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Synergism between the contractile effect of epidermal growth factor and that of des-Arg^9-bradykinin or of α-thrombin in rabbit aortic rings

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1 Rabbit aortic rings were used to test the possible contractile effects of growth factors and their interaction with other stimuli. A rapid potentiation of kinin-induced contraction by epidermal growth factor (EGF) has been previously observed in this preparation.

2 EGF (5–1500 ng ml^{-1}) and the isoform BB of platelet-derived growth factor (PDGF-BB; 1–126 ng ml^{-1}) exerted modest but sustained contractile effects in rabbit aortic rings.

3 EGF pretreatment (100 ng ml^{-1}) potentiated the contractile responses to des-Arg^9-bradykinin (des-Arg^9-BK), an agonist of the B_1 receptors for kinin found in this preparation, and to human α-thrombin but not to several other contractile stimuli. The interaction appeared also relatively selective for the growth factor, because PDGF-BB pretreatment potentiated neither des-Arg^9-BK nor α-thrombin-induced contraction.

4 EGF, applied on a contraction plateau induced by des-Arg^9-BK or α-thrombin, exerted a synergistic contractile effect, with a time course and a half-maximal concentration for EGF-induced contraction similar to the ones recorded in resting tissues (between 67 and 220 ng ml^{-1}, depending on the series of experiments).

5 The direct or synergistic contractile effects of EGF were not modified by the removal of the endothelium or by treatment with indomethacin. However, the tyrosine kinase inhibitors, erbstatin or genistein, inhibited the synergistic effect of EGF with des-Arg^9-BK. The small direct contractile effect of EGF was significantly reduced by genistein. The synergistic effect of EGF with α-thrombin was comparatively more resistant to the tested tyrosine kinase inhibitors.

6 An inhibitor of the catalytic activity of α-thrombin, D-Phe-Pro-Arg-CH_2Cl, prevented the contractile effect of α-thrombin in the aortic rings. In this system, a tetradecapeptide derived from a recently cloned α-thrombin receptor was a contractile stimulus at and above 10 μM. Consistent with the hypothesis that this peptide could behave as an α-thrombin receptor agonist, its contractile effect was potentiated by EGF pretreatment. Pharmacological evidence was provided to show that the receptors for α-thrombin were distinct from the B_1 receptors for kinins. Together, these findings suggest that a model of a cleavable receptor recently elaborated to account for α-thrombin effects on human platelets is valid in blood-free vascular smooth muscle preparations such as the rabbit isolated aorta.

7 The synergism between EGF and kinin- or α-thrombin-induced contractions constitutes a novel mode of myotropic action for growth factors. The synergism is probably dependent on the tyrosine kinase activity of receptors for EGF. These combinations of stimuli could occur in various types of vascular disease and account for abnormal vascular reactivity often associated with atheroma lesions or vascular wound healing.

Keywords: des-Arg^9-bradykinin; B_1 receptors for kinins; rabbit aorta; α-thrombin receptor; epidermal growth factor

Introduction

Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are two potent mitogenic principles for cultured vascular cells (Schwartz *et al.*, 1990). Both factors also elicit mechanical responses when applied to isolated blood vessels *in vitro* (reviewed by Berk & Alexander, 1989). These findings have generated interest because adhering platelets present in advanced atheroma lesions can secrete both PDGF and transforming growth factor-α (TGF-α), a material stimulating the same receptors as EGF (Oka & Orth, 1983; Ross, 1986). In addition, activated macrophages within the lesion constitute a potential source of TGF-α (Jonasson *et al.*, 1986; Madtes *et al.*, 1988). Injuries to the vessel wall, such as coronary atherosclerosis or angioplasty performed with a balloon catheter, are accompanied by platelet deposition (Ross, 1986; Cowley *et al.*, 1987) and may be complicated by vasospasm in man (Kalsner & Richards, 1984; Fischell *et al.*, 1988). Thus, the vascular reaction to injuries is a possible application for growth factor-induced vascular contractility.

The mechanisms of the contractile responses to EGF or PDGF are not fully understood and may vary from one vascular model to another. Proposed mechanisms include the increased production of vasoactive prostaglandins (Muramatsu *et al.*, 1985) or the direct activation of membrane signalling mechanisms by the activated receptors for the growth factors. These factors may activate diacylglycerol and inositol triphosphate production and calcium mobilization (Berk & Alexander, 1989), just as other conventional contractile agonists do (Campbell *et al.*, 1985). Stimulation of intracellular signalling pathways by EGF or PDGF is associated with the intrinsic tyrosine kinase activity of the activated growth factor receptors (Ullrich & Schlessinger, 1990).

A possible link between this activity and the phosphatidylinositol cycle is the tyrosine phosphorylation, accompanied with increased activity, of the isoform γ1 of phospholipase C by EGF or PDGF receptors (Nishibe *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991). This common effector pathway for both EGF- and PDGF-BB-activated receptors constitutes

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the rationale for comparing the vasomotor effects of the growth factors in the present study.

We have previously observed that EGF potentiates the contractile effect of bradykinin (BK) and of des-Arg^9-bradykinin in the rabbit isolated aorta (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989). This preparation possesses the B_1 type receptors for kinins, which implies that des-Arg^9-BK is more potent than BK, and that both peptides are antagonized by the analogue [Leu^8]des-Arg^9-BK (Regoli & Barabé, 1980). The potentiation of the effect of kinins by EGF was a very rapid event that could be observed within 15 min of exposure to EGF. This phenomenon differs from the slow up-regulation of B_1 receptors under the effects of immunological stimuli such as lipopolysaccharide and interleukin-1 (deBlois *et al.*, 1989; 1991). Noradrenaline-induced contractions were not potentiated by EGF (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989), suggesting a possible selectivity of EGF for the contractile stimulus. This observation may indicate a novel mechanism for growth factor-induced vasoconstriction: the potentiation of the contractile responses to other specific agents.

One of the objectives of the present study was to determine the selectivity of the interaction between kinins and EGF in the rabbit aortic preparation. A number of other stimuli have been screened and we have also tried to substitute the growth factor PDGF-BB for EGF. The isoform BB of PDGF was used because it does not discriminate between the various receptor subtypes for this growth factor (Seifert *et al.*, 1989). Possible small direct contractile effects of the growth factors were also investigated. The role of the tyrosine kinase activity of the EGF receptor has been evaluated by use of selective inhibitory drugs for this enzyme activity. Other possible mechanisms of EGF action involving the endothelium or the arachidonate cascade were also examined.

