranulation, which occurs at the onset of blood clotting. Thus platelet-derived products may play predominantly an initial role rather than a sustained role in the VSMC proliferation and increased vasoreactivity which is characteristic of arterial injury. Thrombin, however, may be chronically generated at sites of vascular injury by release during clot lysis, by continued activation of the

coagulation pathways in the presence of dysfunctional endothelium (Wilner *et al.*, 1981), and by release from extracellular matrix (Bar Shavit *et al.*, 1990). Thus α -thrombin may be important in the long-term alterations in VSMC function following platelet deposition.

Various thrombin forms are growth factors for fibroblasts and other cells (Stiernberg *et al.*, 1984; Bar Shavit *et al.*, 1986; Huang & Ives, 1987). α -Thrombin stimulates several intracellular events associated with growth factors, including phosphoinositide hydrolysis, increased free intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and Na^+ influx in platelets (Brass *et al.*, 1986; Zavoico *et al.*, 1986) and fibroblasts (Carney *et al.*, 1985; Stiernberg *et al.*, 1984). Because of its many growth-related signals and high concentrations in clots, it is reasonable to hypothesize that α -thrombin may have growth-promoting actions on VSMC. Work by Huang *et al.* (1987) has demonstrated that α -thrombin is mitogenic for smooth-muscle cells isolated from neonatal-rat heart. The present studies were performed with VSMC cultured from rat aorta to examine the nature of α -thrombin signalling in VSMC. Specifically, we measured α -thrombin-stimulated changes in protein and DNA synthesis, phospholipid hydrolysis, $[Ca^{2+}]_i$, Na^+/H^+ exchange and *c-fos* mRNA expression to analyse the signal pathways by which thrombin exerts its growth-promoting effects.

Abbreviations used: BCECF, 2',7'-bis(carboxyethyl)-5(6)-fluorescein; $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; DMA, 5'-(*NN*-dimethyl)amiloride; DME, Dulbecco's Modified Eagle's Medium; PDGF, platelet-derived growth factor; pH_i , intracellular pH; VSMC, vascular smooth-muscle cell(s).

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MATERIALS AND METHODS

Cell culture

VSMC were isolated from rat thoracic aorta (200–300 g Sprague–Dawley male rats) by enzymic dissociation as previously described (Berk *et al.*, 1989). These cells were characterized as VSMC on the basis of the following characteristics: growth in typical 'hill-and-valley' pattern, staining (> 85% positive at passage 5) with a monoclonal antibody specific for smooth-muscle α -actin (Franke *et al.*, 1980), and presence of high-affinity angiotensin II receptors (Berk *et al.*, 1989). The primary isolates were harvested twice a week with trypsin/EDTA and passaged at a 1:4 ratio in 75 cm² culture flasks. For experiments, cells at passages 4–20 were replicate-plated into 100 mm- or 35 mm-diameter culture dishes (2×10^4 cells/cm²), refed every other day and used at confluence. VSMC were maintained in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% (v/v) heat-inactivated calf serum, 2 mM-L-glutamine, 100 units of penicillin/ml and 100 μ g of streptomycin/ml. Human dermal fibroblasts, kindly given by Dr. Nicola Longo (Emory University, Atlanta, GA, U.S.A.), were maintained in the same medium as VSMC, except that 10% fetal-calf serum was substituted for calf serum.

Cell number, DNA and protein synthesis

Cells grown in 24-well Costar cluster dishes were harvested with trypsin and counted in a Coulter counter with a 100 μ m aperture. DNA and protein synthesis were determined as described previously (Berk *et al.*, 1989). Briefly, cells were grown in DME to 70% confluence as above, and growth-arrested by incubation for 48 h in DME containing 0.4% calf serum. The medium was aspirated and replaced with fresh DME containing 1 μ Ci of [³H]thymidine (6.7 Ci/mmol, New England Nuclear) or [³H]leucine (5 Ci/mmol, New England Nuclear), 0.4% calf serum and thrombin as indicated. After 24 h, the medium was aspirated, and cells were washed, fixed with 10% trichloroacetic acid, and washed with 95% ethanol. Protein was solubilized with 0.2 M-NaOH, and the incorporated radioactivity was measured by liquid-scintillation spectrometry. All experiments were performed in triplicate. Results are presented as means $\pm$ S.E.M. Differences were compared by Student's *t* test. *P* values < 0.05 were considered significant.

