Kallikrein rK10-induced kinin-independent, direct activation of NO-formation and relaxation of rat isolated aortic rings

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1 rK10, a weak T-kininogenase isolated from the rat submandibular gland, is a protein belonging to the rat kallikrein family. In the present work, we have studied the biological effects of rK10 with respect to its ability to alter vascular resistance, either directly like rK9, *i.e.*, another kallikrein-like protein, trypsin and thrombin, or through the release of kinins like tissue kallikrein (rK1). The direct effect was studied by its vasomotor activity on rat isolated aortic rings since this preparation was insensitive to the action of kinins. Its ability to induce altered vascular resistance through kinin-generation was investigated by blood pressure studies in whole animals. The studies were performed in comparison to rK1.

2 Unlike rK1, which induces hypotension when administered intravenously to rats ($\Delta BP = -56 \pm 5$ mmHg, $5 \mu\text{g kg}^{-1}$), rK10 did not have any effect on systemic blood pressure ($\Delta BP = -3 \pm 1$, $5 \mu\text{g kg}^{-1}$, *i.v.*)

3 rK10 was without effect on uncontracted aortic rings, but showed a concentration-dependent (10^{-8} – 10^{-6} M) relaxant effect on tissue precontracted with phenylephrine (10^{-6} M). After removal of endothelial cells, no relaxation was observed. The relaxant response to rK10 was transient. rK1 (with and without endothelium), bradykinin and T-kinin (with endothelium) had no effect on contracted or uncontracted aortic rings.

4 The relaxant effect of rK10 was dependent on its enzymatic activity since preincubation with aprotinin (1.02 mM) significantly reduced vasorelaxation from $74 \pm 4\%$ to $24 \pm 3\%$.

5 The relaxant effect was not inhibited by the kinin antagonist Hoe 140 (10^{-7} M; $34 \pm 4\%$ without, versus $30 \pm 2\%$ with Hoe 140), but was totally inhibited by the NO-synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (2.5×10^{-4} M; $27 \pm 3\%$ without and $2 \pm 1\%$ with L-NAME).

6 These results show that rK10 has the ability to induce vascular relaxation by a specific, direct effect on endothelial cell NO-synthesis, dependent on rK10 proteolytic activity, but independent of its ability to generate kinin. This effect, or its T-kininogenase activity in blood, was not sufficient for rK10 to have an effect on peripheral vascular resistance since intravenous injections of rK10, unlike rK1, did not induce hypotension. Thus, rK10 does not seem to play a role in blood pressure homeostasis but may have a local effect on vascular resistance.

Keywords: Kallikrein rK10; NO; kinin; isolated aortic rings of rat

Introduction

The submandibular gland (SMG) of the rat contains several enzymes belonging to the kallikrein family (Ashley & MacDonald, 1985; Berg *et al.*, 1992). The biological functions of these enzymes are largely unknown. Two have been shown to alter vascular resistance, *i.e.*, vasorelaxation by rK1 (tissue kallikrein) through the release of kinins (Erdős, 1966; Berg *et al.*, 1987), and vasoconstriction by rK9 through a direct effect on vascular smooth muscles (Yamaguchi *et al.*, 1991; Berg *et al.*, 1992). Also, other proteolytic non-kallikrein enzymes such as trypsin and thrombin have been shown to induce vasorelaxation through a direct effect on endothelium-derived relaxing factor (EDRF) release (Thomas & Ramwell, 1987; Johns *et al.*, 1987). Another enzyme of the kallikrein family, rK10 (Berg *et al.*, 1992), previously named antigen gamma (Berg *et al.*, 1987), endopeptidase K (Gutman *et al.*, 1988), proteinase B (Kato *et al.*, 1987), or T-kininogenase (Xiong *et al.*, 1990), so far found in the rat SMG only, resembles rK1 in its ability to release kinin. However, rK10 differs from rK1 in that it releases T-kinin (Ile-Ser-bradykinin) from T-kininogen (Okamoto & Greenbaum,

