British Journal of Pharmacology (1995) 115, 903-908

Mode of action of thrombin in the rabbit aorta

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1 Thrombin is a vasoactive protease that elicits the contraction of the rabbit aorta by activating a G-protein coupled receptor through cleavage of its N-terminal extracellular domain. Synthetic peptides corresponding to the newly exposed N-terminus, following thrombin cleavage, have been shown to reproduce some of the activities of thrombin in the rabbit aorta.

2 Intracellular pathways involved in the contractile response of the rabbit aorta to thrombin and synthetic peptides were examined by use of a series of inhibitors. A similar method was applied to characterize the mitogenic effect of thrombin on cultured smooth muscle cells (SMCs) derived from the same tissue.

3 Results from this study indicate that the contractile response of the rabbit aorta to thrombin is dependent on the activation of protein kinase C (PKC) and independent of extracellular calcium. The contractile response to thrombin can be fully reproduced by peptide agonists related to the N-terminal receptor sequence. However, subtle differences seem to exist between the mechanism of the contractile effect of thrombin and of the synthetic peptides, as both PKC activation and extracellular calcium were found to participate in the contractile effect of the synthetic peptides.

4 In cultured SMCs, both thrombin and the synthetic peptides increased inositol phosphate turnover; however, only thrombin elicited a mitogenic effect, which occurs at thrombin concentrations well below those needed to increase inositol phosphate turnover significantly. Activation of a tyrosine kinase pathway is involved in the mitogenic effect of thrombin on aortic SMCs.

5 Altogether these results suggest the existence of subtle differences between the mode of action of thrombin and of synthetic peptides related to the N-terminal thrombin receptor sequence, in the rabbit aorta.

Keywords: Thrombin; thrombin cleavable receptor; smooth muscle cells; rabbit aorta

Introduction

α-Thrombin is a key enzyme involved in blood coagulation and although its activity on soluble substrates in the circulation has been extensively studied, thrombin also exhibits hormone-like activity in many cell types (for review see Fenton, 1988 and Coughlin *et al.*, 1992). The cloning of a receptor mediating some of these activities has revealed a new class of G-protein coupled receptors that are activated by limited proteolysis of the N-terminal extracellular domain (Vu *et al.*, 1991); a second receptor that is activated in a similar way has recently been described (Nystedt *et al.*, 1994). These receptors can be activated by synthetic peptides, as short as five or six amino acids, such as Ser-Phe-Leu-Leu-Arg-Asn-NH_2 (NAT_6-NH_2) for the thrombin receptor (Hui *et al.*, 1992), corresponding to the newly generated aminoterminus receptor sequence following proteolytic cleavage.

Thrombin is a modulator of vessel tone that can act by either endothelium-dependent or -independent mechanism and the relaxation or contraction induced by thrombin can be reproduced by synthetic agonists of the cleavable receptor. In the intact rat aorta peptide agonists are relaxing agents (Yang *et al.*, 1992); however, contractile responses are observed when the endothelium is removed (Antonaccio *et al.*, 1993). Stimulation of the dog coronary arteries by an agonist of the thrombin cleavable receptor also produces an endothelium-dependent relaxation that is transformed into a contractile response in the absence of endothelium (Ku & Zaleski, 1993). In the rabbit aorta thrombin, NAT_6-NH_2 and an aminopeptidase resistant analogue, [Sar[1]]NAT_6-NH_2 (Godin *et al.*, 1994), produce a dose-dependent contraction suggesting that the cleavable receptor is involved in this activity.

Thrombin has also been found to exert mitogenic activity in several cell types (Fenton, 1988). However, intracellular signals generated by peptide agonists of the cleavable receptor seem insufficient to induce mitosis in hamster fibroblasts (Vouret-Craviari *et al.*, 1992). In an effort to characterize further the second messengers involved in the contractile response of the rabbit aorta, following stimulation by either thrombin or agonist peptides, we have evaluated the effect of different inhibitors in the presence of these contractile agents. We have also used these inhibitors to characterize second messengers activated following stimulation by thrombin or agonist peptides of cultured vascular smooth muscle cells (SMCs) derived from the rabbit aorta. Our results reveal a difference in the way contractile responses are obtained through activation by thrombin or peptide agonists and that a proliferative response of SMCs derived from the aorta is obtained only by stimulation with thrombin.