As the contractile effect of α-thrombin was also shown to be potentiated by EGF, new concepts on thrombin pharmacology have been verified. α-Thrombin is a known contractile stimulus of the rabbit aortic preparation (Haver & Namm, 1984), and a catalytically active protease is required for this action. A cleavable receptor for α-thrombin from megakaryocyte-like cell lines has been cloned and sequenced recently (Vu *et al.*, 1991). This receptor is a member of the rhodopsin superfamily of signalling proteins. It was proposed that a typical thrombin cleavage site present in the N-terminal region of the chain would expose a 'new amino terminus' (NAT) domain that would act as a 'tethered' agonist of the receptor. Vu *et al.* (1991) tested this model by showing that a synthetic tetradecapeptide (NAT_{14}) corresponding to the NAT domain was an agonist of the cellular receptor for α-thrombin in human platelets and in *Xenopus* oocytes expressing the cloned receptor. In the present experiments, we used NAT_{14} to test whether it would behave as a contractile agonist in a blood-free vascular bioassay and whether the relatively selective potentiation of α-thrombin effects by EGF would apply to this oligopeptide.

Methods

Pharmacological preparation

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 (composition as in Marceau *et al.*, 1991). Their responses to agents were isometrically recorded as described previously (Bouthillier *et al.*, 1987).

Protocols

The direct contractile effects of EGF and PDGF-BB were verified on tissues equilibrated for 1 h. Single concentration or a cumulative scale of concentrations (cumulative concentration-effect curve) were applied.

Various agents were screened for potentiation of their contractile effects by EGF (100 ng ml^{-1}). Several agents known to contract the rabbit aortic preparation were applied approximately at half-maximal concentrations, except for des-Arg^9-BK which was applied at a nearly maximal concentration (1.7 μM) and for α-thrombin, which was applied at two concentration levels. The effect of EGF on the whole concentration-effect curve to this kinin has been described previously (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989). The screening of agonists was performed on control tissues or tissues continuously exposed to EGF (100 ng ml^{-1}). After 1 h of equilibration, up to 4 agonists were injected at 90 min intervals and following a random sequence. An exception was des-Arg^9-BK, which was injected at the time 6 h, because the maximal effect of this agent changes considerably as a function of the incubation time. The mechanism for the increase of tissue response to B_1 receptor agonists has been studied previously (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1989; 1991).

Cumulative concentration-effect curves for EGF were obtained after 4.5 h of tissue incubation *in vitro* in aortic rings precontracted or not with des-Arg^9-BK. The kinin was applied at a threshold (1.7 nM) or a maximal (1.7 μM) concentration. Tissues from the same aorta and stimulated with des-Arg^9-BK alone were used as controls to evaluate quantitatively the stability of the kinin-induced plateau of contraction over time (control plateau). The rate of tone loss from control plateaus, expressed as a percentage over the period required for the completion of the concentration-effect curve for EGF, was used to determine the baseline upon which the contraction to EGF were superimposed in the paired aortic ring. Similar experiments were conducted with another contractile agonist potentiated by EGF, α-thrombin.

The direct or synergistic contractile responses of growth factors and other agonists were analyzed in terms of dependence on tyrosine kinase activity, on arachidonate cascade activation, or on the presence of endothelium. Some tissues were treated for 1 h before stimulation with the tyrosine kinase inhibitors, erbstatin (Imoto *et al.*, 1987), genistein (Akiyama *et al.*, 1987) or tyrphostin-51 (Gazit *et al.*, 1989), or with the cyclo-oxygenase blocker, indomethacin. Because erbstatin was not available in large quantities, several tissues were grouped together and exposed for 1 h to this inhibitor in a small volume of Krebs solution. The tissues were then mounted separately in tissue baths and allowed to equilibrate for an additional hour before recording contractility. In some experiments, the endothelial lining of the aortic rings was removed by gently rubbing the intimal surface with a round wooden stick; the loss of acetylcholine-induced relaxation was monitored as an indication of a successful procedure as endothelial cells are necessary for this response in the rabbit isolated aorta (Furchgott & Zawadzki, 1980).

As α-thrombin interactions with EGF were found to be important, the oligopeptide NAT_{14}, which behaves as a thrombin receptor agonist on human platelets (Vu *et al.*, 1991), was tested as a contractile agonist in the isolated aortic rings in combination or not with EGF. The requirement for a proteolytically active form of α-thrombin for the myotropic effects was verified with an irreversible inhibitor of the enzyme.

Finally, pharmacological criteria have been used to show that receptors for α-thrombin and B_1 receptors for kinins are distinct pharmacological entities.

Drugs

Des-Arg^9-BK was purchased from Bachem (Torrance, CA, U.S.A.). Epidermal growth factor (EGF; receptor grade, from mouse submaxillary glands) was from Sigma Chemicals (St-Louis, MO, U.S.A.), as well as cycloheximide, [Leu^8]des-Arg^9-BK, (−)-noradrenaline, 5-hydroxytryptamine (creati-

nine sulphate complex), histamine dihydrochloride, indomethacin, angiotensin II and phorbol 12-myristate 13-acetate (PMA). Human recombinant interleukin-1β was a gift from Biogen S.A. (Geneva, Switzerland). Highly purified human α-thrombin (2863 NIH units per mg) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Human recombinant PDGF (BB isoform, produced in yeast) was a gift from Abbott Laboratories. Erbstatin (Institute of Microbial Chemistry, Tokyo, Japan) was purified from *Streptomyces*. Tyrphostin-51, one of the most potent available inhibitors of the tyrosine kinase activity of EGF receptors (Gazit *et al.*, 1989), was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). D-Phe-Pro-Arg-CH_2Cl (PPACK), an irreversible blocker of the protease activity of α-thrombin (Kettner & Shaw, 1979), and genistein, a tyrosine kinase inhibitor (Akiyama *et al.*, 1987) were purchased from Calbiochem.