1983a; Barlas *et al.*, 1987; Gutman *et al.*, 1988; Berg *et al.*, 1991) a substrate present in the rat only (Gutman *et al.*, 1988), whereas rK1, in the rat, releases bradykinin from high and low molecular weight kininogen (Alhenc-Gelas *et al.*, 1981; Kato *et al.*, 1985). T-kinin, like bradykinin, contracts rat uterus and guinea-pig ileum, and induces hypotension (Okamoto & Greenbaum, 1983b). However, kinetic studies have shown that rK10 is not a very efficient kininogenase (Gutman *et al.*, 1988; Berg *et al.*, 1991) compared to human urinary kallikrein (Maier *et al.*, 1983), and its role in vasomotor regulation may be questionable. In the present study, we therefore wanted to characterize further the biological effects of rK10 with respect to its ability to alter vascular resistance, either directly like rK9, trypsin, and thrombin, or through the release of kinins, like rK1. Since it may be assumed that there will be a marked difference in substrate availability between *in vitro* and *in vivo* experiments, and since preliminary studies showed no effect of kinins on isolated aortic rings, the direct effect of rK10 was studied in isolated aortic rings, whereas the vasomotor activity through the presumed release of T-kinin, was studied by its ability to alter arterial blood pressure. The difference between a direct and kinin-mediated response was also tested by the use of a bradykinin-receptor antagonist.

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Methods

Effect of rK10 and rK1 on isolated aortic rings

Experimental set up Thoracic aortic rings (3 mm length) were obtained from male Wistar rats (250–350 g), killed by a sharp blow to the head. The rings were set up for isometric recording of force at 37°C in a modified Krebs-Henseleit buffer (composition, mM: Na⁺ 143, K⁺ 5.9, Ca²⁺ 0.25, Mg²⁺ 1.2, Cl⁻ 128, H₂PO₄⁻ 2.2, HCO₃⁻ 24.9, SO₄²⁻ 1.2, dextrose 10), and gassed with 95% O₂:5% CO₂. The low concentration of Ca²⁺ was chosen due to the observation of less noise in the phenylephrine concentration-response curve than with the 2.5 mM Ca²⁺ which is normally used, although with no loss of sensitivity to phenylephrine. The aortic rings were stretched with an initial preload of 3 g and washed four times with Krebs-Henseleit buffer over the following hour. The α -adrenoceptor agonist, phenylephrine was added, and contraction allowed to develop. The concentration of phenylephrine was established from a cumulative concentration-response curve where phenylephrine (10^{-8} – 10^{-3} M) was added until the contraction reached its maximum (100%). A $76 \pm 5\%$ ($n = 8$) contraction was obtained with a concentration of 10^{-6} M phenylephrine for aortic rings with intact endothelium. In subsequent studies this concentration of phenylephrine was used to precontract intact rings. Similarly, in denuded aortic rings, phenylephrine (10^{-6} M) gave a $80 \pm 2\%$ ($n = 13$) of maximum contraction, which was not significantly different from intact rings. In intact aortic rings treated with L-NAME (2.5×10^{-7} – 2.5×10^{-3} M) maximum contractile response to L-NAME was obtained at 2.5×10^{-4} M. From a cumulative concentration-response curve for phenylephrine performed on tissues pretreated with L-NAME (2.5×10^{-4} M), a dose of 3×10^{-7} M phenylephrine was found to produce $73 \pm 4\%$ ($n = 8$) of the maximum contraction (NS as compared to intact rings or denuded rings). In experiments where tissue was pretreated with L-NAME, 3×10^{-7} M phenylephrine was used for precontraction. The tension of phenylephrine-induced precontraction was set at 100% for calculation of relaxant or contractile response in the subsequent experiments. The preparations were then either washed 5 times in Krebs-Henseleit buffer over the next hour (uncontracted preparations) or maintained in a contracted state (contracted preparations). Additions of Krebs-Henseleit buffer amounting to 5% v/v of bath volume elicited no effect. Drugs were therefore added in a volume up to this limit. Removal of the endothelium was carried out before setting up the preparations by gentle rubbing of the intimal surface with a roughened 0.5 mm diameter stainless steel rod. At the end of each experiment, the muscarinic receptor agonist, 5-methylfurmethide (10^{-6} M) was added to test if the endothelium was intact. In rings with intact endothelium, concentration-response experiments showed that this concentration of 5-methylfurmethide produced a relaxation response of $26 \pm 3\%$, whereas in denuded rings, relaxation was not observed ($3 \pm 2\%$). Endothelium-independent relaxation was subsequently tested by addition of the β -adrenoceptor agonist, isoprenaline (4×10^{-6} M) which produced a $33 \pm 10\%$ relaxation. By choosing these concentrations of 5-methylfurmethide and isoprenaline, the integrity of endothelium and smooth muscle function in the preparation could be tested subsequent to the rK10-induced relaxation. The observed additional relaxation after addition of these two drugs following rK10, agreed with the relaxations obtained with 5-methylfurmethide and isoprenaline alone.