Methods

Organ bath pharmacology

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.5-2 kg). The vessels were cut into rings (2-3 mm width), suspended between a metal hook and a thread loop under a basal tension of 2 g in 5 ml organ chambers containing oxygenated (95% O_2:5% CO_2) and warmed (37°C) Krebs solution (Marceau *et al.*, 1991). Their responses to agents were recorded isometrically as described previously (Bouthillier *et al.*, 1987).

The mechanism of the contractile effect of the thrombin receptor agonist, [Sar[1]]NAT_6-NH_2 was analysed by applying a submaximal concentration (40 μM) of this peptide to the tissues after an equilibration period of 2 h. The concentration-effect relationship for this agent can be found elsewhere (Godin *et al.*, 1994). Inhibitory drugs were applied 30 min before each agonist and paired control tissues were used to

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evaluate the effect, if any, of the drug vehicle. The same tissues were also challenged at 4 and 6 h by thrombin (60 nM) and KCl (20 mM), respectively, for purposes of comparison. The drugs used in this study were nifedipine, an L-type Ca^{2+} channel blocker (Murad, 1990), H-7, a protein kinase inhibitor (Hidata *et al.*, 1984), diclofenac, a cyclo-oxygenase inhibitor (Lewis & Furst, 1987), erbstatin, a tyrosine kinase inhibitor (Imoto *et al.*, 1987) and D-Phe-Pro-Arg-CH_2Cl (PPACK), an irreversible inhibitor of the catalytic activity of thrombin (Kettner & Shaw, 1979). The concentrations of the drugs were chosen according to their reported activity in various *in vitro* systems (Levesque *et al.*, 1993). The effect of the kinase inhibitor, H-7, was further documented over a wide range of concentrations of the thrombin receptor agonists; cumulative concentration-effect curves for [Sar^1]NAT_6-NH_2 and thrombin were constructed. In order to evaluate the contribution of the endothelium in the contractile response to each agent, the endothelium was carefully removed by rubbing with a metal spatula. The tissues were controlled by assessing the loss of the relaxant effect of acetylcholine (Furchgott & Zawadzki, 1980). We also replaced the Krebs solution by a calcium-free form to evaluate the contribution of extracellular calcium to the contractile effect of each agent. The Krebs solution was replaced by the calcium-free form 10 min before each contractile stimulation and the tissue preparation was maintained in normal Krebs solution between tests to avoid depletion of intracellular Ca^{2+} pools.

Cell culture

Several distinct lines of rabbit SMCs were established from rabbit aorta aseptically removed from freshly killed animals. The tissues (approximate weight 200 mg) were incubated at 37°C for 1.5 h in medium 199 (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), antibiotics (streptomycin, 50 $\mu g\ ml^{-1}$, and penicillin 50 u ml^{-1}; Gibco), bacterial collagenase (type IA, 3 $mg\ ml^{-1}$) and elastase (type III, 1 $mg\ ml^{-1}$); all enzymes were obtained from Sigma (St-Louis, MO, U.S.A.). Dispersed cells and partly digested tissue fragments were centrifuged and resuspended in medium 199 supplemented with 10% FBS and antibiotics. This suspension was seeded directly in 25 cm^2 flasks coated with 0.2% gelatin. Cells were passaged on gelatin-coated surfaces at confluency with a brief trypsin (0.05%)/EDTA (0.53 mM) treatment. The cells were studied at passages 3 to 7 only.

[^{3}H]-thymidine incorporation

Cells, in Medium 199 containing 10% FBS, were plated at an initial density of 3×10^4 cells per well in gelatin-coated 12-well disposable plastic plates (Costar). The cells were growth-arrested, by incubation for 72 h in medium 199 containing 0.4% FBS, before being exposed to thrombin, NAT_6-NH_2, [Sar^1]-NAT_6-NH_2, FBS or to various drugs for 48 h. Enhanced thymidine incorporation was assessed after a 24 h exposure to [^{3}H]-thymidine (Dupont, Mississauga, Ont. Canada; 0.1 $\mu Ci\ ml^{-1}$; specific activity 84 Ci $mmol^{-1}$). The cells were then washed twice with saline, harvested with trypsin (0.05%)/EDTA (0.53 mM) and collected in borosilicate test tubes. The wells were rinsed once with 1 ml Triton X-100 1%/saline and nucleic acids precipitated with 1 ml trichloroacetic acid 40%/water. After a 20 min centrifugation at 2,700 *g*, the supernatant was withdrawn and the pellet resuspended in 1 ml distilled water, placed into scintillation vials containing 10 ml Ecolite + scintillation fluid (ICN Biomedicals, Irvine CA, U.S.A.) and counted in a liquid scintillation counter (Beckman LS 8000).

