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# CHARACTERIZATION OF RECEPTOR-MEDIATED ACTIONS OF T-KININ

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**Abstract**—T-Kinin (Ile-Ser-bradykinin) is unique to the rat. This study characterizes the receptors involved in T-kinin activity on both the intact isolated rat uterus and membrane receptor preparations of the rat uterus. The results show that T-kinin acts through kinin $B_2$ receptors in the rat uterus as demonstrated by $B_2$ receptor–antagonist inhibition. While the potency of T-kinin on rat uterus contraction was similar to that of bradykinin, binding studies showed that the affinity of T-kinin to the receptor was 10-fold lower than that of bradykinin. On the other hand, the D isomer of T-kinin, D-Ile-Ser-bradykinin, had an affinity for the receptor greater than that of T-kinin and was more potent in causing contraction. Comparing this finding with our previously published report that D-Ile-Ser-bradykinin is not active on the kinin receptor for vascular permeability indicates that the kinin receptors in the rat uterus are not the same as those previously reported in the smooth muscle of the vasculature, i.e. there exists subclasses of kinin $B_2$ receptors. The data from binding studies on a variety of T-kinin analogues show that the substitution of hydroxyproline (Hyp) for $Pro^5$, together with the D-configuration at $Ile^1$ and/or $Ser^2$ may be useful for the development of selective T-kinin antagonists. Studies involving pretreatment of the tissue with indomethacin demonstrated that prostaglandin release was more of a component of T-kinin's activity on the rat uterus than that of bradykinin.

Bradykinin is released from high molecular weight (HMW)§ and low molecular weight (LMW) kininogen by plasma kallikrein and tissue kallikrein, respectively. Okamoto and Greenbaum [1, 2] found a third species of kininogen in rat plasma, known as T-kininogen. T-Kinin (Ile-Ser-bradykinin) may be generated from T-kininogen by the action of T-kininogenase found in the rat submandibular gland [3]. T-Kininogen, unlike HMW- and LMW-kininogen, is an acute phase protein. Upon an inflammatory challenge, hepatic synthesis of mRNA of T-kininogen is increased within 24 hr, which is followed by augmentation of plasma levels of T-kininogen as well as that of circulating T-kinin [4, 5].

As a pharmacologically active peptide, T-kinin, like bradykinin, causes contraction of smooth muscle [6], modification of blood pressure [6, 7], and an increase in vascular permeability [8]. Peripheral administration of T-kinin, like bradykinin, reduces the blood pressure of the rat [6]. Both peptides exhibit hypertensive activity following intracerebroventricular injection [7]. Studies on isolated vascular smooth muscles [9] showed that T-kinin causes relaxation of dog carotid and renal arteries and contraction of rabbit jugular vein, but is less potent in producing these effects than bradykinin. In contrast, T-kinin showed higher potencies than bradykinin in increasing the vascular permeability of rat and guinea pig skin capillaries [8], and in changing short-circuit current across the rat colon [10]. T-Kinin, like bradykinin, is also able to induce non-vascular smooth muscle contraction, for example in the rat uterus [6].

The current investigation was designed: (1) to determine whether T-kinin binds to the bradykinin receptor; and (2) to examine the structure–activity relationships of T-kinin to receptors in both intact tissues and partially purified membrane preparations. These studies would help clarify the role of T-kinin in the rat and also aid in comparing bradykinin antagonists for inhibition of bradykinin and T-kinin.

## MATERIALS AND METHODS

*Animals and agents used.* Female Sprague–Dawley rats weighing 200–250 g were used in all experiments. Each rat was injected with diethylestilbesterol (200 μg) subcutaneously 20 hr before the experiments.

