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Kallikrein-induced uterine contraction independent of kinin formation

(serine proteinases/smooth muscle/kininogen/kininogenases/kinin antibodies)

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ABSTRACT **Responses of smooth muscle to kallikreins (EC 3.4.21.8) are generally considered to result from kinin formation. This premise was reexamined with the isolated rat uterus. Rat urinary kallikrein or bradykinin produced dose-dependent contractions of rat uterus but kallikrein was 5-fold more potent than bradykinin. Kallikrein caused an immediate series of rythmic contractions which could be increased gradually with subsequent addition of kininogen substrate. Kallikrein-induced contractions were unaffected by carboxypeptidase B or a bradykinin antiserum whereas bradykinin-induced contractions were attenuated or abolished. Other serine proteinases, including trypsin, either did not induce contraction in the absence of added kininogen or did so minimally. Although small amounts of kininogen-like substrate were found in uterine tissue, detectable kinin levels (>4 pg) could not be found in bathing media during maximal kallikrein-induced contractions or after uterine tissue was incubated with high concentrations of the enzyme in the presence of SQ 20881, a kininase II inhibitor. The data suggest that uterine contraction produced by a homologous kallikrein does not involve kinin formation but results from an action of this serine proteinase upon other accessible systems coupled to the contractile response.**

Glandular kallikrein (EC 3.4.21.8) liberates lysylbradykinin (kallidin) from kininogen substrates by limited proteolysis. Although this serine proteinase can attack proinsulin or prorenin *in vitro* (1, 2), responses of smooth muscle to this kallikrein are attributed to kinin formation and a subsequent peptide–receptor interaction (3–5). However, Beraldo (6, 7) reported that rat glandular kallikrein produced rat uterine contractions in the absence of added kininogen or after pretreatment with actinomycin D or puromycin. It was suggested, but not shown, that these agents might have inhibited endogenous kininogen synthesis, and thus the effects of kallikrein were "direct." Conversely, the uterine contractile response to glandular kallikrein was suggested to depend on a detected kininogen substrate in the tissue (8, 9).

In the present study we have found that rat glandular kallikrein can cause contraction of the rat uterus without detectable kinin liberation.

MATERIALS AND METHODS

Materials. Rat urinary kallikrein B was purified to homogeneity with ammonium sulfate fractionation and chromatography on DEAE-cellulose, CM-cellulose, and Sephadex G-100. "Vertical slab" polyacrylamide gel electrophoresis produced a single band after Coomassie blue staining. The preparation was also homogeneous when analyzed by polyacrylamide isoelectric focusing (10). An antiserum to pure kallikrein B was raised in a sheep (10). Human urinary kallikrein was purified to homogeneity with a modification of the scheme described for rat urinary kallikrein and with aprotinin-agarose affinity chromatography as described (11). Another rat urinary alkaline esterase with kininogenase activity, called rat urinary esterase A (12, 13), was separated from rat urinary kallikrein by DEAE-cellulose column chromatography at pH 7.0 and further purified by aprotinin-agarose affinity and Sephacryl S-200 column chromatography (12).

Trypsin (bovine pancreas, type III, twice crystallized) and 17β-estradiol were purchased from Sigma; carboxypeptidase B was from Calbiochem–Behring. Bradykinin was obtained from the Peptide Institute–Protein Research Foundation (Osaka, Japan) and its antiserum was prepared as described (14). Kininogen substrate was obtained from dog plasma treated for 1–3 hr at 60°C as described (15).

Isolated Rat Uterus Preparation and Bioassay. Virgin female Sprague–Dawley rats (100–150 g) were injected subcutaneously with 17β-estradiol (0.2 mg/ml in 95% ethanol) at 200 μg/kg 24 hr prior to sacrifice by cervical dislocation. The uterine horns were removed into ice-cold de Jalon's solution (containing the following [g/liter]: NaCl, 9; KCl, 0.42; glucose, 0.5; $NaHCO_3$, 0.5; and $CaCl_2$, 0.03). The mesentery was removed and horns were trimmed to 2 cm long. They were attached by a central suture to a tissue hanger and placed in a double-walled bath containing 10 ml of de Jalon's solution and aerated with 95% O_2/5% CO_2 at 27°C. After a 1-hr equilibration period, tension was readjusted to 1 g and isometric responses were recorded with a Grass force displacement transducer (FTO3C) and polygraph.