The tetradecapeptide NAT_{14} (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH) was synthesized and purified in our laboratory by use of a solid-phase method (general methods outlined by Drapeau & Regoli, 1988). Analytical high performance liquid chromatography (h.p.l.c.) profile and mass spectroscopy indicated a pure compound of the expected molecular weight (1740 Da).

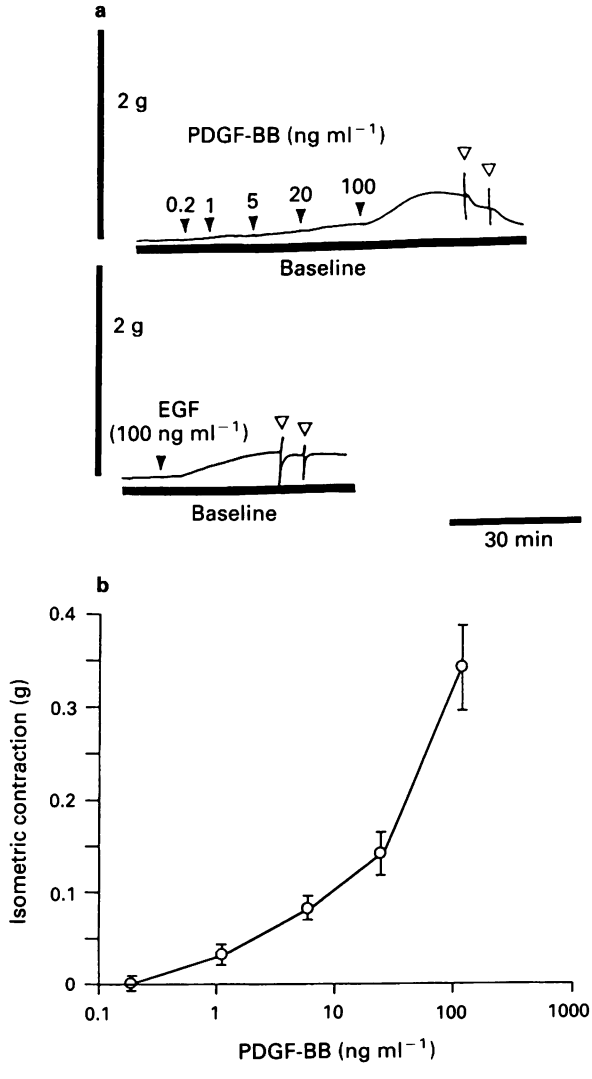

Figure 1 Contractile effect of the isoform BB of platelet-derived growth factor (PDGF-BB) and epidermal growth factor (EGF) on rabbit aortic rings. Vascular tissues were stimulated after 1 h of *in vitro* equilibration. (a) Tracing showing the building of a cumulative concentration-effect curve for PDGF (0.2–100 ng ml^{-1}) and the time course of the development of a contraction induced by a single concentration of EGF (100 ng ml^{-1}). Abscissa scale: time (min). Ordinate scale: isometric contraction (g). Closed symbols indicate the application of agents and open symbols the washout of stimulants. (b) Concentration-effect curve for PDGF-BB. Results are the means of 11 determinations; s.e. shown by vertical bars

Results

Direct contractile effects of growth factors

Both EGF and PDGF elicited contractile responses in rabbit aortic rings (Figure 1), but these responses were comparatively very slow to develop and of modest amplitude when compared with many other vasoactive agents. The contractile response to a single application of EGF (100 ng ml^{-1}) was 0.19 ± 0.045 g ($n = 6$). The effect of PDGF was concentration-dependent in the range of concentration 1–126 ng ml^{-1} (Figure 1b).

Screening of various agonists for synergism with EGF

Various contractile agents effective in the rabbit aortic preparations were tested for potentiation by EGF. Responses of tissues continuously exposed to EGF 100 ng ml^{-1}, were compared to control tissues from the same animals. As found previously, (Bouthillier *et al.*, 1987), exposure to EGF increased the contractile effect of des-Arg^9-BK (at 1.7 μM: from 1.75 ± 0.26 g to 3.90 ± 0.90 g, $n = 4$, $P < 0.001$ by Student's t test), but not that of noradrenaline (at 100 nM: 1.95 ± 0.11 g without EGF; 1.84 ± 0.43 g with EGF, $n = 8$). Other stimuli, the effects of which were not significantly modified by EGF treatments, were angiotensin II (2 nM), histamine (10 μM), 5-hydroxytryptamine (400 nM), KCl (30 mM) and PMA (700 ng ml^{-1}) (not shown). Human α-thrombin (6 or 60 nM) was the other contractile agent besides des-Arg^9-BK that was significantly potentiated by the EGF treatment. Responses to thrombin at 6 nM were 0.46 ± 0.05 without EGF, and 0.64 ± 0.06 g in the presence of EGF ($n = 6$, $P < 0.01$ by Student's t test). With 60 nM thrombin, contractile responses of 1.02 ± 0.10 g were recorded in control tissues, and of 1.50 ± 0.09 g in EGF-treated tissues ($n = 6$, $P < 0.01$). In this series of experiments, the baseline tension presumably included the direct contractile effect of EGF, as the growth factor was continuously present.

The agents potentiated by EGF, des-Arg^9-BK and α-thrombin, were not significantly potentiated in tissues continuously exposed to PDGF-BB (Table 1), indicating a relative selectivity for the growth factor in this system. The concentration of PDGF used in these experiments was biologically active in the rabbit isolated aorta (Figure 1b).

Concentration-effect relationship for EGF

The cumulative concentration-effect curve for the contractile effect of EGF has been established with resting tissues and also with tissues precontracted with either des-Arg^9-BK or α-thrombin (Figures 2–4). The responses attributed to EGF were subtracted from contraction plateaus induced by des-Arg^9-BK (1.7 nM or 1.7 μM) or by α-thrombin (6 or 60 nM) and were plotted in Figures 3 and 4, respectively. The small contractile effect of EGF was increased significantly in tissues pretreated with the higher concentration level of either stimulus (Figures 3a and 4a), but the time course of the contraction was similar, as seen from the tracings (Figure 2). It can be seen that the amplitude of the EGF-induced contraction increased concentration-dependently in the presence of either des-Arg^9-BK or α-thrombin (Figures 3a, 4a), but when these data were plotted as a percentage of maximal EGF-induced effects, the half-maximal concentration of EGF did not change significantly when the other contractile stimulus was present (approximately 220 ng ml^{-1} in the series of experiments with des-Arg^9-BK and 70 ng ml^{-1} in the thrombin series; Figures 3b, 4b).