T-Kinin, bradykinin, and [Des-$Arg^9$]-bradykinin were purchased from Peninsula Lab., Inc., Belmont, CA. [D-$Arg^0$,$Hyp^3$(hydroxyproline), D-$Phe^7$]-Bradykinin was supplied by Dr. Regoli, University of Sherbrooke, Canada. The analogues of T-kinin were synthesized by Drs. Stewart and Vavrek. 1,10-Phenanthroline, indomethacin, trimethylaminoethanesulfonic acid (TES), bacitracin, and bovine serum albumin were obtained from the Sigma Chemical Co., St. Louis, MO. Dithiothreitol (DTT)

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§ Abbreviations: DTT, dithiothreitol; HMW, high molecular weight; Hyp, hydroxyproline; LMW, low molecular weight; and TES, trimethylaminoethanesulfonic acid.

was purchased from the Bio-Rad Corp., Richmond, CA. Captopril was a gift from E. R. Squibb & Sons, Inc., Princeton, NJ. [$^3$H]-Bradykinin was purchased from Dupont-NEN, Boston, MA.

*Bioassay of kinins on isolated tissues.* The method used was described by Freer *et al.* [11]. After removal of the rat uterus under ether anesthesia, the isolated intact tissue was trimmed of fascia, suspended in a 10-mL volume tissue bath, and bathed in de Jalon's solution (NaCl, 0.15 M; KCl, 5.6 mM; $CaCl_2$, 0.4 mM; $MgCl_2$, 25 $\mu$M; $NaHCO_3$, 6 mM; glucose, 3 mM) at 24°. The bathing solution was bubbled continuously with 97% oxygen and 3% carbon dioxide. The rat uterus was placed under 1 g of tension until a stable baseline was obtained (approx. 1 hr). Changes in tension after addition of kinins and their agonists or antagonists were recorded using a polygraph (Grass model 79D) with force-displacement transducers (Grass model FT03C). In the assays of antagonist activities, the antagonist was preincubated in the tissue bath for 20 sec before the addition of T-kinin, bradykinin or agonists. In the studies of the effects of indomethacin on T-kinin- and bradykinin-induced contractions of the rat uterus, the tissue was incubated with indomethacin for 60 min before it was exposed to kinins.

*Binding assay of kinins on rat uterus.* The method used was modification of that of Manning *et al.* [12]. After removal of the rat uterus under ether anesthesia, the myometrium was separated from the parametrium and endometrium. The tissue was placed in cold 25 mM TES buffer, pH 6.8, adjusted with $NH_4OH$, containing 1 mM 1,10-phenanthroline and homogenized using a Polytron homogenizer at setting 6 for 20 sec at 4°. The homogenate was centrifuged at 40,000 *g* for 10 min at 4°. The pellet was resuspended in the same buffer and homogenized two additional times. After protein determination, the final pellet was suspended in binding assay buffer (25 mM TES, 1 mM 1,10-phenanthroline, 140 $\mu$g/mL bacitracin, 1 $\mu$M captopril, 1 mM DTT and 0.1% BSA, pH 6.8) at a final concentration of 50 mg wet tissue/mL and stored at −70°.

The saturation analysis of [$^3$H]-bradykinin binding to the receptors in rat myometrial plasma membrane was accomplished by incubating the partially purified receptor preparation with [$^3$H]-bradykinin (NEN, 78.4 Ci/mmol) in a total volume of 1 mL. At the end of the incubation carried out in a shaking bath at room temperature for 2 hr, each tube was filtered under reduced pressure through Whatman GF/B glass fiber filters pretreated with 0.1% (v/v) aqueous polyethylenimine. After being washed with 3 × 4 mL of ice-cold 25 mM TES, pH 6.8, the filters were immersed in Fisher ScintiVerse BD liquid scintillation mixture for at least 4 hr before quantitation by liquid scintillation spectrometry. Specific binding was calculated by subtracting the non-specific binding measured in the presence of a 1 $\mu$M concentration of unlabeled bradykinin from the total binding. In the experiments of displacement of kinins on [$^3$H]-bradykinin binding, 100 $\mu$L of kinin-containing solutions was incubated with [$^3$H]-bradykinin and receptor preparation.