Tissue Kininogen and Generated Kinin. Uterine kininogen concentration was measured with a modification of the method of Diniz and Carvalho (16). Rat uterine horns were removed, freed of fat, weighed, minced, and homogenized in 10 ml of ice-cold Tyrode's solution with an all-glass conical homogenizer and subsequently with a Polytron PT ST (Brinkmann) for 15 sec at maximum setting. The homogenates (10 ml) were treated with 0.5% deoxycholate for 30 min at room temperature and then centrifuged at 20,000 × *g* for 30 min. The supernatant was then desalted through Sephadex G-25 via centrifugation (17). Protein concentration was measured by the procedure of Lowry *et al.* (18) with bovine serum albumin as the standard.

Uterine extracts were divided into three groups: untreated, heat-treated (60°C, 60 min), and boiled (100°C, 10 min). A sample (0.2 ml) of each extract was added to 0.2 ml of 0.1 M phosphate, pH 8.5/30 mM sodium EDTA/3 mM 1,10-phenanthroline plus 0.05 ml of trypsin (0.9 μM), rat urinary kallikrein (30 nM), human urinary kallikrein (5 nM), rat urinary esterase A (30 nM), or H_2O; the final volume was made 0.5 ml. Reaction mixtures without uterine homogenates or enzymes were used as blanks. After incubation at 37°C for 60 min, reactions were stopped by boiling for 20 min. Kininogen was measured by radioimmunoassay of generated kinins (14) with bradykinin as the standard. In addition, any *in situ* kinin-generating activity of

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kallikreins was measured by kinin radioimmunoassay of bath media during contractile responses or after uterine strips were incubated with supramaximal enzyme concentrations in the presence of kininase II inhibition (SQ 20881, 100 μg/ml).

RESULTS

The typical and prompt dose-dependent contractile responses of the rat uterus to kallikrein or bradykinin are shown in Fig. 1 (*Upper*). The cumulative dose–response curves to kallikrein and bradykinin at concentrations ranging from 9.0×10^{-11} to 1.4×10^{-8} M. Calculated molar concentrations of kallikrein required to produce 1.0- and 1.5-g increases in contractile force were one-fourth and one-sixth the concentrations of bradykinin required to produce equivalent responses.

Kallikrein typically produced rapid and rhythmic contractions followed by spontaneous and full relaxation (Fig. 2). Subsequent addition of kininogen resulted in more rapid contractile responses of augmented force with incomplete relaxation. Kininogen alone never induced uterine contraction (data not shown). Because the kallikrein preparation used for the studies was free of Mg^{2+}, the observed rhythmic contractions were induced by kallikrein and not by contaminating magnesium.

Carboxypeptidase B [peptidyl-L-lysine (L-arginine) hydrolase, EC, 3.4.17.2], which degrades kinins, did not alter contractile responses to kallikrein but abolished those to bradykinin (Fig. 3). Bradykinin responses were restored after removal of carboxypeptidase B.

A bradykinin antiserum (1:20 to 1:20,000 dilution) was incubated with either bradykinin (0.2 μM) or kallikrein (60 μM) at 37°C for 1 hr prior to addition of 0.1-ml aliquots to the 10-ml organ bath. There was a dose-dependent inhibition of contractile responses to bradykinin by antiserum, with a 50% reduction of response at a 1:2000 antiserum dilution (final bath dilution, 1:200,0000) (data not shown). Responses to kallikrein were never affected by preincubation with bradykinin antiserum. Preimmune serum had no effect on uterine contractions produced by either bradykinin or kallikrein. Kallikrein-induced contractions were not affected by bradykinin antiserum added to the organ bath, but bradykinin-induced contractions were significantly reduced (Fig. 4). Bradykinin responses were restored after removal of antiserum.