Table 1 Lack of effect of PDGF-BB on contractions of the rabbit aorta induced by a kinin or α-thrombin receptor agonist

Contractile agent (concentration)		*n*	*Isometric contraction* (g)[a] *Control*	*PDGF-BB* (100 ng ml^{-1})
α-Thrombin	(6 nM)	9	0.17 ± 0.036	0.20 ± 0.02
	(60 nM)	5	1.11 ± 0.09	1.33 ± 0.09
des-Arg^9-bradykinin	(1.7 μM)	5–6	1.44 ± 0.37	1.48 ± 0.44

[a]Results are the means ± s.e. of the number of determinations indicated by *n*. PDGF-BB-treated tissues and controls were exposed to α-thrombin at 1 or 1.5 h of incubation, and to des-Arg^9-BK at 6 h. No value was significantly different from control as calculated by Student's *t* test.

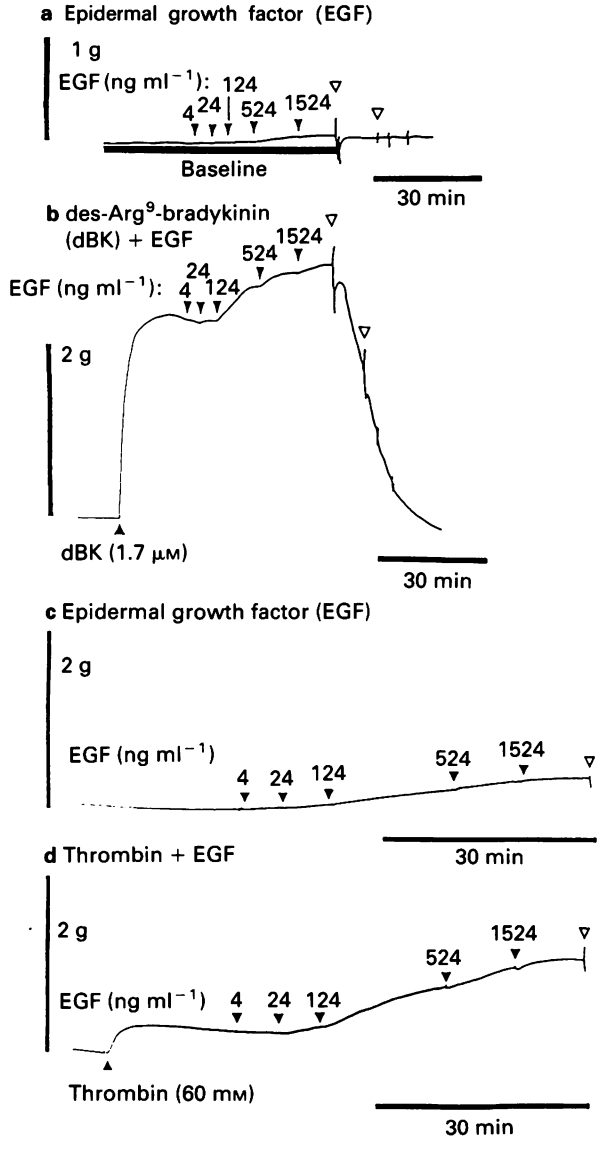

Figure 2 Tracings illustrating the construction of a cumulative concentration-effect curve for the contractile effect of epidermal growth factor (EGF) on rabbit aortic rings. The preparation was resting (tracings a,c) or precontracted with des-Arg^9-BK (1.7 μM; tracing b) or with α-thrombin (60 nM, tracing d). Each pair of tissues was derived from the same animal. Abscissa scale: time (min). Ordinate scale: isometric contraction(g). Closed symbols indicate the application of agents and open symbols the washout of stimulants. The cumulative concentration (ng ml^{-1}) of EGF is indicated

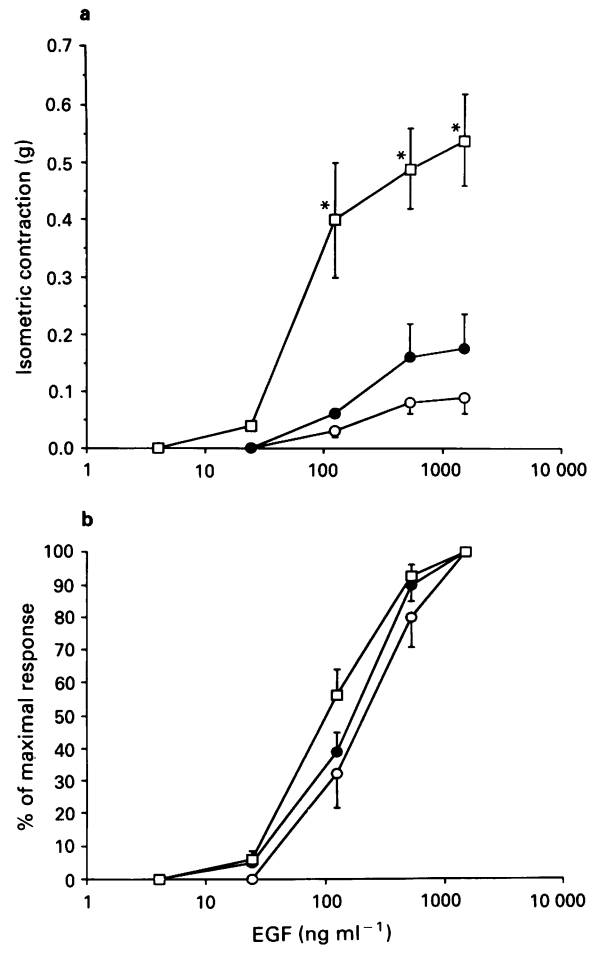

Figure 3 Effect of des-Arg^9-BK pre-stimulation on the contractile responses to epidermal growth factor (EGF) in rabbit aortic rings. (a) EGF concentration-effect curves expressed as grams of developed tension in control preparations (○) or in tissues precontracted with des-Arg^9-BK (1.7 nM, ●; 1.7 μM, □). (b) The same data presented as percentage of the maximal EGF-induced effect. In each case, the contraction plateau induced by des-Arg^9-BK was subtracted, as described in Methods. The peak contractile responses averaged 0.006 ± 0.005 g for the 1.7 nM concentration of des-Arg^9-BK ($n = 9$), and 1.20 ± 0.22 g at 1.7 μM of the peptide. Results are the mean of 5–9 determinations; vertical bars show s.e.. In (a), the contractile responses at each concentration of EGF were compared by one-way analysis of variance, followed by Dunnett's test. Significant differences from the control responses are indicated by $*P < 0.05$

