Bradykinin Receptors in Rat Uterine Smooth Muscle: Studies Using Radiolabeled Ligand Binding

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Direct binding of $^{125}$I-Tyr$^8$-bradykinin to a microsomal fraction prepared from rat uterine smooth muscle, showed an apparent dissociation constant ($K_d$) at 29°C of $5.0 \times 10^{-10}$ M calculated from kinetic studies and $6.6 \times 10^{-10}$ M from Scatchard plot analysis. The binding of $^{125}$I-Tyr$^8$-bradykinin was reversible and saturable, and demonstrated high specificity for Tyr$^8$-bradykinin, bradykinin and Lys-bradykinin, but was not displaced by unrelated peptides angiotensin I, angiotensin II, Arg$^8$-vasopressin and oxytocin. The binding sites were copurified by differential centrifugation and on a discontinuous sucrose density gradient with 5'-nucleotidase activity, a plasma membrane marker enzyme. Prolonged intravenous infusion of bradykinin (5 nmol/h for 2 days) induced a 20% decrease in the number of bradykinin binding sites without a change in the equilibrium dissociation constant. The present results demonstrate that receptors mediating the effect of bradykinin on rat uterine smooth muscle are situated on plasma membranes and the regulation of the receptors is in part under the control of endogenous bradykinin levels.

Bradykinin and other kinins modify cardiovascular hemodynamics by influencing vascular smooth muscle tone. The mechanism of action of bradykinin on vascular smooth muscle remains uncertain as characterisation of the bradykinin-receptor interaction has not been fully documented (Odya and Goodfriend 1979). Receptor binding is the first event in the series of molecular processes leading to hormonal effects in the target tissue and is also important both

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for physiological specificity and sensitivity. The bradykinin-receptor interaction has been characterised by inference from indirect studies using isolated tissues (Regoli et al. 1977; Barabé et al. 1977). These extensive studies of in vitro biological responses to bradykinin and its analogues in rabbit aorta, cat ileum and rat uterus, have provided evidence for the presence of two different types of receptors for bradykinin. However, these pharmacological experiments do not localize the specific subcellular receptors for this hormone, nor are they capable of identifying specific receptor modifications. In vitro direct binding studies using radiolabeled ligands have the advantages of localizing cellular receptors and of characterising specific receptor parameters. There have been few reports on specific bradykinin direct binding studies using a radiolabeled ligand (Reissmann et al. 1977; Odya et al. 1980).

Rat uterine smooth muscle is the tissue most sensitive in vitro to the agonist effect of bradykinin and possesses very little kininase activity (Stewart 1979). Since uterine smooth muscle is a frequently used model of vascular smooth muscle and since it possesses the above two important properties, we have defined the characteristics of receptors for bradykinin using a radiolabeled bradykinin ligand in this tissue.

**Materials and Methods**

Radiolabeled ligands and peptides. Binding studies were performed with moniodinated bradykinin (125I-Tyr8-bradykinin), prepared by the method of Hunter and Greenwood (1962). The labeled ligand was purified using Amberlite resin IRA 400 (BDH Chemicals, Poole, England) ion exchange chromatography to remove unreacted 125I and subsequently purified on Sephadex G25 (Pharmacia Fine Chemicals, Sweden) or CM Sephadex C25 (Pharmacia Fine Chemicals, Sweden) to isolate moniodinated 125I-Tyr8-bradykinin. The specific activity of 125I-Tyr8-bradykinin determined by the self displacement antibody method (Berson et al. 1964) was 100–300 Ci/mmol. 125I-Tyr8-bradykinin retained equal biological activity to the unlabeled Tyr8-bradykinin assessed by the blood pressure dose-response curves in a rat bioassay system. Monoiodination was confirmed by thin layer chromatography (n-butyl alcohol : pyridine : acetic acid : water, 15 : 10 : 3 : 10). Sodium iodide-125 was supplied from New England Nuclear Corporation (Boston, USA). 3H-Bradykinin with specific activity of 21 Ci/mmol was a gift of Dr. J.L. Morgat (Centre D' Energie Atomique, Département de Biologie, Service de Biochimie, Saclay, France). Synthetic bradykinin, Tyr8-bradykinin, Lys-bradykinin, Met-Lys-bradykinin, Tyr8-bradykinin, Tyr-bradykinin, and des-Arg9-bradykinin were obtained from Protein Research Foundation, Osaka. All other reagents were of the standard grade available and purchased from commercial sources.