Measurement of phospholipid and inositol phosphate hydrolysis

Labelling, extraction and separation of the inositol lipids and inositol phosphates were performed as described previously (Alexander *et al.*, 1985). Briefly, VSMC cultures in 35 mm dishes were incubated with *myo*-[³H]inositol (15 μ Ci/ml) for 24 h in standard growth medium. Unincorporated label was removed by washing cultures in a warm balanced salt solution (termed Na⁺ solution) of the following composition (mM): 130 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, and 20 Hepes (buffered to pH 7.4 with Tris base). Cells were incubated for 20 min in 1 ml of Na⁺ solution at 37 °C and then stimulated with 40 nM- α -thrombin in 1 ml of Na⁺ solution for the indicated time periods. The reaction was terminated by rapid aspiration of the buffer and addition of 1 ml of chloroform/methanol/HCl (20:40:1, by vol.). Organic and aqueous phases were separated by sequential addition of 400 μ l of distilled water and 400 μ l of chloroform. After centrifugation (500 g for 5 min) and two chloroform washes, the organic phases were pooled and evaporated to dryness under N₂. PtdInsP and PtdInsP₂ were resolved by t.l.c. as previously described (Brock *et al.*, 1985). Inositol phosphates in the aqueous phase were analysed by ion-exchange chromatography on Dowex AG-1X8 resin as described by Alexander *et al.* (1985). All lipids were quantified by liquid-scintillation spectrophotometry.

Measurement of intracellular pH (pH_i)

The fluorescent pH indicator 2',7'-bis(carboxyethyl)-5(6)-fluorescein (BCECF) was used to measure changes in pH_i as previously described (Berk *et al.*, 1987a). For experiments performed on coverslips, cells at 90% confluence were washed twice with Hanks balanced salt solution and incubated for 30 min in a 37 °C water bath with 3 μ M-BCECF acetoxymethyl ester in Hanks solution. Coverslips were then washed twice with Na⁺ solution and incubated for 5 min with Na⁺ solution containing 2.5 mg of BSA/ml. The coverslip was placed in a modified quartz cuvette containing a stirring bar and allowed to reach equilibrium for 30 min before assay. All fluorescence measurements were performed in a SPEX Fluorolog-2 spectrofluorimeter (model CM-1) with excitation of BCECF at 500 and 438 nm, and emission at 530 nm. Calibration of the pH_i relative to fluorescence intensity was performed at the end of each experiment with 30 μ M-digitonin in Na⁺ solution and Mes for cell suspensions, or with 10 μ M-nigericin in 135 mM-KCl/10 mM-Hepes and Mes for coverslips (Grinstein *et al.*, 1984). Both calibration techniques gave a linear relationship between pH_i and fluorescence intensity over the pH range 7.4–6.4. Calculation of intracellular buffering power was carried out with 5 mM-NH₄Cl (Grinstein *et al.*, 1984), and was 30 mmol of H⁺/litre of cells per pH unit for cells on coverslips and 65 mmol of H⁺/litre of cells per pH unit for cell suspensions at pH_i 7.25 and 7.10 respectively.

Measurement of [Ca²⁺]_i

Cells were prepared for measurement of [Ca²⁺]_i by using the fluorescence indicator fura-2 as described above for BCECF, with the following changes. Cell suspensions were incubated with 4 μ M fura-2 acetoxymethyl ester for 20 min. Excitation wavelengths were set at 340 nm and 380 nm and the emission wavelength at 505 nm. Most of the experiments were performed in suspension rather than on coverslips (although the results were similar), because the suspension technique was both more rapid and more reproducible.