Mechanism of EGF direct or synergistic effects

The synergism between EGF (100 ng ml^{-1}) and des-Arg^9-BK appears to be dependent on the tyrosine kinase activity of the EGF receptors, as suggested by the inhibitory effect of erbstatin and genistein (Table 2). The same drugs, plus tyrphostin-51, another tyrosine kinase inhibitor, partially decreased the potentiating effect of EGF on α-thrombin-induced contraction (to a non-significant level for erbstatin and tyrphostin-51; Table 2), but this system was more resistant than the EGF-des-Arg^9-BK synergy to the action of these metabolic inhibitors. Erbstatin significantly inhibited the direct contrac-

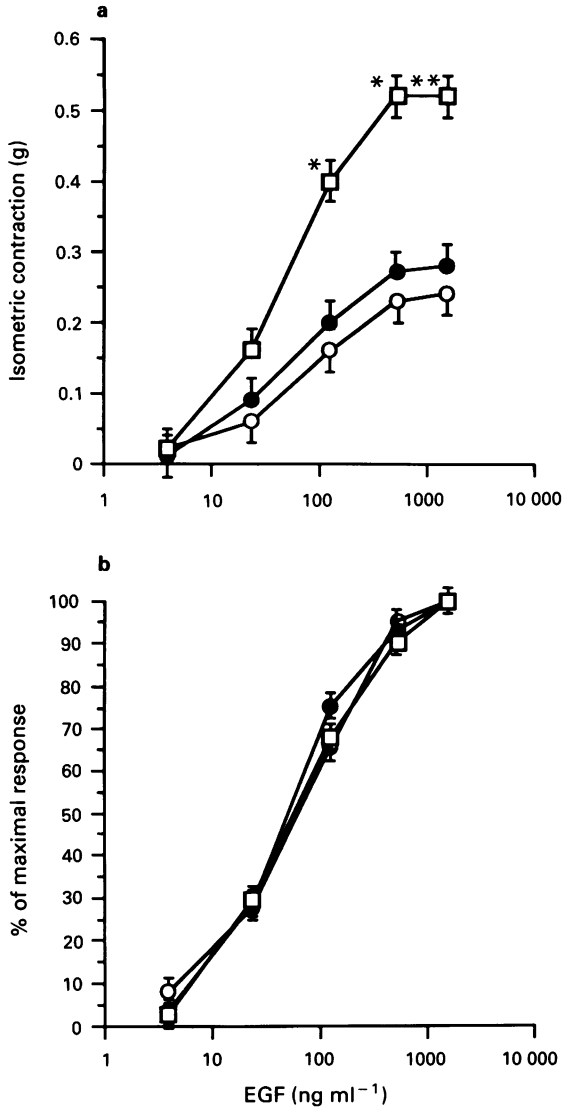

Figure 4 Effect of α-thrombin pre-stimulation on the contractile responses to epidermal growth factor (EGF) in rabbit aortic ring. (a) EGF concentration-effect curves expressed as grams of developed tension in control preparations (○) or in tissues precontracted with thrombin (6 nM, ●; 60 nM, □). (b) The same data presented as percentage of the maximal EGF-induced effect. In each case, the contraction plateau induced by α-thrombin was subtracted, as described in Methods. The peak contractile responses averaged 0.37 ± 0.08 g for the 6 nM concentration of α-thrombin ($n = 5$), and 0.86 ± 0.12 g at 60 nM of the protease. Results are the mean of 5–10 determinations; vertical bars show s.e. In (a), the contractile responses at each concentration of EGF were compared by one-way analysis of variance, followed by Dunnett's test. Significant differences from the control responses are indicated by $*P<0.01$; $**P<0.001$

tile response to PDGF (100 ng ml^{-1}: 0.22 ± 0.04 g of contraction in controls, 0.09 ± 0.02 g in drug-treated tissues, $n = 16$, $P<0.01$ by Student's t test). However, erbstatin exerted no significant effect against the direct effect of EGF (100 ng ml^{-1}: 0.08 ± 0.01 g in controls, 0.07 ± 0.03 g in drug-treated tissues, $n = 16$). Tyrphostin-51 also failed to modify the small contractile effect of EGF (not shown), and only genistein was active in this respect (0.12 ± 0.02 g in controls, 0.06 ± 0.02 g in drug-treated tissues, $n = 11$, $P<0.01$). The contractions induced by des-Arg^9-BK (Table 2), α-thrombin (Table 2) or noradrenaline (not shown) were not affected by the tyrosine kinase inhibitors, suggesting the selectivity of these drugs for effects related to growth factors.

Treatment of aortic rings with the cyclo-oxygenase inhibitor, indomethacin, failed to inhibit either the synergistic effects of EGF (Table 2) or its direct contractile effect (0.13 ± 0.03 g in controls, 0.17 ± 0.05 g in drug-treated tissues, $n = 15$). The removal of the endothelium from the aortic rings also failed to modify the effects of EGF (Table 2).

Effect of α-thrombin on vascular smooth muscle

The concentration-effect relationship for the contractile action of human α-thrombin on the rabbit aortic preparation has been described elsewhere (Haver & Namm, 1984). We established a cumulative concentration-effect curve for NAT_{14}, which was found to be active at and above 10 μM (Figure 5). The contraction induced by NAT_{14} exhibited a temporal profile similar to the one elicited by α-thrombin. The NAT_{14} contractile effect was not tachyphylactic when stimulations were applied at 20–30 min intervals and the contraction amplitude did not vary as a function of incubation time *in vitro* (not shown). A partial tachyphylaxis has been reported when α-thrombin was repeatedly applied to this preparation (Haver & Namm, 1984). The NAT_{14}-induced contraction (100 μM) was significantly potentiated by EGF (100 ng ml^{-1}): 0.94 ± 0.20 g of contraction was recorded in EGF-treated tissues, as compared to 0.51 ± 0.12 in paired controls ($n = 8$; $P<0.01$ by Student's t test). This is in agreement with the hypothesis that the peptide is a α-thrombin receptor agonist.

