Characterization of Gaddum's substance R

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- 1 When the isolated small intestine of the rat is perfused via the mesenteric artery, an oxytocic principle (Gaddum's substance R) is released which is detectable in the perfusate after 30 min and is present in samples collected 8 h later.
- 2 The oxytocic activity of substance R is lost after boiling but is unaffected by treatment with thioglycolate. Furthermore, atropine, methysergide and indomethacin failed to antagonize uterine contractions to substance R.
- 3 Neither substance R nor urinary kallikrein alone induce a contraction of the guinea-pig isolated ileum. However, in the presence of kininogen both substance R and urinary kallikrein produce a slow and prolonged contraction of the guinea-pig ileum.
- 4 The oxytocic and kininogenase properties of both substance R and urinary kallikrein are inhibited by Trasylol.
- 5 Soy bean trypsin inhibitor (SBTI) selectively inhibited both the oxytocic and the kininogenase activities of substance R but not those of urinary kallikrein.
- 6 Gel filtration of substance R resolved a single peak of oxytocic activity with an estimated molecular weight of 40 kDa.
- 7 We conclude that substance R is a kininogenase enzyme which may be distinguished from plasma kallikrein by its molecular weight and from urinary kallikrein by its susceptibility to SBTI. The exact nature of this enzyme remains to be elucidated.

Keywords: Substance R; kallikrein; perfused small intestine; kininogenase

Introduction

In 1953 Gaddum published a highly cited paper in which he described for the first time, the superfusion technique. Less well-known is that in this paper Gaddum also reported the production by the perfused rat intestine of a factor with oxytocic activity. The results he obtained suggested that this factor was a protein. We have recently confirmed these observations (Douglas *et al.*, 1988). Furthermore, we observed that the spectrum of pharmacological actions of 'substance R' was similar, but not identical, to those of a tissue kallikrein.

Methods

Collection of substance R

Male Wistar rats (200–300 g) were first anaesthetized with ether, and heparin (1000 u) was administered by a tail vein to prevent clotting during the perfusion. Following laparotomy the small intestine was ligated at the duodenal level, distal to the pancreas and also at the ileo-caecal junction. The superior mesenteric artery was cannulated and all other vessels, with the exception of the hepatic portal vein, were tied off. The intestine was sectioned anterior to the duodenal ligature and distal to the caecal ligature. The intact mesenteric bed was then removed, care being taken to exclude all pancreatic tissue, suspended in a heated jacket and perfused with oxygenated Tyrode solution (37°C, 0.4 ml min⁻¹) through the mesenteric artery. Effluent from the hepatic portal vein was either collected for consecutive 20 min periods over 6 h and aliquots of each fraction were assayed for oxytocic activity, or, after a 30 min equilibration period, perfusate, collected over 6 h, was pooled, lyophilized and stored at –20°C. For preparative purposes pooled perfusates from three experiments were used.

The pooled perfusates from three such preparations were then dialysed overnight against 10 volumes of ammonium bicarbonate (50 mM, pH 7.8). The high molecular weight residue was freeze-dried and stored at –20°C. The lyophilized perfusate from nine intestinal preparations was redissolved in 6 ml of Trisbuffer (50 mM, pH 7.5) and filtered before application to the ion-exchange column.

Assay of oxytocic activity

Segments of stilboestrol-primed rat uterus superfused with de Jalon's solution (30°C, 5 ml min⁻¹) were used for the assay of oxytocic activity of samples. Volumes of between 10 and 150 µl were injected into the stream of superfusion fluid above the tissue.

Assay of kinin-releasing activity

The kinin-releasing activity of substance R and kallikrein was assayed on the guinea-pig isolated ileum. In these experiments a 2 cm length of ileum was suspended in an 8 ml organ bath and bathed in oxygenated Tyrode solution containing captopril (100 µg ml⁻¹ at 37°C). Bradykinin was used as a standard.

Ion-exchange chromatography

Ion-exchange chromatography of the soluble intestinal extract was performed by use of DEAE-Sephacrose. The sample was applied to the column (2.6 × 30 cm), equilibrated in Tris buffer (50 mM, pH 7.5). Fractions of 5 ml were eluted at a flow rate of 0.4 ml min⁻¹ by a linear salt gradient of 0–1 M sodium chloride and assayed for oxytocic activity before the absorbance at 280 nm was measured. Fractions containing oxytocic activity were then pooled, dialysed against 50 volumes of ammonium bicarbonate (50 mM, pH 7.8) for 12–18 h and lyophilized.