Preparation of subcellular fractions from rat uterine smooth muscle. Subcellular fractions of rat myometrium were prepared using the methods of Kidwai et al. (1971) and Janis et al. (1977) with some modifications. Female Sprague-Dawley rats weighing 150–200 g were injected subcutaneously for 2 days with 500 µg/kg of stilbesterol dipropionate (May and Baker Aust. Pty. Ltd., Victoria, Australia) dissolved in peanut oil. They were killed by cervical dislocation and the uteri were rapidly removed, placed in 0.25 M mannitol and 1 mM Tris-EDTA, pH 7.1 at 4°C. For each binding experiment uteri from three rats were pooled and fractionated. The myometrium from uterine horns were carefully separated from the endometrium, minced finely and homogenized with a Potter-Elvehjem homogenizer.
Rat Uterine Bradykinin Receptors

at 1,200 rpm and 4°C for 2 min. The homogenate was centrifuged at 700×g for 10 min and the supernatant then centrifuged at 5,000×g for 10 min. The second supernatant was centrifuged, at 115,000×g for 40 min to sediment a microsomal fraction which was resuspended in the assay buffer (100 mM KCl, 5 mM MgCl₂, and 100 mM Histidine, pH 6.8). This resuspended pellet was used routinely as the microsomal fraction for the binding studies.

For subcellular localization of receptors, the resuspended microsomal pellet was layered onto a discontinuous sucrose density gradient (4 ml of 45% sucrose, 3 ml of 33% sucrose, 3 ml of 28% sucrose, and 3 ml of the 8% sucrose loading solution; sucrose solutions w/w), and centrifuged at 115,000×g for 90 min. After the centrifugation, the layers were harvested and centrifuged at 115,000×g for 40 min at 10 mM Tris-HCl buffer, pH 7.4.

Radiolabeled binding assay. Aliquots of freshly prepared microsomal fraction were incubated in the assay buffer together with ¹²⁵I-Tyr⁸-bradykinin in a final volume of 0.5 ml. The protein concentration of the fraction in the incubation medium determined by the method of Lowry et al. (1951) was 100–500 µg/ml. Incubation was performed at 29°C, after which bound and unbound radioactivity was separated by filtration through Millipore filters HAWP 0.45 µm. Radioactivity trapped by filters was counted in an automatic gamma spectrometer at a counting efficiency of 68%. Specific radiolabeled ligand binding was determined by the difference between the radioactivity bound in the absence and in the presence of 1,000-fold excess of unlabeled bradykinin.

The stability of ¹²⁵I-Tyr⁸-bradykinin during the incubation with the microsomal fraction of rat myometrium was determined by serial thin layer chromatography of the incubate (n-butyl alcohol : pyridine : acetic acid : water, 15 : 10 : 3 : 10). The integrity of the radiolabeled ligand was also confirmed by showing no alteration in binding in the presence of the specific kininase II inhibitor, captopril (SQ 14225) up to 10⁻³ M (E.R. Squibb & Sons, Inc., Princeton, USA). The stability of binding sites under experimental conditions was checked by preincubation of the microsomal fraction.

The activity of 5'-nucleotidase (E.C.3.1.3.5.), used as a marker enzyme of plasma membrane, was measured as described by Song and Bodansky (1967).