Although α-thrombin and kinins that are agonist for B_1 receptors share the capability of contracting the aortic rings in synergism with EGF, they probably do not stimulate the same population of receptors. This conclusion is supported by several experimental approaches (Table 3). The inhibitor of α-thrombin catalytic activity, PPACK, prevented completely the contractile effect of α-thrombin (6 nM) without influencing that of des-Arg^9-BK (1.7 μM). Incidentally, PPACK could also partially relax a tissue precontracted with α-thrombin, indicating the need for a continuous proteolytic action for the maintenance of a contraction plateau (Figure 5b). PPACK had no effect on contractions induced by NAT_{14} at 100 μM (Figure 5b; statistical analysis: contraction of 0.59 ± 0.10 in tissues pretreated with PPACK, compared to a control of 0.51 ± 0.12, $n = 8$).

The competitive agonist of B_1 receptors, [Leu^8]des-Arg^9-BK (Regoli & Barabé, 1980), did not inhibit α-thrombin-induced contraction (Table 3). In addition, two types of treatment that increase the level of response to B_1 agonists over several hours, namely pulse exposure to cycloheximide or to interleukin-1β (deBlois *et al.*, 1991), failed to modify the contractile effect of α-thrombin.

Discussion

In this study, we investigated the possible interactions between the growth factors EGF or PDGF-BB and other stimuli in the regulation of vascular smooth muscle tone. Our data indicate a novel mode of myotropic action for EGF and, possibly, other growth factors: the potentiation of the contractile responses to other specific agents. When applied alone, EGF and PDGF-BB exerted only modest direct contractile effects on the rabbit aortic preparation. The temporal profile of growth factor-induced contraction consisted of a relatively slow onset, a sustained plateau and a slow relaxation after washing. All these features were observed previously on the rat isolated aorta (Berk *et al.*, 1985; Berk & Alexander, 1989). The concentrations of EGF needed to contract the rabbit aorta are similar to those needed to induce contraction in the rat aortic preparation (Berk *et al.*, 1985) or proliferation in calf aortic smooth muscle (Bhargava *et al.*, 1979). The concentrations of PDGF-BB needed to elicit a contractile response in the rabbit aorta are somewhat higher than those reportedly active in contracting the rat

Table 2 Studies on the mechanism of epidermal growth factor (EGF) potentiation of des-Arg^9-BK-induced or of α-thrombin-induced contraction in rabbit aortic rings

	Response to des-Arg^9-BK, 1.7 μM (g)		*Response to α-thrombin*[b] (g)	
Treatment[a]	*Paired control without EGF*	*EGF* (100 ng ml^{-1})	*Paired control without EGF*	*EGF* (100 ng ml^{-1})
Experiment A				
Control	1.08 ± 0.15 (12)[c]	1.59 ± 0.20 (12)**[d]	0.46 ± 0.05 (9)	0.64 ± 0.05 (9)**
Erbstatin (5 μg ml^{-1})	0.82 ± 0.13 (12)	0.95 ± 0.19 (12)	0.36 ± 0.05 (9)	0.51 ± 0.07 (9)
Experiment B				
Control	1.47 ± 0.24 (6)	2.31 ± 0.25 (6)*	0.64 ± 0.18 (5)	1.00 ± 0.21 (5)**
Genistein (50 μg ml^{-1})	1.01 ± 0.14 (6)	0.97 ± 0.09 (6)	0.61 ± 0.10 (5)	0.87 ± 0.16 (5)*
Experiment C				
Control	NT[e]	NT	1.26 ± 0.14 (5)	1.70 ± 0.21 (5)*
Tyrphostin-51 (5 μM)	NT	NT	1.20 ± 0.08 (5)	1.57 ± 0.21 (5)
Experiment D				
Control	1.19 ± 0.29 (10)	1.63 ± 0.38 (10)*	1.04 ± 0.12 (5)	1.53 ± 0.11 (5)*
Indomethacin (2.8 μM)	1.13 ± 0.26 (10)	1.49 ± 0.33 (10)*	0.95 ± 0.05 (5)	1.54 ± 0.17 (5)*
Experiment E				
Endothelium removal	2.16 ± 0.55 (4)	3.07 ± 0.14 (4)**	1.60 ± 0.11 (9)	1.87 ± 0.15 (9)*

[a]EGF was applied 15 min before the application of the major contractile stimulus, des-Arg^9-BK (applied in tissues preincubated for 6 h) or thrombin (applied either at 6 or 60 nM in tissues preincubated for 1.5 h).
Tyrosine kinase inhibitors (erbstatin, genistein or tyrphostin-51) were applied 1 h before stimulation with the contractile agent and indomethacin, 30 min before. Tissues with the endothelium removed did not relax when acetylcholine (100 nM) was applied on a contraction induced by noradrenaline or phenylephrine (not shown).
[b]Thrombin concentration was 6 nM in experiment A and 60 nM in the other ones.
[c]Values are the means ± s.e.mean of the number of determinations indicated in parentheses.
[d]EGF potentiation was evaluated by comparing the values from EGF-treated tissues from the paired controls using Student's *t* test for paired data. Levels of significance: $*P<0.05$; $**P<0.01$.
[e]NT, not tested.