Gel filtration

Gel filtration of the oxytocic principle was carried out routinely on a calibrated Sephacryl S-300 column (2.6 × 90 cm). The column was equilibrated with phosphate-buffered saline

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(50 mM, pH 7.5 (PBS) and 5 ml of soluble intestinal extract was applied to the column. The sample was eluted at a flow rate of 0.4 ml min^{-1} and 5 ml fractions were collected. Fractions were then assayed for oxytocic activity and the absorbance at 280 nm measured.

Estimation of the molecular weight of the oxytocic principle was made on a Sephacryl S-200 column ($2.6 \times 90 \text{ cm}$), calibrated with molecular weight markers, under the conditions described above and the fraction containing the peak oxytocic activity was determined by assay on the superfused isolated uterus of the rat.

Urinary kallikrein

Initially, crude preparations of rat urinary kallikrein were made by desalting rat urine on a Sephadex G-25 (PD-10) column equilibrated with PBS. Later, for comparative purposes, rat urinary kallikrein was prepared from urine collected overnight from male Wistar rats. The urine from individual rats was pooled and filtered before an overnight dialysis against PBS. The dialysed sample was then stored at -20°C . A partially purified preparation of urinary kallikrein was obtained by the same preparative and assay techniques employed in the isolation of substance R.

Materials

Reagents and drugs were obtained from the following sources: aprotinin (Trasylol), (Bayer UK Ltd); H-D-Val-L-Leu-L-Arg-p-nitroanilide dichloride (S-2266), (Kabi Vitrum), D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin, (Novabiochem); PD-10 columns, Sephacryl S-200, Sephacryl S-300 and DEAE-Sephacryl (Pharmacia Ltd); methysergide hydrogen maleate, (Sandoz UK Ltd), atropine sulphate, bradykinin, carbamylcholine, α - and δ -chymotrypsin, diethylstilboestrol, heparin sulphate, indomethacin, soy bean trypsin inhibitor (SBTI), thioglycolic acid, Trizma base, Trizma hydrochloride, and trypsin, (Sigma); captopril, (Squibb). Human plasma kininogen was kindly donated by Dr K.D. Bhoola, University of Bristol.

Statistical analysis

Data are expressed as mean $\pm$ s.e.mean. Statistical significance was computed by the paired *t* test or analysis of variance (ANOVAR) where appropriate.

Results

The effluent from perfused rat small intestines was found to contract the superfused rat uterus in a dose-dependent manner. Contractions were delayed in onset (30 s) when compared to those produced by carbachol or bradykinin. When consecutive fractions of perfusate were collected, at least 30 min elapsed before oxytocic activity was detectable (Figure 1). However, oxytocic activity was released in increasing amounts thereafter, reaching a maximum concentration at 3–4 h, which was sustained for a further 2–3 h. Protein concentration, estimated by the Lowry-Folin method (Lowry *et al.*, 1951), remained between $1\text{--}2 \text{ mg ml}^{-1}$ throughout the period of perfusion with no correlation between protein concentration and oxytocic activity.

The characteristics of the oxytocic principle isolated are very similar to those described by Gaddum (1953) for substance R. As Gaddum himself observed, these properties are similar to those of (then recently described) kallikrein. To compare the activities of the two substances further, we used crude preparations of rat urinary kallikrein as a source of tissue kallikrein, and compared its properties with those of substance R.

The oxytocic principle from the intestinal perfusate was non-dialysable (mol. wt. cut off $10\text{--}15 \text{ kDa}$) and oxytocic activ-

ity was not inhibited by addition of atropine (10^{-6} M), methysergide ($5.7 \times 10^{-7} \text{ M}$) or indomethacin ($1 \times 10^{-5} \text{ M}$) to the superfusate. Samples of perfusate possessing substantial oxytocic activity were inactive when tested on the isolated superfused guinea-pig ileum or rat fundic strip preparations. The perfusate lost oxytocic activity on heating but was unaffected by treatment with thioglycolate which inactivates oxytocin (Ames *et al.*, 1950). The material was stable when frozen at -20°C for at least 8 weeks.

Trypsin and chymotrypsin contract the superfused rat uterus in a similar way to substance R (Gaddum, 1953). Gaddum (1953) found that a preparation of substance R was more active, weight for weight, on the rat uterus than crystalline preparations of trypsin or chymotrypsin. It has since been shown that both trypsin and chymotrypsin sensitize certain smooth muscle preparations to bradykinin (Aarsen, 1968; Edery & Grumfeld, 1969). In the present study it was found that trypsin, α -chymotrypsin and δ -chymotrypsin induced sensitization to bradykinin of both the superfused rat uterus and guinea-pig ileum. However, in each of three experiments, substance R and urinary kallikrein failed to induce sensitization to bradykinin in either of these preparations.