*Data analysis.* Values are expressed as means ± SEM. Student's unpaired *t*-tests were used for determining the difference between means of different groups. A level of probability $< 0.05$ was considered to represent a statistically significant difference. The $EC_{50}$, $IC_{50}$, and $K_D$ values were obtained using nonlinear least squares regression of curves (Michaelis–Menten equation). $K_i$ values were calculated from the $IC_{50}$.

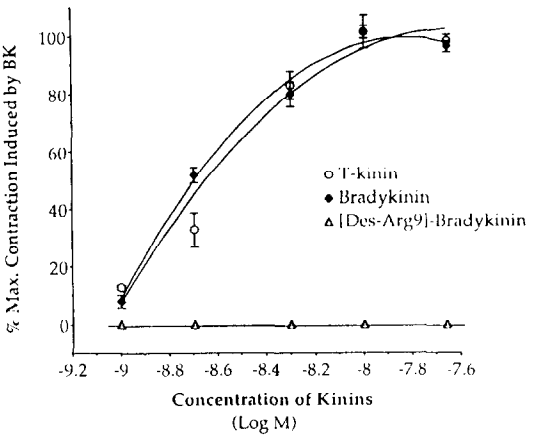


Fig. 1. Response of the rat uterus to T-kinin, bradykinin, and [Des-Arg$^9$]-bradykinin. Two hundred microlitres of T-kinin, bradykinin, or [Des-Arg$^9$]-bradykinin, a $B_1$ agonist, was added into the tissue bath. Data are expressed as the percentage of the maximal contraction induced by bradykinin (means ± SEM, N = 4). The curves were created by using the Michaelis–Menten equation.

## RESULTS

*Responses of the rat uterus to kinins.* T-Kinin and bradykinin were tested on intact isolated rat uterus smooth muscle. The dose–response curves of bradykinin and T-kinin on rat uterus showed that T-kinin and bradykinin had very similar potencies for contraction of rat uterus smooth muscle (Fig. 1). There were no significant differences in $EC_{50}$ values between T-kinin ($EC_{50}$: 2.3 ± 0.2 nM) and bradykinin ($EC_{50}$; 2.0 ± 0.2 nM). The maximal contractions of the rat uterus induced by T-kinin and bradykinin were similar (T-kinin: 2.8 ± 0.3 g; bradykinin: 2.8 ± 0.3 g). [Des-Arg$^9$]-Bradykinin, a bradykinin $B_1$ agonist, did not exhibit activity on the rat uterus.

*Effects of bradykinin antagonists on activities of T-kinin and bradykinin on rat uterus contraction.* Following treatment with a bradykinin $B_2$ antagonist, [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin, responses of the rat uterus to T-kinin were reduced (Fig. 2). Comparison of the effects of [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin on the activities of T-kinin and bradykinin showed that the $pA_2$* was 6.69 for the $B_2$ antagonist on T-kinin activity and 6.27 for bradykinin activity. Therefore, T-kinin was more sensitive to this $B_2$ antagonist than bradykinin. [Des-Arg$^9$, Leu$^8$]-

* The negative logarithm of the molar concentration of antagonist which reduces the effect of a double dose of agonist to a single dose.

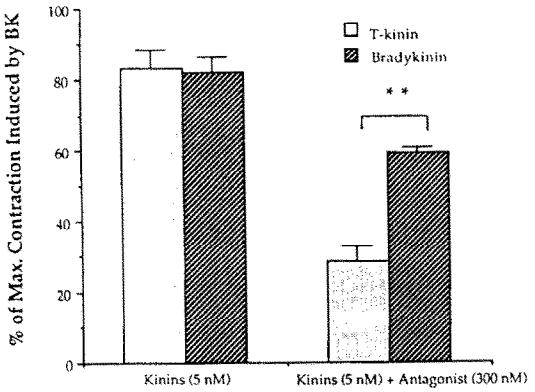


Fig. 2. Effect of [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin, a $B_2$ antagonist, on the response of rat uterus to T-kinin. The $B_2$ antagonist (300 nM), [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin, was added to the tissue bath 20 sec prior to the addition of T-kinin. Results are expressed as the percentage of the maximal contraction induced by bradykinin (means ± SEM, N = 4). Key: (**) $P < 0.01$.