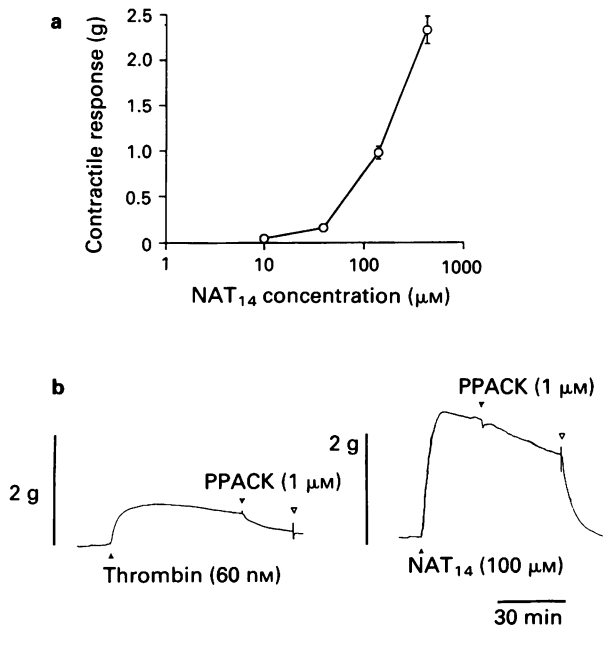

Figure 5 Effect of stimuli related to receptors for α-thrombin on rabbit aortic rings. (a) Concentration-effect relationship for NAT_{14}, a peptide derived from a cloned receptor for α-thrombin. Values are expressed as the means of 5 determinations. (b) Representative tracings of tension changes induced by α-thrombin (60 nM) or NAT_{14} (100 μM) and acute effect of the α-thrombin inhibitor, D-Phe-Pro-Arg-CH_2Cl (PPACK; 1 μM), on the contraction induced by these agents. Abscissa scale: time (min). Ordinate scale: isometric contraction (g). Closed symbols indicate the application of agents and open symbols the washout of stimulants

aorta (Berk *et al.*, 1985) or stimulating proliferation in human fibroblasts (Seifert *et al.*, 1989).

In addition, and as previously observed (deBlois *et al.*, 1989), the kinin-induced contractility in the rabbit aortic preparation was rapidly potentiated by EGF. In this study the interaction with EGF was shown to be relatively selective, being shared only by α-thrombin. The order of application of the growth factor and the other agonist was not important, as EGF applied on a plateau induced by des-Arg^9-BK or by α-thrombin also resulted in a contractile effect greater than the sum of the responses to each agent (Figures 2–4). Considering the small direct contractile effect of EGF and the fact that the concentration of either des-Arg^9-BK or α-thrombin resulted in a further increase in the contractile effect of EGF, the interaction of EGF with des-Arg^9-BK or α-thrombin could be called synergism.

The rat superior mesenteric artery responded to EGF by an apparent prostaglandin-mediated contraction (Muramatsu *et al.*, 1985). However, in the present study, indomethacin did not inhibit direct or synergistic contractile effects of EGF, excluding the role of cyclo-oxygenase products. Endothelium removal did not influence the direct contractile effect of the growth factor, nor its synergistic effect with α-thrombin. This was attempted because some of the effects of α-thrombin in various vascular preparations are endothelium-dependent (DeMey & Vanhoutte, 1981; Hatake *et al.*, 1990; Boulanger & Lüscher, 1991). The contractile effect of des-Arg^9-BK was not influenced by endothelium removal in the rabbit aortic preparation (Bouthillier *et al.*, 1987).

The primary signalling mechanism of EGF and of PDGF is believed to be the activation of a protein tyrosine kinase domain present in their respective receptors (Ullrich & Schlessinger, 1990). Therefore appropriate blockers of tyrosine kinase activity could theoretically inhibit the effects of growth factors. We observed that the three competitive inhibitors used, erbstatin, genistein and tryphostin-51, behaved differently in reference to different effects of growth factors on the rabbit aorta at the concentrations tested. Erbstatin inhibited the direct contractile effect of PDGF-BB and the EGF/des-Arg^9-BK synergism, but was a weak inhibitor of the thrombin/EGF synergism and failed to inhibit the direct contractile effect of EGF. Genistein inhibited all effects of EGF, but again, the inhibition of EGF/thrombin synergism was less extensive. Tyrphostin-51, one of the most potent inhibitors of EGF receptor tyrosine kinase in cell-free

Table 3 Differential effects of several treatments on the contractile response to des-Arg^9-BK and to α-thrombin

Treatment (concentration; duration)[a]	n	*Contractile response* (g) *des-Arg^9-BK* (1.7 μM)	*α-Thrombin* (6 nM)
Experiment A			
Control	4	0.58 ± 0.02	0.39 ± 0.06
PPACK (1 μM, 0–6.5 h)	4	0.61 ± 0.05	0**[b]
[Leu^8]des-Arg^9-BK (17 μM, 20 min)	4	NT[c]	0.42 ± 0.11
Experiment B			
Control	7	1.84 ± 0.30	0.47 ± 0.05
Cycloheximide (17 μM, 0–3 h)	7	2.88 ± 0.28*	0.58 ± 0.09
Experiment C			
Control	4	0.78 ± 0.26	0.23 ± 0.07
Interleukin-1β (5 ng ml^{-1}, 0–3 h)	4	1.72 ± 0.20*	0.19 ± 0.03

[a]Rabbit aortic rings were challenged with des-Arg^9-BK after 6 h, or with α-thrombin after 1 h of *in vitro* incubation in Krebs solution. Treatments consisted of exposing tissues to a drug for a definite period of time: cycloheximide and interleukin-1β were given as a 'pulse' during the first 3 h of incubation, [Leu^8]des-Arg^9-BK was given 20 min before the 6 h recording and D-Phe-Pro-Arg-CH_2Cl (PPACK) was applied continuously to treated tissues. The statistical weight of each animal involved in these experiments was the same in control and treated tissue groups.
[b]Values from treated groups were compared to controls by Student's *t* test: $*P<0.05$; $**P<0.01$.
[c]Not tested.

systems (Gazit *et al.*, 1989), was only marginally active against EGF/thrombin synergism and inactive against the direct contractile effect of EGF. The tyrosine kinase activities of various growth factor receptors are sufficiently distinct to be inhibited differentially by tyrosine analogues, such as erbstatin and tyrphostins, but both types of drugs are reported to inhibit cell responses to EGF (Gazit *et al.*, 1989; Powis, 1991). These compounds are known to be relatively unstable in biological systems (Powis, 1991). The inhibitors may be much less potent in our system than in cell-free biochemical assays (Gazit *et al.*, 1989) and it is plausible that different concentration levels are required to inhibit the phosphorylation of different substrates (Enright & Booth, 1991). If this is the case, it is possible that the thrombin/EGF synergism is dependent on the action of tyrosine kinase on a high affinity substrate, because the competitive kinase inhibitors are comparatively less efficient to prevent it.