To investigate further the similarity between substance R and tissue kallikrein, the effect of inhibitors of kallikrein on the oxytocic activities of substance R and urinary kallikrein were studied. Addition of the dual plasma and tissue kallikrein inhibitor aprotinin (Trasylol, $50\text{--}100 \text{ Kiu ml}^{-1}$), to the fluid superfusing the isolated uterus completely inhibited the activity of both substance R and urinary kallikrein in six experiments (Figure 2). However, the plasma kallikrein inhibitor, soy bean trypsin inhibitor (SBTI, $100 \mu\text{g ml}^{-1}$), completely inhibited the action of substance R on the isolated uterus ($99.4 \pm 0.3\%$) whereas the oxytocic activity of urinary kallikrein was only reduced by $12.6 \pm 2.5\%$ ($n = 6$) (Figure 3). A reduction of $9.9 \pm 1.8\%$ and $3.0 \pm 0.8\%$ was seen in response to bradykinin and carbachol respectively ($n = 4$). The inhibition of the oxytocic activity of substance R by SBTI was significant ($P < 0.01$, ANOVAR).

Both urinary kallikrein and substance R were found to contract the guinea-pig isolated ileum in the presence of crude human plasma kininogen. Use of the selective bradykinin receptor antagonist, D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin (Regoli *et al.*, 1986; Schachter *et al.*, 1987) inhibited the contractions induced by substance R plus kininogen indicating that the generated factor was a kinin. The kinin releasing activity was assayed on the guinea-pig isolated ileum in the

Figure 1 Appearance of oxytocic activity in perfusate from the mesenteric bed. The contractions of the superfused rat uterus induced by $100 \mu\text{l}$ aliquots of intestinal perfusate from samples collected over consecutive 20 min periods. No oxytocic activity was detectable during the first 20 min of perfusion, but thereafter the samples contained increasing amounts of activity, reaching a maximum concentration at 3–4 h. The lower time scale refers to the time of collection of the perfusate.

Figure 2 The effect of aprotinin on the oxytocic activities of urinary kallikrein (UK), substance R (R) and bradykinin (BK). Aprotinin completely inhibited uterine contraction to both kallikrein and substance R but did not affect responses to bradykinin.

Figure 3 The effect of soy bean trypsin inhibitor (SBTI) on the oxytocic activities of urinary kallikrein (UK), substance R (R) and carbachol (CCh). SBTI reversibly inhibited the response to substance R without a significant reduction in the response to kallikrein or carbachol.

Figure 4 The kininogenase activity of substance R. (a) Addition of substance R (R) alone to guinea-pig isolated ileum elicited no response. However, when kininogen (K) was added, followed by substance R, a slow contraction was seen which was blocked by the presence of soy bean trypsin inhibitor (SBTI). (b) Urinary kallikrein (UK) induced no effect unless kininogen was also present. The contraction induced by kininogen plus urinary kallikrein was not markedly inhibited by SBTI.

Figure 5 Ion-exchange chromatography of crude intestinal perfusate on DEAE-Sephacrose. The sample was applied at $V = 0$ and was washed with 100 ml Tris buffer (50 mM, pH 7.5). Substance R was eluted with a linear salt gradient at approximately 0.2 M NaCl indicated by the solid bar above the elution profile. The absorbance of fractions was measured at 280 nm and oxytocic activity determined on the superfused rat uterus.

presence of captopril (4.6×10^{-4} M). The ileum was unresponsive to either kallikrein, substance R, or kininogen alone (Figure 4a and b). However, in the presence of kininogen, addition of kallikrein or substance R to the bath resulted in kinin liberation (Figure 4a). Aprotinin (100 Kiu ml^{-1}) completely inhibited the kinin-releasing activity of both urinary kallikrein and substance R ($n = 3$). In contrast, SBTI ($100 \mu\text{g ml}^{-1}$) produced a small inhibition of the kinin liberation by urinary kallikrein of $13.2 \pm 6.3\%$ but significantly ($P < 0.001$, paired t test) reduced the release of kinin by substance R by $80.4 \pm 5.0\%$ ($n = 6$) (Figure 4b).

When the selective BK_2 bradykinin receptor antagonist D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin was added to the superfusate (1×10^{-7} M), oxytocic responses to bradykinin, kallikrein and substance R were reduced by $63.8 \pm 10.7\%$, $69.5 \pm 11.7\%$, $85.9 \pm 8.6\%$, respectively ($P < 0.01$, ANOVA) ($n = 8$). Responses to carbachol were not reduced in the presence of the antagonist.