Experimental protocols After preparation of uncontracted and contracted aortic rings with and without endothelium as described above, rK10 or rK1 (10^{-8} , 10^{-7} , 3×10^{-7} and 10^{-6} M) was added in a cumulative manner at 5 min intervals, and any contractile or relaxant activity measured isometrically. The 5 min interval was shown in preliminary

studies to be sufficient to observe the full relaxant effect of rK10.

After an endothelium-dependent relaxant effect of rK10 had been established, further experiments were carried out on contracted aortic rings with the endothelium intact. To determine if the relaxant effect of rK10 was dependent on its enzymatic activity, rK10 was incubated with aprotinin for 30 min (22°C) before adding it to the organ baths, giving a final concentration of rK10 of 1.5×10^{-6} M and aprotinin of 1.02 mM ($n = 8$) (Berg *et al.*, 1987). Three sets of controls were used: rK10 similarly incubated with Krebs-Henseleit buffer ($n = 8$), Krebs-Henseleit buffer incubated with aprotinin ($n = 8$), and Krebs-Henseleit buffer only. When enzyme activity was tested in samples from the rK10- and the rK1 with aprotinin-incubates using the chromogenic substrate S-2266 as previously described (Berg *et al.*, 1985), rK10 enzyme activity inhibition was confirmed found ($96.3 \pm 0.1\%$ inhibition).

To determine if bradykinin or T-kinin induced a relaxant effect on contracted aortic rings with intact endothelium, bradykinin (3×10^{-8} – 2×10^{-6} M, $n = 8$) or T-kinin (10^{-7} – 10^{-6} M, $n = 8$) were added in cumulative manner at 5 min intervals.

In another set of experiments, the tissues were preincubated with the kinin antagonist Hoe 140 (10^{-7} M, $n = 8$) or Krebs-Henseleit buffer ($n = 8$), with the addition of rK10 (1.5×10^{-6} M) to all preparations 5 or 10 min later. The concentration of Hoe 140 was established in separate studies by its ability to inhibit kinin-induced uterine contraction.

The role of endothelium-derived relaxing factor (EDRF), later identified as nitric oxide (NO) (Ignarro *et al.*, 1987; Rees *et al.*, 1990), in the rK10-induced vasorelaxation was tested in similar experiments by addition of NO-synthase inhibitor (L-NAME; 2.5×10^{-4} M, $n = 8$) or Krebs-Henseleit buffer ($n = 8$), followed by the addition of rK10 (1.5×10^{-6} M) to all preparations.