RNA preparation and blot hybridization

Total RNA was extracted and purified from VSMC by the guanidinium isothiocyanate/CsCl procedure. RNA (10 μ g/lane) was size-fractionated by electrophoresis on 1% agarose gels in 200 mM-Mes, pH 7.4, 1 mM-EDTA and 3% formaldehyde. Transfer to nitrocellulose and hybridization to ³²P-labelled DNA were as described by Taubman *et al.* (1989a). Filters were hybridized with the *c-fos* cDNA probe at 42 °C for 14 h in a cocktail containing 50% formamide, 5 $\times$ SSC (1 $\times$ SSC = 0.15 M-NaCl/0.015 M-sodium citrate), 2 $\times$ Denhardt's solution, 0.1% SDS, 0.025 M-sodium phosphate and 50 mg of calf thymus DNA/ml. The probe was derived from the 4.5 kb *Bam*H1/*Hind*-III fragment of the mouse genomic plasmid pc-*fos*-3 labelled with [³²P]dCTP by random oligomer priming. After hybridization, filters were washed at 55 °C in 0.2 $\times$ SSC/0.2% SDS for 1 h. Filters were exposed to Kodak XAR-5 film for 3 days at –70 °C with one intensifying screen. As a control, all filters were also hybridized with cDNA encoding a myosin heavy chain (SMHC 29) isolated from rabbit aortic smooth muscle (Nagai *et al.*, 1988). Hybridization and wash conditions were the same as for the *c-fos* cDNA. This mRNA is constitutively expressed in VSMC, and levels were equal in all lanes.

Thrombin and other materials

Human α -thrombin (> 98% α -thrombin; 100% active enzyme, specific clotting activity of 2163 units/mg of protein) was prepared and characterized as previously described (Fenton *et al.*, 1977). On the basis of this specific activity and an *M_r* of

36600, 10 units of clotting activity/ml was equivalent to 130 nM active enzyme. Choline chloride was from Calbiochem. Trypsin/EDTA and DME were from Gibco. 5'-(*NN*-dimethyl)amiloride (DMA) was prepared as previously described by Cragoe *et al.* (1967). BCECF and fura-2 were obtained from Molecular Probes, Eugene, OR, U.S.A. Recombinant *c-sis* (PDGF B-chain homodimer) was from Amgen, Thousand Oaks, CA, U.S.A. Anti- α -actin antibody was from Sigma.

RESULTS

Thrombin-stimulated changes in VSMC growth

The growth-promoting abilities of α -thrombin were studied by assaying its effects on VSMC cell number and synthesis of protein and DNA. As shown in Fig. 1, α -thrombin stimulated a significant increase in protein synthesis assayed by [3 H]leucine incorporation, with a maximal $52 \pm 18\%$ increase after exposure to 130 nM- α -thrombin ($P < 0.05$). There was a $41 \pm 9\%$ increase in protein content at this concentration. However, there was no increase in [3 H]thymidine incorporation at the same concentration ($-2 \pm 4\%$), and a small decrease ($-14 \pm 3\%$) at 0.013 nM. As expected from the [3 H]thymidine data, there was no increase in VSMC cell number in response to α -thrombin (Fig. 2). The failure of α -thrombin to stimulate DNA synthesis was not due to binding or inactivation of serum components. Under the same conditions utilized for VSMC growth, human dermal fibroblasts showed a $166 \pm 27\%$ increase in [3 H]thymidine incorporation in response to 13 nM- α -thrombin. Furthermore, in a defined medium composed of DME supplemented with $5 \mu\text{g}$ of insulin/ml, $5 \mu\text{g}$ of transferrin/ml and 5 ng of selenium sulphide/ml, [3 H]thymidine incorporation by VSMC in response to 13 nM- α -thrombin was $4 \pm 7\%$. Similar negative results were obtained with several preparations of α -thrombin and several different primary VSMC isolates.