Chromatography of crude intestinal perfusate preparations on DEAE-sephacrose resulted in elution of a single peak of activity with approximately 0.2 M NaCl (Figure 5). When the active ion-exchange fractions were applied to the Sephacryl S-200 column, a single peak of oxytocic activity was resolved with a molecular weight of approximately 40 kDa (Figure 6). There was no detectable oxytocic activity in fractions either side of this peak. Preliminary studies with SDS-PAGE confirm this estimate of molecular weight. Urinary kallikrein was found to have very similar chromatographic properties to substance R.

The kininogenase activity of fractions eluted from the Sephacryl column was determined by hydrolysis of the chromogenic substrate S-2266 using the endpoint method. A single peak of kininogenase activity was found which coincided with that of oxytocic activity.

Discussion

Perfusion of the rat isolated small intestine resulted in the liberation of a non-dialysable oxytocic principle which Gaddum

Figure 6 Gel filtration on Sephacryl S-200 of pooled oxytocic fractions previously subjected to ion-exchange chromatography. The sample was applied at $V = 0$ and was eluted with 50 mM PBS, pH 7.5. The absorbance of fractions was measured at 280 nm and the elution profile is shown by the solid line. Oxytocic activity of the eluted fractions was determined on the isolated superfused uterus of the rat, indicated by the dashed line, and is expressed as % maximum contraction per μ l eluate. The molecular weight of the substance R peak (●) corresponded to 40 kDa when compared to calibrating proteins (○).

(1953) named substance R. Release of substance R from the perfused intestine increased with time from 20 min to approximately 3 h after which the oxytocic activity of samples collected remained high and fairly constant. Contraction of the rat uterus by substance R was unaffected by atropine, methysergide or by treatment with thioglycolate. However, boiling samples of substance R for 3–4 min resulted in a substantial loss of activity. These results are in agreement with the observations of Gaddum (1953).

Substance R and tissue kallikrein share a number of properties, as Gaddum himself remarked (1953). Our results with the guinea-pig isolated ileum support this, as kallikrein and substance R only induce contraction of the ileum when kininogen is also present, resulting in the generation of a kinin.

The polyvalent protease inhibitor, aprotinin (Trautschold *et al.*, 1967) was found to inhibit the oxytocic responses of the

rat uterus to both substance R and urinary kallikrein. However, SBTI, which selectively inhibits plasma kallikrein (Werle & Maier, 1952; Back & Steger, 1968) blocked responses of the uterus to substance R without affecting those to urinary kallikrein. The release of kinin from kininogen by substance R is likewise inhibited by SBTI and aprotinin, whereas kinin release by urinary kallikrein was inhibited by the latter only. These results suggest that substance R possesses kininogenase activity but is unlikely to be a tissue kallikrein. An attempt was therefore made to purify and further characterise substance R.

Gel filtration chromatography of the oxytocic principle obtained from ion exchange chromatography of crude intestinal perfusate resolved a single peak of biological activity with a molecular weight corresponding to approximately 40 kDa. Preliminary experiments with SDS-PAGE confirm this estimate. As plasma kallikreins have been reported to possess a molecular mass of over 100 kDa (see Movat, 1979; Schachter, 1980) it is unlikely that substance R is a plasma kallikrein. The site of release of substance R from the perfused intestine is at present unknown. The presence of kininogenase in rat vascular tissues has previously been reported (Nolly & Lama, 1982; Nolly *et al.*, 1985; 1986). However, when the arterial vasculature of the mesentery alone was perfused, none of the 20 min fractions collected over 6 h contained oxytocic activity. The kininogenase reported by Nolly and colleagues was not inhibited by SBTI. It thus seems probable that substance R is released from the intestinal tissue during perfusion, rather than the arterial vasculature (unpublished data). The physiological significance of substance R in the gut is not fully understood but is the subject of further investigation.

It has recently been demonstrated that contraction of the rat isolated uterus by glandular kallikrein is dependent on the presence of kininogen within the uterine tissue (Figueiredo *et al.*, 1990). This supports the evidence presented by Orce *et al.* (1989) who suggested that the oxytocic activity of glandular kallikrein is mediated, at least in part, by kinins generated from uterine kininogen. In the present study uterine contractile responses to both urinary kallikrein and substance R were inhibited by the kinin antagonist D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin. There was no significant difference between the inhibition of responses to bradykinin, urinary kallikrein or substance R by this antagonist, suggesting that the oxytocic activity of each is mediated by the BK₂ kinin receptor. It is proposed that substance R shares with glandular kallikrein the same mechanism in the contraction of the rat isolated uterus.

We conclude that substance R is a proteolytic enzyme with a molecular weight of approximately 40 kDa which is biochemically related to the tissue kallikrein family of enzymes but has an anomalous sensitivity to SBTI. The reasons for this discrepancy require further elucidation.

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