The effect of rK10 on arterial blood pressure (BP) in comparison to rK1 Male Wistar rats (300–350 g) were anaesthetized with Nembutal (70 mg kg⁻¹, *i.p.*), and tracheotomized. BP was recorded continuously through a catheter in the femoral artery connected to a Statham pressure transducer and to a Hewlett Packard polygraph. After a control period of 5 min, a sham injection with PBS followed by rK10 ($n = 5$) or rK1 ($n = 4$) administered as bolus injections (0.15 ml) in increasing doses (0.05, 0.5, 2.5, 5.0 μ g kg⁻¹ in PBS) at 5 min intervals were given through a catheter in the femoral vein. After each dose, the catheter was flushed with 0.05 ml PBS.

Reagents

Pentobarbitone (Nembutal Sodium) was obtained from Abbott Laboratories, Chicago, IL, U.S.A.; Hoe 140 (D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]bradykinin) was a kind gift from Hoechst, Frankfurt/Main, Germany. 5-Methylfurmethide was obtained from Wellcome Research Laboratories, Langley Court, Beckenham, Kent, U.K.; phenylephrine hydrochloride and isoprenaline sulphate from Norsk Medisinaldepot, Oslo, Norway; T-kinin and bradykinin from Sigma Chemical Co., St. Louis, MO, U.S.A.; N^ω-nitro-L-arginine methylester hydrochloride (L-NAME) from Sigma Chem. Co., St. Louis, U.S.A.; aprotinin from Bayer AG, Leverkusen, Germany, and heparin from Apothekernes Laboratorium A.S., Oslo, Norway. The chromogenic substrate S-2266 (D-valyl-L-leucyl-L-arginine-4-nitroanilide) was from Kabi Diagnostica, Stockholm, Sweden. rK10 and rK1 were purified from the rat SMG as previously described (Berg *et al.*, 1987; Johansen *et al.*, 1987). All other chemicals were of analytical laboratory reagent grade. All drugs and enzymes were dissolved in phosphate-buffered saline (PBS; 0.01 M Na-phosphate, pH 7.4, 0.14 M NaCl).

Statistics

The results are expressed as mean values $\pm$ s.e.mean. Dose-dependency was tested by two-way analysis of variance (ANOVA). Differences between dose-response curves were tested by one-way ANOVA. When differences were observed, two-sample Student's *t* tests were used to test for differences between the groups at a particular dose. A *P* value less than 0.05 was considered significant.

Results

Effects of rK10, rK1, and kinins on isolated aortic rings

rK10 had no effect on uncontracted aortic rings but showed a concentration-dependent relaxant effect on phenylephrine-contracted tissues ($P < 0.0001$). At the highest concentration tested (10^{-6} M), the effect of rK10 induced a $40 \pm 6\%$ relaxation. When the endothelium was removed, the vessels failed to relax (Figure 1). The ability of the tissue to relax was confirmed at the end of the experiment, in intact vessels by 5-methylfurmethide ($58 \pm 7\%$ total relaxation) and in vessels

denuded of endothelium by isoprenaline ($64 \pm 9\%$ total relaxation). Typical recordings are shown in Figure 2. The relaxant effect of rK10 was transient. Tissue kallikrein (rK1) showed no effect on uncontracted tissue with endothelium, or contracted tissue with or without endothelium (Figure 1). Furthermore, no relaxant effect was observed in response to bradykinin or T-kinin with intact endothelium (Figure 1).

The relaxant effect of rK10 on contracted aortic rings was significantly reduced by aprotinin from $74 \pm 4\%$ to $24 \pm 3\%$ ($P < 0.0001$). Aprotinin alone induced a $6 \pm 3\%$ relaxation, which was not significantly different from that observed after addition of Krebs-Henseleit buffer.