In comparison with α -thrombin, 10 ng of PDGF/ml elicited increases in both [3 H]leucine and [3 H]thymidine incorporation ($89 \pm 7\%$ and $92 \pm 9\%$ respectively). PDGF was mitogenic, as indicated by a 285% increase in cell number after 5 days of treatment compared with cells maintained in 0.4% calf serum. No synergism was observed between α -thrombin and several VSMC growth factors, including calf serum, PDGF and epidermal growth factor, for either protein or DNA synthesis (results not shown). Thus, although α -thrombin stimulates protein synthesis in VSMC to nearly the same extent as PDGF, it fails to stimulate mitogenesis.

Thrombin-stimulated changes in pH_i and Na^+/H^+ exchange

In fibroblasts (Paris & Pouyssegur, 1986) and in VSMC derived from neonatal-rat hearts (Huang & Ives, 1987; Huang *et al.*, 1987), α -thrombin has been shown to be mitogenic, and in these cells it stimulates Na^+/H^+ exchange and cellular alkalization. To investigate whether the inability of α -thrombin to stimulate mitosis in these adult rat VSMC was associated with a change in intracellular pH_i , we studied the effect of α -thrombin on pH_i and Na^+/H^+ exchange.

The pH_i of cultured VSMC suspended in Na^+ solution at pH 7.4 and 37°C was 7.1 ± 0.08 ($n = 50$), whereas VSMC grown on glass coverslips incubated in Na^+ solution had a basal pH_i of 7.25 ± 0.12 ($n = 25$). The addition of α -thrombin caused a concentration-dependent biphasic shift in pH_i (Fig. 3) similar to that reported by Huang *et al.* (1987). Initially, there was a brief fall of 0.01–0.1 pH unit that began within 10 s and was maximal at 60 s. This was followed by a more slowly developing alkalization, which persisted for at least 20 min and resulted in a final pH_i 0.1–0.2 unit above resting pH_i . To determine whether

Fig. 1. Effect of α -thrombin on VSMC growth

VSMC and human dermal fibroblasts were growth-arrested by 48 h incubation in DME supplemented with 0.4% calf serum. Fresh DME containing 0.4% serum and the indicated concentrations of thrombin plus either $1 \mu\text{Ci}$ of [3 H]leucine/ml or $1 \mu\text{Ci}$ of [3 H]thymidine/ml were then added for 24 h. Results are means $\pm$ S.D. of 3 determinations for fibroblasts and 6 determinations for VSMC. Key: $\circ$, VSMC, leucine; $\bullet$, VSMC, thymidine; $\blacktriangle$, fibroblasts, thymidine.

Fig. 2. Effect of α -thrombin on cell number

VSMC were growth arrested as described in Fig. 1 legend. Fresh DME containing 0.4% serum ($\bullet$) and 13 nM- α -thrombin ($\blacktriangle$) or 10% serum ($\blacksquare$) was added on day 3. Fresh media and agonists were added every 48 h. Cell counts were performed as described above on triplicate wells. Data are representative of three separate determinations (S.D. of measurements was $< 5\%$).

Fig. 3. Effect of α -thrombin on VSMC pH_i

BCECF-loaded VSMC on coverslips in Na^+ solution were treated with the concentrations of thrombin indicated. All tracings are drawn on the same pH_i scale. Calculated values for initial pH_i and maximal pH_i are indicated on the left and right respectively. Results are typical of three experiments.

Fig. 4. α -Thrombin-stimulated increases in $[Ca^{2+}]_i$.

α -Thrombin was added to suspensions of fura-2-loaded VSMC after 2 min of baseline equilibration. Calculated values for basal and peak $[Ca^{2+}]_i$ for each tracing are indicated at the left. Results are representative of 8 experiments.