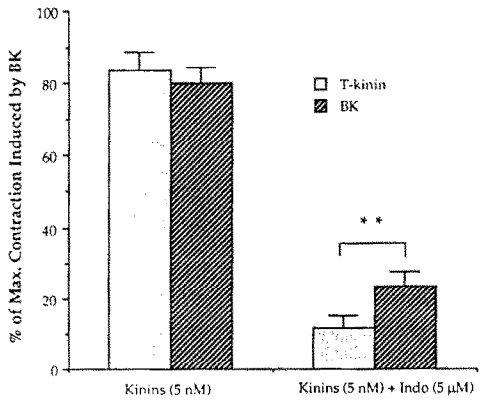


Fig. 3. Response of the rat uterus to T-kinin and bradykinin in the presence of indomethacin. The isolated tissue was incubated with indomethacin (5 μM) for 60 min before the application of 5 nM T-kinin or bradykinin. Data are expressed as the percentage of the maximal contraction induced by bradykinin (means ± SEM, N = 4). Key: (**) $P < 0.01$.

Bradykinin, a bradykinin $B_1$ antagonist, did not interfere with the actions of T-kinin and bradykinin on rat uterus contraction (data not shown).

*Response of the rat uterus to kinins in the presence of indomethacin.* Kinins are known to function partially through actions of prostaglandins. Therefore, the effects of indomethacin, a cyclo-oxygenase inhibitor, on rat uterus contractions by T-kinin and bradykinin were compared (Fig. 3). At the concentration tested (5 μM), indomethacin reduced the activity of T-kinin by 80.3 ± 7.02%, while it reduced bradykinin activity by 65.1 ± 5.44% (the means of the differences were statistically significant from each other $P < 0.01$).

Table 1. Inhibition constants of kinins and their antagonists on [$^3$H]-bradykinin binding

| Kinins | $K_i$ (nM) |
|---|---|
| Bradykinin | 1.4 |
| [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-Bradykinin | 7.7 |
| T-Kinin | 13.2 |
| [Des-Arg$^9$]-Bradykinin | >1250 |
| [Des-Arg$^9$,Leu$^8$]-Bradykinin | >1250 |

Partially purified receptors in rat myometrial plasma membrane were labeled by incubating the tissue with 1 nM [$^3$H]-bradykinin. Then kinins were added into the incubation mixture to compete with [$^3$H]-bradykinin for the binding sites. A 1 μM concentration of unlabeled bradykinin was used to determine non-specific binding, which was 25.4% of total binding (1200 cpm). Inhibition constants were calculated using the equation: $K_i = IC_{50}/(1 + [[^3H]BK]/K_D)$.

*Receptor binding assays of kinins on rat uterus.* The saturation binding of [$^3$H]-bradykinin was obtained by incubating $^3$H-labeled bradykinin at different concentrations (0.01 to 2 nM) with a partially purified receptor preparation (1 mg wet tissue/mL). The specific binding exhibited Michaelis–Menten kinetics with a maximal binding ($B_{max}$) of 3.76 pmol/mg wet tissue; the dissociation constant ($K_D$) was 0.33 ± 0.073 nM. Scatchard analysis indicated one component of binding. This is in agreement with the previous findings of Farmer *et al.* [13] and Engstrom *et al.* [14], but disagrees with Liebmann *et al.* [15], who proposes two receptor sites.