Inositol phosphate production

Labelling and stimulation of rabbit aorta SMCs were carried out according to Balla *et al.* (1988) with some modifications. Briefly, cells in medium 199 supplemented with 10% FBS and antibiotics, were seeded at a density of 2.5×10^5 in 2 cm^2 wells and cultured for 24 h. The cells were then washed three times with serum-free medium. The incorporation medium (500 μl) consisted of medium 199 containing 20 $\mu Ci\ ml^{-1}$ of *myo*-[^{3}H]-inositol (Amersham, specific activity 82 Ci $mmol^{-1}$), was added to the wells for a labelling period of 16–20 h. After incubation at 37°C in a humidified atmosphere containing 5% CO_2, each well was washed twice with Earle's balanced salt solution (EBSS). The cells were further incubated for 20 min in EBSS with 10 mM LiCl then challenged with thrombin or NAT_6-NH_2 at different concentrations for exactly 5 min. Stimulation was terminated by the addition of perchloric acid (final concentration 5%).

Inositol phosphates were extracted and separated as previously described (Guillemette *et al.*, 1989). At the end of the experiment, the cells were kept on ice for 30 min and then scraped from the wells and transferred into tubes. The wells were rinsed with additional perchloric acid (5% W/V) and the resulting media was centrifuged at 10,000 *g* for 5 min. Perchloric acid was extracted from the supernatant with a 1 : 1 mixture of Freon and tri-n-octylamine (Downes *et al.*, 1986; reagents from Sigma). After neutralization, the samples were applied to anion exchange resin (Dowex AG 1-X8 formate, 200–400 mesh, Bio-Rad No. 140-1450) columns (1 ml bed volume) for the separation of the inositol phosphates according to Berridge *et al.* (1983). The inositol phosphates were sequentially eluted with 3 × 5 ml of distilled water (free inositol), 3 × 5 ml of 0.2 M ammonium formate/0.1 M formic acid (inositol monophosphates, IP_1), 3 × 5 ml of 0.5 M am-

Table 1 Pharmacological analysis of the mode of action of thrombin and the thrombin receptor agonist [Sar^1]NAT_6-NH_2 in the rabbit aortic preparation

		Contractile responses (g)		
Treatment	n	*[Sar^1]NAT_6-NH_2* (40 μM)	*Thrombin* (60 μM)	*KCl* (20 μM)
Intimal damage	6	2.21 ± 0.12	1.69 ± 0.35	2.34 ± 0.58
Control	6	1.81 ± 0.19	1.64 ± 0.46	2.59 ± 0.53
Nifedipine 100 nM	8	1.12 ± 0.09*[a]	0.77 ± 0.09	0.31 ± 0.05***
Vehicle (Ethanol)	8	1.59 ± 0.13	0.75 ± 0.09	2.74 ± 0.31
Calcium-free Krebs	6	1.16 ± 0.19*	0.91 ± 0.24	0.51 ± 0.13***
Control	6	1.75 ± 0.10	0.97 ± 0.14	2.92 ± 0.24
Diclofenac 0.5 μM	8	1.63 ± 0.14*	0.64 ± 0.03	2.55 ± 0.28
Vehicle (Na_2CO_3)	8	1.41 ± 0.13	0.67 ± 0.05	2.42 ± 0.24
Erbstatin analogue	4	2.19 ± 0.31	1.75 ± 0.36	3.41 ± 0.42
Vehicle (DMSO)	4	2.28 ± 0.24	1.80 ± 0.47	3.34 ± 0.20
PPACK 1 μM	8	1.51 ± 0.28	0.03 ± 0.02*	1.25 ± 0.17
Vehicle (saline)	8	1.11 ± 0.23	1.30 ± 0.41	1.22 ± 0.18