the thrombin-stimulated rise in pH_i was mediated by Na^+/H^+ exchange, cells were placed in Na^+ -free solution (iso-osmotic replacement with 130 mM-choline chloride, referred to as choline $^+$ solution). In the absence of extracellular Na^+ , addition of 130 nM α -thrombin caused only a rapid and sustained fall in

pH_i , with no subsequent alkalization (results not shown). To corroborate further the role of Na^+/H^+ exchange, the potent amiloride derivative DMA was added 5 min before α -thrombin. DMA (30 μ M) alone caused an acidification as previously reported (Berk *et al.*, 1987b). Upon addition of α -thrombin, the acidification response was significantly enhanced, whereas the alkalization phase was delayed and attenuated, failing to rise above the baseline pH_i (results not shown). Thus α -thrombin stimulates a biphasic pH_i response in VSMC: an initial acidification independent of Na^+/H^+ exchange, and a subsequent alkalization which is dependent on extracellular Na^+ and inhibited by DMA, consistent with mediation by the Na^+/H^+ exchanger (Huang *et al.*, 1987). The lack of a mitogenic effect therefore cannot be attributed to the inability of α -thrombin to activate Na^+/H^+ exchange.

Thrombin-stimulated changes in $[Ca^{2+}]_i$

In previous studies, we have shown that increases in VSMC $[Ca^{2+}]_i$ are necessary for growth-promoting activities of angiotensin II, including induction of *c-fos* mRNA (Taubman *et al.*, 1989a) and stimulation of protein synthesis (Berk *et al.*, 1989). To study the effects of α -thrombin on VSMC $[Ca^{2+}]_i$, we measured changes in $[Ca^{2+}]_i$ using cells loaded with the Ca^{2+} -sensitive fluorescent dye fura-2.

The baseline $[Ca^{2+}]_i$ of stirred VSMC suspensions was 90 ± 10 nM ($n = 50$) at 37 °C in Na^+ solution. Addition of α -thrombin caused a rapid dose-dependent increase in $[Ca^{2+}]_i$, as shown in Fig. 4. In these experiments, 0.13 nM α -thrombin caused a transient increase in $[Ca^{2+}]_i$ after a brief lag, which reached a maximum value at 2 min and remained elevated for > 5 min. In contrast, 130 nM α -thrombin induced a rapid increase, which reached a peak within 10 s, followed by a rapid decline to a plateau of approx. 2–3 times resting level. The concentration-response curve for α -thrombin-stimulated increases in $[Ca^{2+}]_i$ exhibited a threshold at 0.01 nM, was half-maximal at 2 nM, and was maximal (1280% increase) at 130 nM.

To determine the source of Ca^{2+} mobilized by α -thrombin, we preincubated cells for 5 min in a Ca^{2+} -free 2 mM-EGTA/ Na^+ solution before addition of α -thrombin. After this treatment, the peak response to 13 nM α -thrombin was decreased by 25% (results not shown). This small degree of inhibition is partially explained by the slightly lower resting $[Ca^{2+}]_i$ after the 5 min

Fig. 5. Time course of $InsP_3$ formation induced by 39 nM α -thrombin

Cultured VSMC were labelled with *myo*- $[^3H]$ inositol for 24 h and assayed for $InsP_3$ as described above. Data are expressed as percentage change from control levels. Each point is the mean of four experiments performed in triplicate.

Table 1. Thrombin-stimulated inositol phospholipid breakdown and inositol phosphate formation

Cultured VSMC were prelabelled with *myo*-[³H]inositol (15 μ Ci/ml) for 24 h, and treated with 39 nM- α -thrombin. Each point represents the mean of triplicate determinations from 2–4 experiments, and is expressed as percentage of control values (mean $\pm$ S.E.M.).

Time	Inositol lipids (% of control)		Inositol phosphates (% of control)	
	PtdIns(4,5) P_2	PtdIns(4) P	Ins P_3	Ins P_2
15 s	62 $\pm$ 10	62 $\pm$ 3	226 $\pm$ 45	387 $\pm$ 79
5 min	108 $\pm$ 8	131	101 $\pm$ 5	122 $\pm$ 8

EGTA incubation (70 versus 90 nM). However, at least 75 % of the response persisted in the absence of extracellular $[Ca^{2+}]_o$, indicating that the primary source of Ca^{2+} is intracellular. As has been observed with many agonists (Brock *et al.*, 1985), the plateau phase of the response is primarily dependent on extracellular Ca^{2+} , as it was abolished by EGTA treatment.