The relaxant effect of rK10 on contracted aortic rings was not inhibited by the kinin antagonist Hoe 140. The relaxant effect of rK10 was $34 \pm 4\%$ without, versus $30 \pm 2\%$ with addition of Hoe 140 (5 min preincubation), and $23 \pm 6\%$ without, and $26 \pm 7\%$ with addition of Hoe 140 (10 min preincubation). Hoe 140 alone had no significant effect on vasomotor tone ($3 \pm 1\%$ versus $3 \pm 1\%$ for Krebs-Henseleit buffer).

The relaxant effect of rK10 was totally inhibited by L-NAME. The relaxation following rK10 alone was $31 \pm 6\%$ and after preincubation with L-NAME $2 \pm 1\%$ ($P < 0.0024$). Validation of the inhibitory effect of L-NAME on NO-dependent relaxation was provided by the observation that L-NAME subsequently inhibited the response to 5-methylfurmethide ($6 \pm 2\%$ and $53 \pm 9\%$, with and without L-NAME, respectively, $P < 0.0014$), but not the effect of

Figure 1 Concentration-response curves for the effect of rK1, rK10, bradykinin (BK) and T-kinin on isolated, contracted aortic rings of the rat. Contraction was developed by adding phenylephrine to the bath (10^{-6} M). In aortic rings with endothelium intact (E+), rK10 induced a significant dose-dependent relaxation ($n = 8$) whereas no effect was observed when the endothelium was removed (E-, $n = 8$). (●) rK10, E+; (▼) T-kinin, E+; (■) BK, E+; (□) rK1, E+ and E-; (▽) rK10, E-. Relaxation was not observed in response to rK1 with ($n = 8$) or without ($n = 8$) endothelium, or bradykinin or T-kinin with endothelium ($n = 6$).

Figure 3 Blood pressure dose-response curves for the effect of rK10 (●) or rK1 (▽) ($0.05, 0.5, 2.5, 5.0 \mu\text{g kg}^{-1}$ in PBS). rK1 induced a dose-dependent drop in BP ($P < 0.0001, n = 4$) significantly different from the nonsignificant response to rK10 ($n = 5$).

Figure 2 Typical recordings showing the effect of rK10 on contracted aortic rings with (E+) and without (E-) intact endothelium. Contraction was developed by adding phenylephrine to the bath (10^{-6} M). The concentration of rK10 added is indicated above the trace. Addition of 5-methylfurmethide (5-MeFur) (10^{-6} M) and isoprenaline (4×10^{-6} M) demonstrated the ability of the tissue to respond with endothelium-dependent and endothelium-independent relaxation, respectively.

isoprenaline ($84 \pm 3\%$ and $92 \pm 5\%$, with and without L-NAME, respectively, $P < 0.36$).

The effect of rK10 and rK1 on BP

rK1 induced a dose-dependent hypotensive response with a maximum fall of 56 ± 5 mmHg at maximum dose ($5 \mu\text{g kg}^{-1}$, i.v.) ($P < 0.0001$, $n = 5$). Injections of rK10 (0.05, 0.5, 2.5, $5.0 \mu\text{g kg}^{-1}$) did not induce a hypotensive response ($n = 4$) (Figure 3).

Discussion

In the present study, a direct endothelium-dependent vasodilator effect of the kallikrein-like enzyme rK10 on isolated contracted aortic rings was demonstrated. The relaxant effect was found to be dependent on rK10 enzyme activity since preincubation with aprotinin, an inhibitor of rK10 (Berg *et al.*, 1987), significantly reduced relaxation. Some variation in the magnitude of the relaxant response to rK10 was observed throughout the different experiments. These differences were explained by variations in degeneration of the enzyme during storage, since the enzyme activity measurements (S-2266 chromogenic enzyme assay) of the two different rK10-preparations used, showed that variations in the relaxant effect corresponded to variations in specific enzyme activity. The relaxant effect of rK10 on isolated contracted aortic rings was followed by a small contraction. Since rK10 did not have any contractile response on uncontracted vascular rings, this relaxant response was viewed as a transient effect and not as a biphasic response.