To compare affinities of T-kinin, bradykinin, [Des-Arg$^9$]-bradykinin, and their antagonists for the receptors in the rat uterus, the abilities of these peptides to compete with the binding of $^3$H-labeled bradykinin was examined by incubating kinins at different concentrations (1 nM–10 μM) with 1 nM [$^3$H]-bradykinin. Data were from 3–4 separate experiments performed in duplicate with less than 25% intra-assay variability. The calculated $K_i$ values ($K_i = IC_{50}/(1 + [[^3H]\text{-}BK]/K_D)$) of kinins and their antagonists indicated that bradykinin had the highest affinity for the receptor. The affinity of T-kinin was 13-fold lower than that of bradykinin. The affinity of [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin was lower than that of bradykinin but higher than that of T-kinin. The logarithmic curves of inhibition of $^3$H-labeled bradykinin binding by T-kinin, bradykinin, and [D-Arg$^0$, Hyp$^3$, D-Phe$^7$]-bradykinin showed the same slopes. Both the $B_1$ agonist, [Des-Arg$^9$]-bradykinin, and antagonist, [Des-Arg$^9$, Leu$^8$]-bradykinin, had very low affinities for the [$^3$H]-bradykinin-labeled receptor (Table 1).

*Studies on the structure–activity relationships of T-kinin to receptors.* These studies were carried out by studying the inhibition of [$^3$H]-bradykinin binding to the partially purified rat myometrial plasma membrane by analogues of T-kinin and by comparing the agonist activities in contracting the isolated intact rat uterus. The analogues of T-kinin used in this

Table 2. Inhibition constants of T-kinin and its analogues (in ascending order)

| T-Kinin and analogues | $K_i$ (nM) |
|---|---|
| [D-Ile$^1$]-T-Kinin | 4.1 |
| T-Kinin | 13.2 |
| [Hyp$^5$,D-Phe$^9$]-T-Kinin | 56.7 |
| [D-Ile$^1$,D-Ser$^2$,Hyp$^5$,D-Phe$^9$]-T-Kinin | 62.8 |
| [D-Ser$^2$,Hyp$^5$,D-Phe$^9$]-T-Kinin | 83.6 |
| [D-Ile$^1$,D-Phe$^9$]-T-Kinin | 93.8 |
| [D-Ile$^1$,Hyp$^5$,D-Phe$^9$]-T-Kinin | 123.7 |
| [D-Ile$^1$,D-Ser$^2$,D-Phe$^9$]-T-Kinin | 149.5 |
| [D-Phe$^9$]-T-Kinin | 165.8 |
| [D-Ser$^2$,D-Phe$^9$]-T-Kinin | 210.2 |

The experimental conditions were the same as described in the legend of Table 1.

Table 3. Activities of T-kinin and its analogues on rat uterus contraction

| T-Kinin and analogues | $EC_{50}$ (M) |
|---|---|
| T-Kinin | $2.3 \times 10^{-9}$ |
| [D-Ile$^1$]-T-Kinin | $<1 \times 10^{-10}$ |
| [D-Phe$^9$]-T-Kinin | $>1 \times 10^{-7}$ |
| [D-Ile$^1$,D-Phe$^9$]-T-Kinin | $>1 \times 10^{-7}$ |
| [D-Ser$^2$,D-Phe$^9$]-T-Kinin | $>1 \times 10^{-7}$ |
| [D-Ile$^1$,D-Ser$^2$,D-Phe$^9$]-T-Kinin | $>1 \times 10^{-7}$ |
| [Hyp$^5$,D-Phe$^9$]-T-Kinin | $>1 \times 10^{-7}$ |
| [D-Ile$^1$,Hyp$^5$,D-Phe$^9$]-T-Kinin | —* |
| [D-Ser$^2$,Hyp$^5$,D-Phe$^9$]-T-Kinin | — |
| [D-Ile$^1$,D-Ser$^2$,Hyp$^5$,D-Phe$^9$]-T-Kinin | — |

The experimental conditions were the same as described in the legend of Fig. 1.