Thrombin stimulation of phospholipase C activity

Phospholipid hydrolysis and generation of phosphoinositide metabolites appear to be important growth signals (Paris & Pouyssegur, 1986; Carney *et al.*, 1985). α -Thrombin has been shown to induce rapid hydrolysis of membrane polyphosphoinositides in several systems (Brass *et al.*, 1986; Paris & Pouyssegur, 1986; Huang *et al.*, 1987). To investigate whether this mechanism was present in α -thrombin-stimulated VSMC, we monitored inositol phosphate formation and inositol phospholipid hydrolysis in *myo*-[³H]inositol-labelled VSMC. α -Thrombin (39 nM) increased formation of Ins P_3 transiently, reaching a peak at 15 s and returning to control values within 5 min (Fig. 5). There was an average increase in Ins P_3 to 226 ± 45 % of control values and in Ins P_2 to 387 ± 79 % of control within 15 s (Table 1). Concomitantly, PtdIns P and PtdIns P_2 decreased by 30–40 %, consistent with phospholipase C-mediated hydrolysis of polyphosphoinositides. Thus α -thrombin activates phospholipase C, which likely stimulates increases in both $[Ca^{2+}]_i$ and Na^+/H^+ exchange.

Thrombin-induced expression of *c-fos* mRNA

Another signal event common to many growth factors and Ca^{2+} -mobilizing hormones is induction of the proto-oncogene *c-fos*. We have previously shown that angiotensin II, which like α -thrombin stimulates an increase in VSMC protein synthesis, also induces *c-fos* expression (Taubman *et al.*, 1989a). In the following experiments, we examined the effect of α -thrombin on *c-fos* mRNA expression as well as the ionic events required for this induction. There was no constitutive expression of *c-fos* in growth-arrested VSMC. When growth-arrested VSMC were exposed to 13 nM- α -thrombin there was a rapid accumulation of *c-fos* mRNA (Fig. 6a), detectable within 30 min, maximal at approx. 60 min and undetectable by 6 h. Induction of *c-fos* mRNA by α -thrombin was concentration-dependent, with a threshold at 1.3 nM and a maximal response at 130 nM (Fig. 6b). The similarity of the concentration–response relationships for *c-fos* expression and Ca^{2+} mobilization is consistent with the dependence of *c-fos* expression in VSMC on an increase in $[Ca^{2+}]_i$.

To investigate whether alterations in pH_i are important in α -thrombin-induced *c-fos* expression, Na^+/H^+ exchange was blocked by pretreating cells with 30 μ M-DMA. As shown in Fig. 6(c), there was a significant decrease in *c-fos* mRNA accumu-

Fig. 6. Effect of α -thrombin on *c-fos* mRNA accumulation in VSMC

(a) Time course of *c-fos* mRNA induction. Growth-arrested VSMC were treated for the times indicated with DME containing 0.4 % calf serum and 130 nM- α -thrombin. (b) Concentration–response relationship for α -thrombin induction of *c-fos* mRNA. VSMC were exposed for 30 min to α -thrombin at the indicated concentrations and RNA was then prepared. (c) Effect of ionic manipulation on *c-fos* mRNA accumulation in response to α -thrombin. Growth-arrested VSMC were treated for 30 min with 13 nM- α -thrombin for all experiments described (left to right). Quiescent VSMC were treated with α -thrombin in choline⁺ solution (+choline) or in Na^+ solution containing 30 μ M-DMA (+DMA) as described in Fig. 4. Cells were calcium-clamped by incubation for 60 min with 4 mM-EGTA alone (+EGTA) or with 100 μ M-quin2/AM and 4 mM-EGTA (+quin2/EGTA). The upper and lower autoradiographic bands represent hybridization to the SMHC 29 and *c-fos* cDNA probes respectively. Results are representative of two experiments.

lation. This was decreased further when extracellular Na^+ was completely removed and replaced with choline⁺ solution (Fig. 6c). Thus intracellular alkalinization, and perhaps an increase in intracellular Na^+ (since choline caused even greater inhibition than DMA), may play important roles in α -thrombin-stimulated *c-fos* mRNA expression.