Growth factor-associated tyrosine kinase activities also exhibit some substrate selectivities, and there is indication that some kinase substrates are recognized by only one (or few) of these activities (Pandiella *et al.*, 1989; Powis, 1991). The synergism that we observed between EGF and two other agonists must fall in this category, because PDGF-BB exhibited no such interactions. The activation of phospholipase Cγ1 is a common effect of EGF and PDGF (Goldschmidt-Clermont *et al.*, 1991) and consequently, it is not a likely explanation of the observed synergisms in the rabbit aortic rings. It is of interest that the synergism between EGF and α-thrombin has been documented previously: the mitotic rate of human cultured endothelial cells under stimulation with EGF or fibroblast growth factor was markedly increased by α-thrombin (Gospodarowicz *et al.*, 1978). EGF is known to increase the duration of the plateau of the cytosolic calcium increase induced by BK in various cell lines (Olsen *et al.*, 1988; Pandiella & Meldolesi, 1988; Marks *et al.*, 1988). A rapid and selective reinforcement of the transmembrane signalling at BK receptors was postulated; in these systems the B_2 receptor type was involved. Some undetermined interaction of intracellular second messenger pathways was postulated to occur at a level proximal to the kinin receptor. A proposed mechanism was the phosphorylation of the B_2 type receptor, or of a G protein that couples this (but not all) receptor to phospholipase C, by the kinase activity of EGF receptors (Pandiella & Meldolesi, 1988). Such an interaction between B_1 receptors for kinins or α-thrombin receptors and EGF receptors could constitute a reasonable basis to explain the relative selectivity of the EGF synergistic effect in the rabbit aortic tissue. The C-terminal cytosolic domain of the cloned receptor for α-thrombin is rich in tyrosine residues: 6 are present, including a tyrosine triplet apparently unique in the rhodopsin family of receptors (Vu *et al.*, 1991). These are potential sites for phosphorylation by activated growth factor receptors.

α-Thrombin (EC 3.4.21.5) is a serine protease, the principal function of which is to convert the soluble plasma protein, fibrinogen, into insoluble fibrin (Fenton, 1986). Therefore α-thrombin plays a central role in the mechanism of blood coagulation. Incidentally, the concentrations of α-thrombin used in this study were less than the ones found in spontaneously clotting human blood (circa 140 nM; Aronson *et al.*, 1977). In addition, this enzyme also exerts hormone-like effects on a large number of cell types (Shuman, 1986). Both endothelium-dependent and independent actions of α-thrombin have been reported on vascular tissue (DeMey & Vanhoutte, 1981; Hatake *et al.*, 1990; Boulanger & Lüscher, 1991). In the rabbit aortic preparation, we have reproduced the findings of Haver & Namm (1984), which could be summarized as follows: α-thrombin is an endothelium-independent contractile agent (Table 2) the activity of which is abolished by inhibitors of its catalytic function (see the effect of PPACK, Table 3), but not influenced by inhibitors of arachidonate metabolism (lack of effect of indomethacin on the direct effect of α-thrombin, Table 2). The recently cloned receptor for α-thrombin from megakaryocyte-like cell lines also requires a proteolytically active enzyme. A tetradecapeptide from the receptor N-terminal region, NAT_{14}, behaves as an agonist (Vu *et al.*, 1991). We show here that this peptide, a postulated stimulatory domain of α-thrombin receptor, also behaves as a contractile agonist on the rabbit aorta at concentrations similar to those used previously (Vu *et al.*, 1991). Therefore it seems that the cleavable receptor model of Vu *et al.* (1991) also applies to the myotropic effect of α-thrombin in the aortic system. Consistent with an effect of NAT_{14} on the receptors for α-thrombin, the contraction induced by this peptide was also potentiated by EGF pretreatment of the tissues. A noticeable difference between NAT_{14} and α-thrombin is that the effect of the former is not inhibited by PPACK. This is consistent with the model of Vu *et al.* (1991): the peptide is not a protease.

In human platelets, the action of α-thrombin on the cleavable receptor results in the activation of phospholipase C (Rittenhouse-Simmons, 1979), an event that could take place in the rabbit aorta. Proteolytically active α-thrombin exerts several effects on rat vascular smooth muscle cells, including the increased synthesis of DNA and proteins, increased cytosolic concentration of calcium, protein kinase C

activation and Na^+/H^+ exchange (Berk *et al.*, 1990).

The synergism between the contractile effects of EGF and of other agents is a novel mode of the vasomotor actions of growth factors. The relatively selective interaction between the effects of EGF and mediators that are formed during coagulation, namely kinins and α-thrombin, may occur in a wide range of vascular pathologies. For example, in partially occluded human coronary arteries (Fischell *et al.*, 1988), as well as in rat carotid arteries (Clowes *et al.*, 1983), chronic spasm may occur early after mechanical dilatation of the lumen with a balloon catheter.

Removal of the endothelium *in vivo* with a balloon catheter initiates a thrombogenic reaction and platelet adhesion at the rabbit aorta luminal surface (Hatton *et al.*, 1989). Although α-thrombin is a vasodilator in several vascular beds, such as the perfused coronary artery of the dog, the protease becomes a potent vasoconstrictor after the destruction of the endothelium (Ku *et al.*, 1987). Thus, the combination of α-thrombin and EGF-like material derived from platelets (e.g. TGF-α) may contribute to vasospasm, especially in vessels where the endothelium is absent. In contrast, pig isolated coronary artery incubated *in vitro* contracts in response to des-Arg^9-BK independently of the presence of the endothelium (Beny *et al.*, 1987). It is tempting to speculate that vascular injury *in vivo* may also lead to the expression of kinin B_1 receptors mediating vascular contraction and capable of interacting with receptors for EGF. Finally, this type of interaction may not be limited to mechanical responses and may extend to muscle proliferation in response to injury, with pathological remodelling of the vascular wall as a consequence (neointima formation; Schwartz *et al.*, 1990). This hypothesis will be tested by use of a balloon injury model.

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