[a] Values in each treated group were compared to controls from the same animals by Student's *t* test for paired samples: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

monium formate/0.1 M formic acid (inositol biphosphates, IP_2), 3 × 5 ml of 0.8 M ammonium formate/0.1 M formic acid (inositol trisphosphates, IP_3) and 3 × 5 ml of 1.0 M ammonium formate/0.1 M formic acid (inositol tetrakisphosphates, IP_4). The fractions were collected, mixed with Ecolite + scintillation fluid and counted for radioactivity in a liquid scintillation counter.

Reagents

The following drugs were purchased from Sigma (St-Louis MO, U.S.A.): nifedipine, diclofenac, PPACK, PGE_2 (prostaglandin E_2) and PMA (phorbol 12-myristate 13-acetate). Erbstatin analogue and H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine) were purchased from Biomol Res. Lab. Inc. (Plymouth Meeting PA, U.S.A.) Wortmannin was from Calbiochem (San Diego, CA, U.S.A.), iloprost from Berlex Canada Inc. (Lachine QC, Canada) and Thrombostat (bovine thrombin) from Parke-Davis (Scarborough ON, Canada). NAT_6-NH_2 and [Sar^1]NAT_6-NH_2 were synthesized in our laboratory by solid phase peptide synthesis methods. Their homogeneity was assessed by high performance liquid chromatography and their identity confirmed by mass spectrometry.

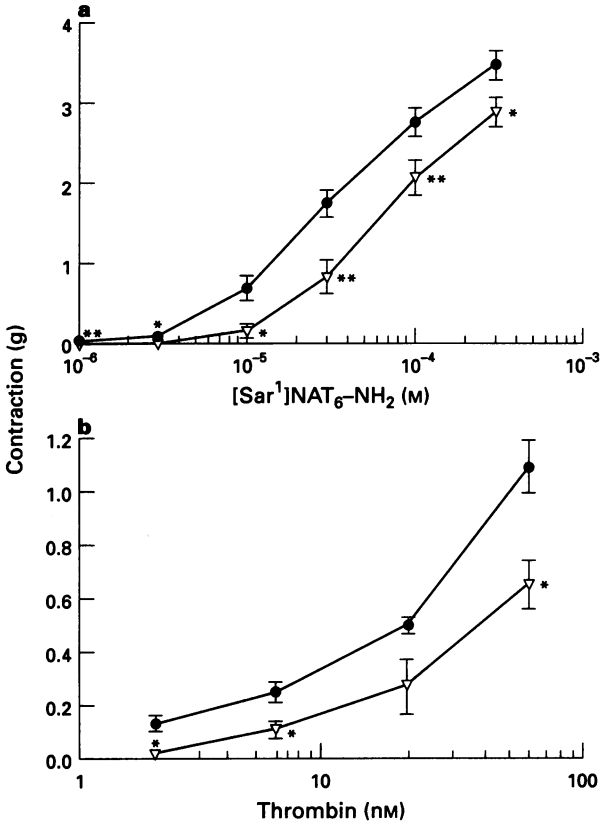

Figure 1 Effect of H-7 (▽ 10 μM) on the cumulative dose-response curve for [Sar^1]NAT_6-NH_2 (a) and thrombin (b). The results are expressed in g of developed contraction. Values are the mean ± s.e.mean of at least 6 determinations. Statistical analysis by Student's *t* test for paired samples: $*P<0.05$; $**P<0.001$ (● control responses).

Results

Contractility studies

A series of experiments using rabbit aortic rings was carried out to compare the mode of action of thrombin with an aminopeptidase resistant agonist of the thrombin cleavable receptor, [Sar^1]NAT_6-NH_2 (Godin *et al.*, 1994), and KCl used as a control agonist. Submaximal concentrations of these agonists were chosen based on previous experiments (Godin *et al.*, 1994; Levesque *et al.*, 1993). In order to avoid any tachyphylaxis, [Sar^1]NAT_6-NH_2 was always the first agonist injected after an equilibration period of 2 h, followed by thrombin and KCl successively with an interval of 2 h between agonists. Paired controls were used to evaluate the effect of solvents. All inhibitory drugs were applied 30 min before recording the response to each agonist.