To study further the importance of ionic events in α -thrombin-stimulated *c-fos* expression, VSMC were calcium-clamped by

using quin2 AM/EGTA to prevent increases in intracellular Ca^{2+} , as previously described (Berk *et al.*, 1987b; Ives & Daniel, 1987). No increase in *c-fos* mRNA was observed in treated cells even in response to 130 nM- α -thrombin (Fig. 6c). This treatment had no effect on the steady-state mRNA levels of the constitutively expressed myosin heavy chain gene (Fig. 6c, upper band). This calcium-clamping technique is specific for certain Ca^{2+} -dependent transduction pathways: induction of the early response gene, JE, in these VSMC by PDGF is not affected by this treatment (Taubman *et al.*, 1989b). Thus α -thrombin induction of *c-fos* mRNA appears to be completely dependent on an increase in $[\text{Ca}^{2+}]_i$.

DISCUSSION

The present work demonstrates that α -thrombin is a potent activator of multiple growth-related signal pathways in VSMC. We discuss below the mechanisms for its stimulation of VSMC protein synthesis, Na^+/H^+ exchange, phospholipase C, *c-fos* expression, and its role in VSMC growth in a variety of conditions.

Thrombin effects on VSMC growth

Our results indicate that in cultured VSMC isolated from adult rat aorta, α -thrombin significantly increases protein, but not DNA, synthesis. Although α -thrombin has been found to be mitogenic for fibroblasts and neonatal VSMC (Carney *et al.*, 1984, 1985; Huang & Ives, 1987), under the conditions of our studies it caused no change in cell number. These findings with α -thrombin and VSMC are similar to previous studies with angiotensin reported by Geisterfer *et al.* (1988) and our laboratory (Berk *et al.*, 1989). Angiotensin II stimulated many early growth signals, such as phospholipase C, protein kinase C, Na^+/H^+ exchange and *c-fos* mRNA accumulation, but caused an increase only in protein synthesis without stimulating cell division.

The mechanism(s) by which angiotensin II and thrombin stimulate protein synthesis is unclear. Several signal-transduction events activated by α -thrombin in VSMC have been associated with increased protein synthesis. We have previously reported that activation of protein kinase C by phorbol esters increases protein synthesis in VSMC (Berk *et al.*, 1989). α -Thrombin stimulates phosphorylation of a 76000-Da protein kinase C substrate which is inhibited after down-regulation of protein kinase C (Berk *et al.*, 1990). Stimulation of Na^+/H^+ exchange has been associated with increased polysome formation and hence increased protein synthesis (Chambard & Pouyssegur, 1986). Finally, there may be direct activation of VSMC ribosomal-protein S6 kinase by α -thrombin, analogous to findings with angiotensin II (Scott-Burden *et al.*, 1988).

Thrombin as an activator of Na^+/H^+ exchange

α -Thrombin causes both acidification and alkalinization responses in platelets (Zavoico *et al.*, 1986) and VSMC (Huang *et al.*, 1987). In cultured VSMC, α -thrombin also elicited a biphasic pH_i response. The initial response was a rapid transient acidification, which paralleled the $[\text{Ca}^{2+}]_i$ response in both its rate and dose-response. Previously we found that angiotensin II and PDGF stimulated an acidification of VSMC which correlated with these agonists' ability to increase $[\text{Ca}^{2+}]_i$ (Berk *et al.*, 1987b). Huang *et al.* (1987) demonstrated that pertussis toxin, which inhibited the α -thrombin-stimulated rise in $[\text{Ca}^{2+}]_i$, also prevented intracellular acidification, but not alkalinization, in neonatal VSMC. We also found that a rise in $[\text{Ca}^{2+}]_i$ was required for acidification, but not for alkalinization (Berk *et al.*, 1990), which

supports the findings by Huang *et al.* (1987) in VSMC and by Zavoico *et al.* (1986) in platelets. Finally, there appear to be both protein kinase C-dependent and -independent pathways for α -thrombin stimulation of Na^+/H^+ exchange in VSMC (Huang *et al.*, 1987; Berk *et al.*, 1990).