Responses to other serine proteinases known to liberate kinins from kininogen substrates were studied. Human urinary kallikrein (11), rat urinary esterase A (12), and trypsin produced the typical uterine contractions when kininogen was preincu-

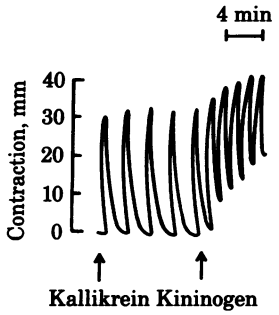

FIG. 2. Effect of added kininogen on kallikrein-induced contractions in the uterus. Kallikrein (0.6 nM) causes repetitive contractions followed by full relaxation. Addition of 0.1 ml of dog plasma kininogen results in a further and time-dependent increase in contractility.

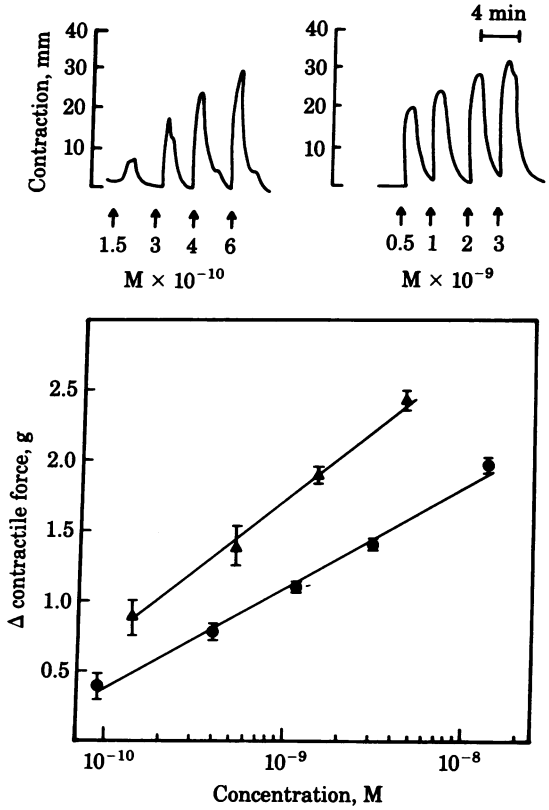

FIG. 1. (*Upper*) Typical dose–response relationships for kallikrein (*Left*) and bradykinin (*Right*) in the rat uterus. In these studies, uteri were washed with fresh medium as soon as the contractile response reached its peak. (*Lower*) Dose–response curves for kallikrein (▲) and bradykinin (●). Data are shown as mean ± SEM (n = 4).

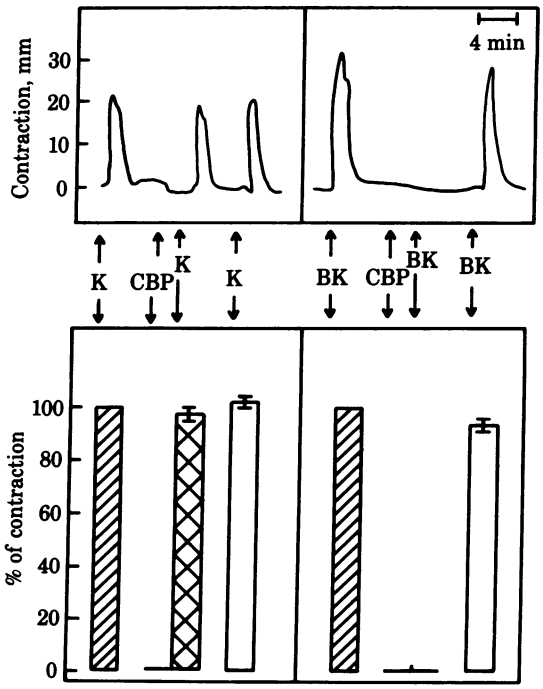

FIG. 3. Effect of carboxypeptidase B on kallikrein- and bradykinin-induced contraction in the rat uterus. (*Upper*) Typical tracings of contractions produced by kallikrein (K) or bradykinin (BK) before, during, and after addition of carboxypeptidase B (0.37 unit/ml, CBP). As soon as the contractile response reached its peak, the bath fluid was removed and the tissue was washed with fresh medium. (*Lower*) Contractile responses as mean (±SEM) percentage of control before (▨), during (▩), and after (□) addition of carboxypeptidase B (n = 7).