Since endothelial denudation and NO-synthase inhibition has been shown to augment phenylephrine-induced contractions (Umans *et al.*, 1993) a comparable phenylephrine-precontractile state (about 75% of maximum contraction) was established for denuded and L-NAME-treated tissue by concentration-response experiments. However, we found no difference in sensitivity to phenylephrine between aortic rings with or without endothelium. On the other hand, after pretreatment with L-NAME, it was necessary to reduce the concentration of phenylephrine by 3.3 times to obtain the desired precontraction tension. This modification ensured that the inhibitory effect of L-NAME was not induced by having reached a supramaximal contraction through the contractile effect of L-NAME, but was indeed due to NO-synthase inhibition.

Two lines of evidence strongly suggest that the relaxant effect of rK10 was not mediated through the formation of kinins. First, the rat aortic rings did not respond to agonists such as bradykinin, T-kinin, or the efficient kininogenase rK1, and second, the relaxant effect was not inhibited by the B_2 kinin receptor antagonist, Hoe 140. Instead, the relaxant effect of rK10 was found to be inhibited by the NO-synthase inhibitor L-NAME, thus indicating that rK10 is mediated

through NO-production. The effect of rK10 appeared to be specific for this particular protein, since a similar effect was not observed for the closely related enzyme tissue kallikrein, rK1. The direct enzymatic effect on vascular tension corresponds to that observed for another enzyme from the rat SMG, *i.e.*, rK9, which has been shown to induce a small, but statistically significant concentration-dependent endothelium-independent vasoconstriction of isolated, uncontracted aortic rings. rK9-induced vasoconstriction did not involve the release of angiotensin II (Yamaguchi *et al.*, 1991; Berg *et al.*, 1992) and may indicate another vasomotor reaction due to direct enzymatic activation of smooth muscle receptors. It is possible that enzymes such as rK10 and rK9, through their proteolytic activity may be responsible for a direct conformational change of membrane receptor(s) leading to the activation of NO-synthesis for rK10 or activation of unidentified receptor(s) in smooth muscle cells for rK9.

At the present time we do not know whether the direct vasomotor effect of rK10 is restricted to large arteries, such as the aorta, or whether, resistance vessels show the same reaction. However, the lack of a depressor response after i.v. injections of rK10, may indicate a limited activation, since rK10, unlike rK1, did not have an effect on systemic BP. This is in agreement with previous results where rK10 was found to be a rather inefficient T-kininogenase, (Gutman *et al.*, 1988; Berg *et al.*, 1991) compared to the high/low-molecular kininogenase rK1 (Maier *et al.*, 1983). This may explain the lack of a systemic effect of rK10, in spite of the fact that T-kininogen is the main kininogen in rat plasma (Okamoto & Greenbaum, 1983b). The BP-response for rK1 and rK10 was compared only for similar doses of the two enzymes, since rK10 so far has been detected only in the rat SMG where it is present in approximately the same order of concentration as rK1 (2% and 8% of total gland protein, respectively, Berg *et al.*, 1987). Furthermore, since it may be expected that rK10 will gain access to the vascular compartment in the same way as rK1 (Rabito *et al.*, 1982; Berg *et al.*, 1985), there is no reason to expect that rK10 should enter the blood stream in a higher concentration than rK1. Thus, since the concentration injected induced a large hypotensive response to rK1, we conclude that rK10 does not play a role in BP homeostasis either through a direct effect on vascular resistance or through an ability to generate T-kinin.

In conclusion, the present study demonstrates that in contrast to rK1, rK10 did not have a general effect on blood pressure, but induced a relaxation of isolated aortic rings precontracted with phenylephrine. Furthermore, the relaxation was dependent on endothelial cells and was abolished by NO-synthase inhibition, but was not dependent on the generation of kinin in spite of the fact that rK10 previously has been shown to act as a T-kininogenase. A similar direct effect has also been observed for other proteolytic enzymes on endothelial cells and smooth muscle cells.

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