Thrombin as a Ca^{2+} -mobilizing agonist

The primary source of Ca^{2+} mobilized in these cultured rat aortic VSMC (> 75%) was of intracellular origin. The hydrolysis of PtdInsP_2 to generate InsP_3 implicates activation of phospholipase C as the mechanism by which α -thrombin mobilizes Ca^{2+} . Several studies have described the effects of α -thrombin on blood-vessel contraction. De Mey & Vanhoutte (1982) found that thrombin caused an endothelial-dependent relaxation of dog femoral, splenic and pulmonary arteries. Ku (1986) reported similar results for dog coronary arteries, as did Rapaport *et al.* (1984) for rat aorta. In the absence of endothelium, however, Haver & Namm (1984) and Walz *et al.* (1985) observed α -thrombin-mediated contraction of rabbit aorta and dog coronary arteries respectively. Haver & Namm (1984) and Ku (1986) reported that the contractile response to α -thrombin in dog coronaries was only minimally inhibited by Ca^{2+} -channel blockers or addition of EGTA to the muscle bath. This suggests that in these vessels thrombin-induced contraction is not mediated primarily by activation of voltage-dependent Ca^{2+} channels. Our data indicate that in cultured VSMC α -thrombin increases $[\text{Ca}^{2+}]_i$ secondarily to mobilization of intracellular Ca^{2+} stores, suggesting that the vasoconstrictor actions of thrombin are mediated by InsP_3 -induced Ca^{2+} release.

Thrombin effects on *c-fos* expression

The ability of α -thrombin to stimulate *c-fos* expression (half-maximal concentration of 1.3 nM), yet not to stimulate cell division, is similar to the effect of angiotensin II on VSMC (Berk *et al.*, 1989; Taubman *et al.*, 1989a). Specifically, an increase in $[\text{Ca}^{2+}]_i$ is required for induction of *c-fos* by α -thrombin, because calcium-clamping prevented the increase in *c-fos* mRNA. In other work we have extended these findings and have shown that α -thrombin induction of *c-fos* is dependent on stimulation of protein kinase C (Berk *et al.*, 1990). Inhibiting Na^+/H^+ exchange by incubating the cells in Na^+ -free media or in the presence of DMA also blunted *c-fos* expression, suggesting a role for increases in intracellular pH or Na^+ concentration. Because addition of monensin to load the cells with Na^+ (B. C. Berk, unpublished work) or NH_4Cl to alkalize the cells failed to induce *c-fos* (Taubman *et al.*, 1989a), it is clear that alterations in Na^+ and H^+ concentrations are necessary, but not sufficient, to induce *c-fos*.

Relationship of thrombin signalling to VSMC growth

In summary, α -thrombin is a potent agonist for cultured VSMC, stimulating increases in $[\text{Ca}^{2+}]_i$, pH_i , PtdInsP_2 hydrolysis and *c-fos* mRNA. Although α -thrombin stimulates many of the same early cellular events as PDGF, it fails to induce cell division. Several possibilities may account for the non-proliferative nature of α -thrombin in these VSMC. PDGF, but not α -thrombin, causes a significant increase in VSMC cyclic AMP (B. C. Berk, unpublished work), which has been suggested by Rozengurt (1986) to be important in mediating the mitogenic response. There may be other positive growth signals (*c-myc*) which α -thrombin fails to stimulate, or negative growth signals (transforming growth factor- β) which α -thrombin stimulates, in contrast with PDGF. Finally, although α -thrombin has been shown to increase tyrosine phosphorylation in platelets (Golden & Brugge, 1989), this tyrosine kinase activity may be lacking in VSMC, or the substrates for tyrosine phosphorylation may be

different from those observed with growth factors such as PDGF. Characterization of the differences in growth-related pathways among various thrombin forms and classic growth factors should provide valuable insights into the mechanisms underlying VSMC growth and vasoreactivity.

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