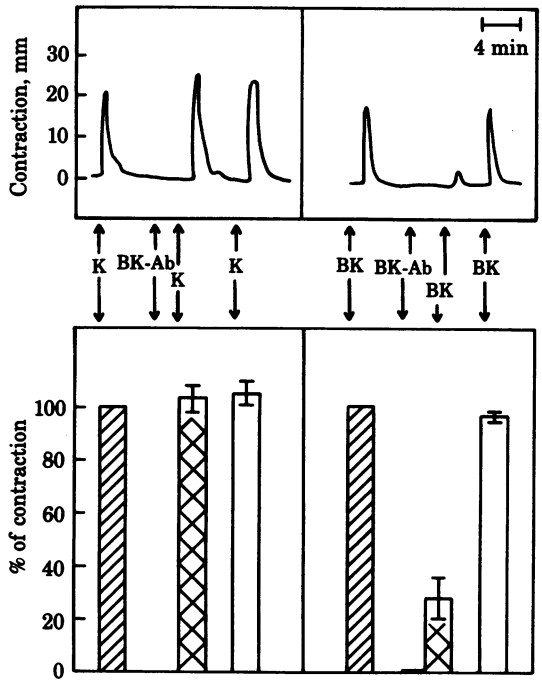

FIG. 4. Effect of bradykinin antiserum on kallikrein- and bradykinin-induced contraction in the rat uterus. (*Upper*) Typical tracings of contractions produced by kallikrein (K) or bradykinin (BK) before, during, and after addition of bradykinin antiserum (BK-Ab) (1:2000, final bath dilution). Uteri were washed with fresh medium as soon as the contractile response reached its peak. (*Lower*) Contractile responses as percentage of control before (▨), during (⊠), and after (□) addition of the antiserum ($n = 6$).

bated with the enzyme, and these responses always were abolished by carboxypeptidase B (0.37 unit/ml). However, these proteases alone either did not cause uterine contraction or produced minimal responses (<10% of that seen with rat kallikrein) in concentrations up to 50-fold greater than that of rat kallikrein (Table 1).

The role of endogenous kininogen in the contractile response to kallikrein was assessed by measurement of uterine homogenate kininogen content and enzyme-induced kinin release. Released kinins were measured by a direct radioimmunoassay (14). Untreated, heat-treated (60°C, 60 min), or boiled (10 min) uterine homogenates were treated with typsin (0.9 μM). Mean (±SEM) kininogen content, in terms of the released kinin measured by radioimmunoassay, was 29.3 ± 1.9, 30.7 ± 0.3, and 26.5 ± 0.7 ng of kinin equivalents/mg protein in untreated, heat-treated, or boiled homogenates, respectively ($n = 4$). Extracts treated with rat urinary kallikrein (30 nM), human urinary kallikrein 50 nM), or rat urinary esterase A (30 nM) contained 2.0, 1.5, or 1.2 ng of kinin equivalents/mg protein, respectively. Similar results were observed with heated and boiled uterine extracts. Thus, the rat kallikrein (and other kininogenases) at supramaximal concentrations with respect to contractile activity released <10% of the kinin released by trypsin.

Table 1. Effect of kininogenases on rat uterine contraction

Enzyme	Bath concentration, $M \times 10^{10}$	% of max contraction
Rat urinary kallikrein	6	100
Human urinary kallikrein	60	0
Rat urinary esterase A	60	0
Trypsin	30–300	<10

Measurements were repeated at least three times in each case. The specific activities of rat urinary kallikrein, human urinary kallikrein, and rat urinary esterase A were 125, 74, and 550 Tos-Arg-OMe esterase units/mg, respectively. One unit is defined as that amount of enzyme which hydrolyzes 1.0 μmol of Tos-Arg-OMe per min at pH 8.0 and 30°C in a standard titrimetric assay (17).