Bradykinin infusion. In order to determine whether elevation of endogenous circulating bradykinin influences ¹²⁵I-Tyr⁸-bradykinin binding sites of rat uterine smooth muscle, rats were infused intravenously for 2 days at a rate of 1 µl/hr with 5 × 10⁻⁵ M solution of bradykinin (n = 4) or vehicle alone (n = 4) delivered via osmotic minipumps (Alza, Palo Alto, California, USA) into the jugular vein. This infusion rate lowered the systolic blood pressure of the rats by 8.7±2.4 mmHg (mean ± s.e.m., p <0.05). The vascular catheter was inserted subcutaneously, and osmotic minipump was implanted in the interscapular region of the rat's back under anesthesia of pentobarbitone sodium (Abbott Laboratories Pty. Ltd., Australia). The binding assay was assessed 2 days after commencement of the infusions.

RESULTS

¹²⁵I-Tyr⁸-bradykinin binding to rat uterine microsomal fraction

Initial experiments indicated that the binding of ¹²⁵I-Tyr⁸-bradykinin to the microsomal fraction from rat myometrium was time and temperature dependent. The time course for the binding of ¹²⁵I-Tyr⁸-bradykinin to the microsomal fraction at 0°C, 15°C, and 29°C is shown in Fig. 1 at an initial concentration of 10⁻⁹ M of ¹²⁵I-Tyr⁸-bradykinin. The equilibrium of binding was reached after 8–10 min at 29°C, after 10–15 min at 15°C and later than 30 min at 0°C.

No change in the thin layer chromatographic patterns of 10⁻⁹ M of ¹²⁵I-Tyr⁸-bradykinin incubated at 29°C for 30 min with uterine microsomal fraction was seen, confirming lack of degradation of the radiolabeled ligand. Degradation
of the binding sites was also not demonstrable after 30 min of preincubation of uterine microsomal fraction at 29°C. Subsequent studies were therefore performed at 29°C since equilibrium of binding was developed rapidly and no breakdown of ligand and binding sites could be identified under these conditions.

Estimation of \(^{125}\)I-Tyr\(^8\)-bradykinin binding parameters

The association rate constant (\(k_1\)) and the dissociation rate constant (\(k_{-1}\)) were calculated from the association curves of \(^{125}\)I-Tyr\(^8\)-bradykinin using final concentrations of \(4.7 \times 10^{-10}\) M and \(9.3 \times 10^{-10}\) M, assuming a second order rate reaction. The association rate constant was \(4.8 \times 10^6/\text{M} \cdot \text{sec}\) and a dissociation rate constant was \(2.4 \times 10^{-3}/\text{sec}\) (Fig. 2). The equilibrium dissociation constant \((K_D)\) calculated from these values was \(5.0 \times 10^{-10}\) M. Assuming a pseudo-first order rate reaction for dissociation, a dissociation rate constant of \(1.3 \times 10^{-3}/\text{sec}\) was calculated from data for the first 5 min of displacement of \(^{125}\)I-Tyr\(^8\)-bradykinin by a 1,000-fold excess of unlabeled bradykinin (Fig. 2). This value is close to that calculated from the association studies.

The concentration dependence of bradykinin binding studied with \(^{125}\)I-Tyr\(^8\)-bradykinin from \(2.0 \times 10^{-10}\) M to \(5.0 \times 10^{-9}\) M, indicated the presence of more than one class of binding sites (Fig. 3). The high affinity binding sites were observed up to equilibrium with initial concentrations below \(1.2 \times 10^{-9}\) M. These high affinity binding sites had an apparent dissociation constant of \(6.6 \times 10^{-10}\) M and a capacity of \(44.2\) fmol/mg protein by Scatchard plot analysis (Scatchard 1949). The low affinity binding sites noted when using initial concentrations
above $1.2 \times 10^{-9}$ M, appeared unsaturable. The $K_D$ for the high affinity binding sites obtained from Scatchard plot analysis of binding data was close to the value of $K_D$ calculated from the $k_1/k_1$.