* No intrinsic activities.

study can be classified into three types: (1) a peptide with a D-configuration at position 1 (Ile$^1$); (2) peptides with D-Phe replacing Pro$^9$ with/without a D-configuration at Ile$^1$ and/or Ser$^2$; and (3) peptides with Hyp substituting for Pro$^5$ in addition to modification of Pro$^9$ with/without D-Phe and D-configuration at Ile$^1$ and/or Ser$^2$.

The results showed that [D-Ile$^1$]-T-kinin, unlike the other analogues, showed a much lower $K_i$ values than T-kinin (Table 2). In the bioassay on isolated intact tissue, this D-analogue of T-kinin also exhibited a higher activity than T-kinin (Table 3). The bradykinin $B_2$ antagonist, [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin, was able to abolish the activities of this T-kinin D-analogue completely (data not shown). In general, the substitution of D-Phe at Pro$^9$ seemed to reduce remarkably the affinity of the T-kinin analogues. In comparison, the peptides with modification of Pro$^5$ with Hyp as well as Pro$^9$ with D-Phe had higher affinities towards the receptors except for [D-Ile$^1$,Hyp$^5$,D-Phe$^9$]-T-kinin and [D-Ile$^1$,D-Phe$^9$]-T-kinin (Table 2). The bioassays of T-kinin analogues on rat uterus demonstrated that some of these peptides showed very weak agonist activities. The peptides with modification of Pro$^5$ with Hyp, Pro$^9$ with D-Phe and D-configuration at position 1 and/or 2 did not exhibit any intrinsic activities (Table 3).

## DISCUSSION

Kinin receptors are classified into two well-established types: the $B_1$ receptor which has high affinity for [Des-Arg$^9$]-bradykinin (the product of carboxypeptidase action on bradykinin) and the $B_2$ receptor which is sensitive to intact bradykinin itself [16]. T-Kinin receptors have not been characterized.

Our current findings showed that the potencies and efficacies of T-kinin and bradykinin were similar in contracting the isolated rat uterus. The action of T-kinin on $B_2$ receptors was confirmed by using the bradykinin $B_2$ antagonist [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin. The inhibitor reduced the activity of T-kinin dramatically. In addition, T-kinin showed greater sensitivity to the $B_2$ antagonist than bradykinin. Our results also confirmed reports of many other studies that $B_1$ receptors are not present in the rat uterus, i.e. the $B_1$ agonist, [Des-Arg$^9$]-bradykinin, did not cause contraction of the uterus; similarly, the actions of T-kinin were not inhibited by $B_1$ antagonists.

T-Kinin has a lower affinity than bradykinin for the $B_2$ receptor. The results from the studies on the inhibition of [$^3$H]-bradykinin binding to rat uterus smooth muscle showed that the potency of T-kinin binding to the receptor was 13 times less than that of bradykinin. Comparison of the binding of the $B_2$ antagonist, [D-Arg$^0$,Hyp$^3$,D-Phe$^7$]-bradykinin, with T-kinin and bradykinin to the $B_2$ receptor demonstrated that the $B_2$ antagonist has a higher affinity than T-kinin, but lower than bradykinin. This explains why the $B_2$ antagonist was more potent against the action of T-kinin than that of bradykinin on rat uterus contraction.