As shown in Table 1, intimal damage did not modify the response to these agonists while it completely blocked the relaxation induced by acetylcholine, used as a control, of tissues pre-contracted with phenylephrine (data not shown). Nifedipine, the voltage-operated calcium channel blocker, was inactive against the thrombin-induced contraction of the rabbit aorta whereas a partial inhibition of the contraction induced by [Sar^1]NAT_6-NH_2 was observed. Extensive inhibition of the depolarizing agent KCl occurred in the same tissues, thus providing an internal control for the effectiveness of the drug in our assay. The contraction induced by KCl was profoundly affected in the presence of a Krebs solution devoid of calcium while the contraction induced by [Sar^1]NAT_6-NH_2 was reduced to 2/3 of the control and the effect of thrombin was left unchanged by calcium removal.

Diclofenac, an inhibitor of fatty acid cyclo-oxygenase, and the erbstatin analogue, an inhibitor of tyrosine kinases, did not have any depressant effect on the contraction induced by the three agonists presented in Table 1. An inhibitor of the catalytic activity of thrombin, PPACK, completely prevented the

Table 2 Inositol phosphates measurements in rabbit aortic smooth muscle cells 5 min after exposure to different concentrations of thrombin or NAT_6-NH_2[a]

	Inositol phosphates (c.p.m. per well)			
Stimulus	IP_1	IP_2	IP_3	IP_4
Control	6995 ± 1381	765 ± 140	328 ± 21	308 ± 66
Thrombin 10 nM	8277 ± 1743	929 ± 97	473 ± 32**	273 ± 24
Thrombin 100 nM	6038 ± 1547	1377 ± 371	509 ± 79	236 ± 39
Thrombin 1000 nM	11077 ± 1552	3074 ± 372**	1263 ± 104***	393 ± 41
NAT_6-NH_2 10 μM	6691 ± 1579	1714 ± 318*	684 ± 124*	271 ± 38
NAT_6-NH_2 100 μM	13504 ± 2531*	4351 ± 636***	1694 ± 145***	392 ± 24
Analysis of variance[b]	NS	$P<0.0001$	$P<0.0001$	$P<0.05$

[a] Smooth muscle cells from 3 different cell lines were used at passage 4 or 5 (values are the means ± s.e.mean of 9 experiments composed of triplicate determinations). The results have been normalized as c.p.m. of IP per 100,000 c.p.m. of the remaining cell-associated *myo*-inositol in each cell well.

[b] One-way analysis of variance was performed on each column (NS, not significant). Mann-Whitney's test was then applied to compare the effect of each treatment with the control cells: $*P<0.05$; $**P<0.01$; $***P<0.001$.

contraction of the rabbit aorta by thrombin at a concentration of 1 μM. The activities of the peptide agonist and of KCl were not influenced by this inhibitor.

Following the observation that single doses of thrombin or [Sar¹]NAT_6-NH_2 were affected by the protein kinase inhibitor H-7, full cumulative concentration-effect curves for these agonists were established in the presence of this inhibitor. As shown in Figure 1, H-7 (10 μM) significantly depressed all concentrations of thrombin and [Sar¹]NAT_6-NH_2. Previous experiments on the same tissue have also shown that H-7 significantly reduced the contraction to PMA while it affected only minimally contractions induced by KCl (Levesque *et al.*, 1993).

Cellular model of vascular effects

Evidence was obtained that cultured SMCs derived from the rabbit aorta respond to thrombin and NAT_6-NH_2 by inducing

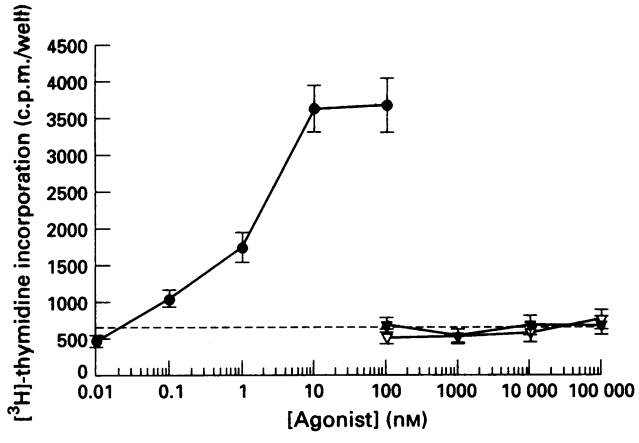

Figure 2 Effect of thrombin (●), NAT_6-NH_2 (▽) and [Sar¹]-NAT_6-NH_2 (▼) on the synthesis of DNA in cultured rabbit vascular smooth muscle cells. Data presented are the mean ± s.e. of several experiments involving triplicate determinations. Dashed line represents basal incorporation (0.4% FBS).