Free kinin was not found in uterine extracts. The de Jalon's solution surrounding isolated rat uteri ($n = 3$), producing 2.5 g of contractile force in response to kallikrein (4 nM), was withdrawn and kinin content was measured by radioimmunoassay. No detectable kinin levels (<4 pg per assay tube) were found in 0.1-ml aliquots of the media; recovery of added kinin was 98%. Finally, 2-cm uterine strips were incubated in de Jalon's solution with rat urinary kallikrein (0.5–4 nM) in the absence or presence of the kininase II inhibitor SQ 20881 (100 μg/ml) for periods of 10, 30, and 60 min. No detectable kinin was present in the incubated media.

DISCUSSION

The present study shows that rat uterine contraction produced by a rat glandular kallikrein occurs without detectable kinin release. This conclusion is supported by the following findings.

First, the enzyme alone produced prompt contractions not typical of those which result from a time-dependent initial rate of enzymatic action and product formation. The enzyme is 4- to 6-fold more potent than the product bradykinin, used for comparison as bradykinin is 1.6-fold more potent that kallidin in the isolated rat uterus (19).

Second, the addition of a kininogen substrate to the organ bath after kallikrein has initiated maximal and rhythmic responses produced a time-dependent further increase in contractility, as would be expected secondary to product formation.

Third, both carboxypeptidase B (a nonspecific kininase) and a specific kinin antiserum abolished the uterine contractile responses to bradykinin but did not affect those to kallikrein.

Fourth, other potent kininogenases, including human urinary kallikrein (11), rat urinary esterase A (12), and trypsin, in amounts much larger than those of the rat enzyme either did not induce uterine contraction or did so minimally

Fifth, there was no relationship between the calculated amount of uterine kininogen detected after enzymatic treatment and the ability of the enzymes to induce contractile responses. That is, amounts of rat urinary kallikrein supramaximal in terms of contractile responses released <10 % of the kinin from uterine extracts that was released by trypsin; the latter produced contractile responses <10% of those of kallikrein in concentrations up to 50-fold higher.

Finally, kinin could not be detected with a sensitive radioimmunoassay while uteri were contracting in response to kallikrein or after prolonged incubation of uteri with excess amounts of enzyme in the presence of kininase II inhibition.

It is known that other rat glandular kallikreins (e.g., pancreatic and salivary) cause the isolated rat uterus to contract (20, 21); these enzymes have been considered to be 500-fold more potent than trypsin insofar as this effect is concerned (22). However, because small but detectable quantities of a kinin-releasing substrate have been found within the organ (23), the mechanism of kallikrein-induced uterine contraction has been uncertain. The possibility exists that this detectable kininogen is residual from plasma which contains 3–10 μg of bradykinin equivalents/ml. On the other hand, the possibility exists that rat glandular kallikrein uniquely releases kinin from some endogenous uterine kininogen to a location not accessible to available kinin antibody or kininase and in quantities too small to be detected by radioimmunoassay. This seems unlikely because the efficiency of trypsin as a kininogenase is not paralleled by contractile efficacy.

Fiedler (24) has pointed out that, although kallikreins are defined by one function (i.e., the ability to liberate a kinin from kininogen), definite proof that this is the only role of these enzymes is lacking. This is especially pertinent because activities of these serine proteinases not related to kinin are now being discovered. Thus, the abilities of plasma or glandular kallikreins to cleave proinsulin (1), activate prorenin (2), release renin (25), attack angiotensinogen (26), and , now, contract the uterus suggest that the enzymes have other important biologic functions.

It is now known that glandular kallikrein exists in rat plasma (27). Aprotinin, an effective inhibitor of glandular kallikrein, has been found to decrease pregnant uterine muscular activity and prolong the duration of parturition (28). Whether the enzyme has some role in parturition—for example, via the adenyl cyclase activation that occurs in response to various serine proteinases in the rat ovary (29)—remains to be determined.

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