The phenomenon that T-kinin had a lower affinity than bradykinin to the $B_2$ receptor but had a similar potency on rat uterus contraction could be explained by the possibility that T-kinin and bradykinin may have similarities but also differences in intracellular transduction mechanisms leading to similar potency of contraction. For example, our experiments showed that the activity of T-kinin was blocked more effectively by indomethacin than the activity of bradykinin, indicating that the actions of T-kinin are much more dependent on prostanoids than are the actions of bradykinin. It is unlikely that T-kinin may exert its activity by being converted to bradykinin by aminopeptidase activity in the uterus since there was no delayed action of T-kinin seen during the experiments. In addition, the D-analogue of T-kinin, [D-Ile$^1$]-T-kinin, which would not be cleaved by aminopeptidases, showed a higher potency than bradykinin in contracting the uterus. Thus, T-kinin need *not* be converted to bradykinin for its activity. In addition, it is not clear whether T-kinin and bradykinin share the exact same binding sites. This can only be determined by using labeled T-kinin. In our experiments, only [$^3$H]-bradykinin was used.

Experiments were carried out in order to further

define the structure–activity characteristics of T-kinin in terms of relationships to its receptors and to explore the possibility of developing a specific T-kinin antagonist. A series of analogues were compared for their affinities to membrane-bound receptor preparations as well as for their potencies in contracting the rat uterus. The results showed that the analogues of T-kinin with a substitution of D-Phe at position $Pro^9$, which is the critical change to develop antagonist activity to bradykinin [17], exhibited reduced affinity for the receptors. In general, except for [D-$Ile^1$,$Hyp^5$,D-$Phe^9$]-T-kinin and [D-$Ile^1$,D-$Phe^9$]-T-kinin, the analogues that had modifications at $Pro^5$ (substitution with Hyp) as well as $Pro^9$, which increase antagonist activity towards bradykinin [18], also showed reduced affinities but to a lesser degree than those having modification solely at $Pro^9$. This implies that substitution of $Pro^5$ with Hyp may enhance the affinity of T-kinin analogues for the receptors. When tested on the rat uterus, neither of the analogues with replacement of D-Phe at $Pro^9$ demonstrated any antagonistic effect against T-kinin. They did, however, have very low agonist activities. The D-configuration at $Ile^1$ and/or $Ser^2$, as well as substitution of $Pro^5$ with Hyp, had a higher affinity but did not show any intrinsic activity. This suggests that the peptides with modification at $Pro^5$ and $Ile^1$ or $Ser^2$ may be important for the development of T-kinin antagonists.

[D-$Ile^1$]-T-Kinin, which binds to the bradykinin $B_2$ receptor, had a higher affinity for the receptors than T-kinin. Comparison of the effects of T-kinin and the D-$Ile^1$-analogue on rat uterus contraction demonstrated that this analogue was also much more potent than T-kinin itself. This can be explained by its greater affinity for the receptors. It would thus appear that affinity for the receptor does not require all the amino acids of T-kinin to be in the L form, at least as far as rat uterus contraction is concerned. It should be noted, however, that our previous studies with T-kinin and its D-$Ile^1$ analogue on vascular permeability showed that kinin receptors on endothelial cells of blood vessels involved in vascular permeability apparently *require* the L-form [8]. This indicates that subclasses of kinin receptors exist in the smooth muscle of rat uterus which are different from those in rat capillary endothelium. This adds to the evidence of multiple $B_2$ receptors also noted by Vavrek and Stewart [19], Llona *et al.* [20], and Steranka *et al.* [21].

Overall, the results from the studies carried out using both the intact rat uterus and a rat uterus membrane receptor preparation indicate that: (a) T-kinin probably functions through bradykinin $B_2$ receptors to cause rat uterus smooth muscle contraction, (b) it has a potency very similar to that of bradykinin, but has a lower affinity for the receptors than bradykinin, (c) T-kinin and bradykinin may differ in their intracellular signal transductions which would result in different rates of synthesis and release of prostaglandins by these two peptides, (d) evidence appears to favor the concept that multiple subclasses of kinin $B_2$ receptors exist in different tissues, and (e) antagonists for T-kinin seem to require modifications at the $Ile^1$/$Ser^2$ position as well as at the $Pro^5$ position of T-kinin. However, antagonists that block T-kinin but not bradykinin are not yet in hand.

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