Table 3 [³H]-thymidine incorporation by rabbit aortic smooth muscle cells[a]

Treatment	*Control*	*Thrombin stim. cells* (1 nM)
Control	661 ± 112	1891 ± 237
Diclofenac 0.5 μM	709 ± 112	2367 ± 301
+ PGE_2 0.3 μM	666 ± 81	1371 ± 130
+ Iloprost 0.3 μM	525 ± 55	1451 ± 195
+ Nifedipine 0.1 μM	871 ± 101*	2995 ± 325**
+ Wortmannin 10^{-7} M	685 ± 90	2162 ± 228
+ PMA 1 μg ml^{-1}	798 ± 74*	NT
+ H-7 10 μM	808 ± 123	2633 ± 432
+ H-7 30 μM	360 ± 66**	676 ± 187***
+ Erbstatin analogue 10 μM	484 ± 147	705 ± 127*
ANOVA	$P < 0.0001$	$P < 0.0001$

[a] Values are the mean ± s.e.mean of at least 12 determinations from 4 separate experiments with different cell lines at passages 4 and 5 (NT: not tested). One-way analysis of variance was performed on each column. Mann-Whitney's test was then applied in each column to compare the effect of each treatment with the baseline or the thrombin-stimulated thymidine incorporation respectively (first line; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The effect of thrombin treatment alone within each treated group was assessed by Student's t test and was significant ($P < 0.0001$) for all groups except H-7 (30 μM) and erbstatin analogue-treated groups.

inositol phosphate (IP) turnover (Table 2). Significant increases in the production of IP_2 and IP_3 were observed in cells stimulated with thrombin. Although a dose-dependent effect is observed for thrombin, significant values were obtained only at concentrations of 10 and 1000 nM for IP_3 formation and 1000 nM for IP_2. NAT_6-NH_2 increased IP_1, IP_2 and IP_3 formation with a significant dose-dependent relation for both IP_2 and IP_3. Thrombin, NAT_6-NH_2 and [Sar¹]NAT_6-NH_2 were also tested for their ability to induce the incorporation of [³H]-thymidine in quiescent cells of the same origin that were maintained in 0.4% FBS. Thrombin stimulated the incorporation of thymidine in these cells in a dose-related manner (Figure 2), with maximum incorporation attained at a dose of 10 nM while NAT_6-NH_2 and [Sar¹]NAT_6-NH_2 at doses in excess of 100 μM failed to produce any significant thymidine incorporation in these cells. Several inhibitors were tested for their ability to modulate the thrombin-induced DNA synthesis into rabbit cultured aortic SMCs (Table 3). Previous experiments had shown that diclofenac, an inhibitor of fatty acid cyclo-oxygenase, was useful in revealing agonist-induced thymidine incorporation in this model (Levesque *et al.*, 1993). As shown in Table 3 diclofenac (0.5 μM) revealed maximum expression of thymidine incorporation following stimulation with thrombin. In the presence of diclofenac, PGE_2 and iloprost (a PGI_2 analogue) produced a partial inhibition of DNA synthesis in cells stimulated by thrombin. Nifedipine produced a significant increase of thymidine incorporation in both control and thrombin-stimulated cells. A slight increase was also observed with PMA while wortmannin, a phospholipase D inhibitor, did not produce any significant inhibition of thrombin-stimulated thymidine incorporation. At a dose of 10 μM the PKC inhibitor, H-7, was without effect on either control or thrombin-stimulated cells, however, H-7, at a higher dose (30 μM) and the erbstatin analogue (a tyrosine kinase inhibitor) reduced the effect of thrombin to baseline values. At the same dose, H-7 also reduced DNA synthesis in control cells.

Discussion

The comparative mode of action of thrombin and a synthetic agonist of the cleavable receptor for thrombin, either NAT_6-NH_2 or [Sar¹]NAT_6-NH_2 was investigated using a fresh rabbit aortic preparation (contractility assay) and cultured SMCs derived from the same tissue (IP turnover, mitosis). Vascular responses to thrombin seem to vary according to species; some vascular beds, such as pig and dog coronary arteries or dog saphenous veins respond to thrombin, or an agonist peptide of the cleavable receptor, with an endothelium-dependent relaxation that is mediated by the release of nitric oxide and a endothelium-indendent contraction (Tesfamariam *et al.*, 1993; Ku & Zaleski, 1993). The contractile response of the rabbit isolated aorta to thrombin and [Sar¹]NAT_6-NH_2 is mediated by smooth muscle since mechanical removal of the endothelium did not affect the response to either agonists or to KCl used as a control agonist (Table 1).

As the contractile response of the rat aorta has been shown to be dependent on extracellular calcium, we investigated whether the rabbit aorta operates in a similar manner. A sustained contraction was observed with thrombin in the absence of extracellular calcium in the bathing fluid, while the contraction to KCl, a depolarizing stimulus dependent on extracellular Ca^{2+} (Cauvin *et al.*, 1983), was inhibited by almost 90% on calcium removal. In the same conditions a modest inhibition of the contraction to the agonist peptide, [Sar¹]NAT_6-NH_2 was observed. The partial inhibition of the contractile activity of this peptide by calcium removal was also in the same order as that produced by the calcium channel blocker nifedipine. While nifedipine had no effect on the contraction to thrombin, it abolished the KCl-induced contraction, which is consistent with the inhibitory effect of this drug on the voltage-operated calcium channel (Murad, 1990). It is worth noting that the responses to thrombin and agonists of the cleavable receptor of the rabbit and rat aorta differ in

several respects. First of all, human or rat thrombin have no effect on the rat aorta, which on the other hand responds to synthetic agonists of the thrombin cleavable receptor (Antonaccio *et al.*, 1993). Aside from showing an endothelium-dependent relaxation, which is not observed in the rabbit aorta, contractile responses of the rat aorta to peptide agonists rely on extracellular Ca^{2+} while in the rabbit preparation the effect of thrombin is independent of extracellular Ca^{2+} and the contraction to [Sar^1]NAT_6-NH_2 is only partially affected by Ca^{2+} removal or blockade of Ca^{2+} channels by nifedipine. The partial inhibition of [Sar^1]-NAT_6-NH_2-induced contractile responses by Ca^{2+} removal or nifedipine in the rabbit aorta may indicate that receptor activation by the peptide agonist, which does not involve proteolysis, may be more dependent on extracellular Ca^{2+} than activation following cleavage of the receptor by thrombin. It may also be due to the activation of another receptor that is insensitive to the proteolytic action of thrombin. A second G-protein coupled receptor that is proteolitically activated has recently been cloned from a mouse genomic library (Nystedt *et al.*, 1994). Although the endogenous protease that presumably activates this receptor has not been identified, this receptor is insensitive to thrombin but may be activated by trypsin. Like the thrombin receptor, it is also activated by a synthetic hexapeptide, Ser-Leu-Ile-Gly-Arg-Leu corresponding to the newly exposed N-terminus following proteolytic cleavage. Also structure-activity studies on rat and guinea-pig tissues using a series of peptide analogues suggest the existence of thrombin receptor subtypes for peptides related to NAT_6-NH_2 (Hollenberg *et al.*, 1993).

Data obtained in rabbit aortic rings also suggest the involvement of protein kinase(s) in the contractions provoked by thrombin or [Sar^1]NAT_6-NH_2. Protein kinase C is inhibited by H-7 (Hidata *et al.*, 1984), and accordingly has been shown to reduce the contractile effect of PMA, a direct activator of protein kinase C, in aortic rings (Levesque *et al.*, 1993). H-7, in the same assay, also shifted the concentration-response curve to both [Sar^1]NAT_6-NH_2 and thrombin (Figure 1). However, the use of H-7 as a protein kinase C inhibitor has some limitations owing to other inhibitory effects of this drug, as for example, on cyclic nucleotide-dependent protein kinases (Hidata *et al.*, 1984). While the contractile response of the rabbit aorta to thrombin has been shown to act synergistically with that of EGF the receptor for which is a tyrosine kinase (Deblois *et al.*, 1992), erbstatin, an inhibitor of this type of receptor did not block the responses of any of the agonists in Table 1. As previously observed (Deblois *et al.*, 1992), PPACK, in accordance with its inhibition of the catalytic site of thrombin, did block the contractile effect of the latter while it had no effect on [Sar^1]NAT_6-NH_2-induced contraction.

SMCs derived from the rabbit aorta and cultured over weeks retained the capacity to respond to both the agonist of the cleavable receptor, in this case NAT_6-NH_2, and to thrombin by the generation of phosphoinositides. The increased IP concentration after 5 min exposure to either agonist, evidence of the activation of phospholipase C, was obtained at concentrations comparable to those used for the contractile response of isolated tissues (Table 1; Godin *et al.*, 1994). The activation of phospholipase C following stimulation of the cleavable receptor by NAT_6-NH_2 is consistent with the participation of diacylglycerol-regulated protein kinase C in the contractile effect of this peptide and of thrombin as referred to above with the use of the protein kinase inhibitor H-7 (Figure 1). Significant increases in IP_2 and IP_3 occurred at both concentrations of NAT_6-NH_2 tested and a graded response is observed. A dose-dependent IP production was also observed in cells stimulated with thrombin although significant only for IP_3. Here also the doses used to obtain this response are similar to those necessary to contract the rabbit aorta (Godin *et al.*, 1994).

Although the response of cultured rabbit SMCs to thrombin and NAT_6-NH_2 is similar, with regard to IP breakdown, only thrombin could induce DNA synthesis in these cells (Figure 2). NAT_6-NH_2 or [Sar^1]NAT_6-NH_2 at doses up to 100 μM did not provoke any significant [^{3}H]-thymidine incorporation whereas with thrombin, a full concentration-effect curve was obtained with a threshold dose as small as 0.1 nM while a full effect was obtained at 10 nM. Interestingly the concentrations of thrombin needed to produce the incorporation of thymidine seem well below those needed to induce IP breakdown or contraction of the isolated aorta (Table 2; Godin *et al.*, 1994). There are also other indications that the intracellular pathways involved in DNA synthesis in rabbit SMCs are at least partly different from those mediating the contractile response to thrombin. DNA synthesis was blocked by the erbstatin analogue while this tyrosine kinase inhibitor had no effect on the contractile response. H-7, at a concentration of 10 μM, can shift the contractile dose-response curve to both [Sar^1]NAT_6-NH_2 and thrombin but does not affect DNA synthesis. At 30 μM the effect of H-7 on thymidine incorporation is probably of a toxic nature as control cells were also affected.

Within the limitations of our pharmacological approach, the contractile response of the rabbit aorta to thrombin seems to be mediated by the activation of PKC and can be reproduced with synthetic agonists. However mitogenic responses to thrombin, of cultured vascular SMCs derived from this tissue, require a tyrosine kinase signal that is not generated by stimulation of the cleavable receptor with peptide agonists. These findings agree with observations in hamster fibroblasts where peptide agonists of the thrombin receptor do not reproduce the mitogenic effect of thrombin (Vouret-Craviari *et al.*, 1993). The inhibition of the mitogenic effect of thrombin in our cultured cells by erbstatin indicates that a tyrosine kinase signal is essential for cell proliferation in this model. Tyrosine kinase activity is also required for the mitogenic activity of thrombin on neonatal rat vascular SMCs but is not needed for intracellular calcium release (Weiss & Nuccitelli, 1992). How this signal is generated in rabbit vascular SMCs whether through another type of receptor or through the autocrine release of a growth factor (Weiss & Maduri, 1993) remains to be established.

Supported by the Medical Research Council of Canada (grant MT-12570) and by the Québec Heart Foundation. F.M. and G.D. are Scholars of the 'Fonds de la recherche en Santé du Québec' and D.G. is a recipient of a Studentship from the same agency.

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(Received February 22, 1995
Accepted March 27, 1995)