Comparison of binding parameters of $^{125}$I-Tyr$^8$-bradykinin and of $^3$H-bradykinin

To confirm the validity of the values of $K_D$ and binding capacity, the concentration dependence of bradykinin binding was studied with $^3$H-bradykinin. $^3$H-Bradykinin ranging from $2.0 \times 10^{-10}$ M to $1.2 \times 10^{-9}$ M was incubated with the same microsomal fraction from rat myometrium under identical conditions. The high affinity binding sites had $K_D$ of $6.7 \times 10^{-10}$ M and a capacity of 51.5 fmol/mg protein. The binding parameters in the same preparation using $^{125}$I-Tyr$^8$-bradykinin as radiolabeled ligand were very similar with a $K_D$ of $7.1 \times 10^{-10}$ M and a capacity of 45.1 fmol/mg protein.
Specificity of $^{125}$I-Tyr$^8$-bradykinin binding

The specificity of the bradykinin binding sites was indicated by the lack of binding inhibition in the presence of the unrelated peptides angiotensin I, angiotensin II, Arg$^8$-vasopressin and oxytocin, and by the relationship between binding-inhibition potency and biological activity of a variety of bradykinin analogues. The results are summarized in Fig. 4. The inhibition of binding by Lys-bradykinin was nearly as efficient as by Tyr$^8$-bradykinin and bradykinin, whereas Met-Lys-bradykinin, Tyr-bradykinin and Tyr$^5$-bradykinin had significantly less inhibitory effect. The des-Arg$^9$-bradykinin had no inhibitory effect on the binding of $^{125}$I-Tyr$^8$-bradykinin up to a concentration of $3 \times 10^{-8}$ M.

Subcellular fractionation and localization of $^{125}$I-Tyr$^8$-bradykinin binding

Fig. 5 shows the distribution of protein, 5’-nucleotidase activity and specific $^{125}$I-Tyr$^8$-bradykinin binding among the subcellular fractions. The results are illustrated following the method of De Duve et al. (1955). The greatest concentrations of both 5’-nucleotidase activity and specific $^{125}$I-Tyr$^8$-bradykinin binding, occurred in the 115,000 x g pellet after differential centrifugation of 700 x g supernatant. The patterns of 5’-nucleotidase activity and specific $^{125}$I-Tyr$^8$-bradykinin binding were also closely related after discontinuous sucrose density gradient of a 115,000 x g pellet. These results suggest that the bradykinin
binding sites are in the plasma membrane enriched material.

**Influence of bradykinin infusion on \(^{125}\text{I-Tyr}^8\)-bradykinin binding**

Binding parameters by Scatchard plot analysis of \(^{125}\text{I-Tyr}^8\)-bradykinin binding, ranging from \(2 \times 10^{-10}\) M to \(1.2 \times 10^{-9}\) M, to the uterine membranes from rats infused with bradykinin and with vehicle alone, are summarized in Table 1. \(^{125}\text{I-Tyr}^8\)-bradykinin binding capacity was decreased after continuous infusion of bradykinin for 2 days. There were no significant changes in the affinity of binding sites. No significant changes were found in wet weight of uterus, protein content and enzymatic activity of the uterine membranes between rats infused with bradykinin and with vehicle (Table 1).

**DISCUSSION**

Most studies on bradykinin receptors have relied on indirect information obtained from agonist dose-response curves obtained using in vitro biological preparations. Only limited reports using a direct binding technique are available (Reissmann et al. 1977; Odya et al. 1980). Direct binding techniques, similar to those reported here, have many advantages in characterising hormone-receptor...
Fig. 5. De Duve plots of the distribution of the constituents both of 700 × g supernatant from rat myometrial homogenate on differential centrifugation (left) and of microsomal fraction (115,000 × g pellet) from 5,000 × g supernatant on discontinuous sucrose density gradient centrifugation (right). In this experiment, the initial concentration of $^{125}$I-Tyr$^8$-bradykinin was $10^{-9}$ M. The data as shown correspond to the mean from 3 individual determinations. Vertical bar indicates S.E.M.

### Table 1. Binding parameters of uterine myometrial bradykinin receptors and uterine characteristics after continuous intravenous infusion of bradykinin for 2 days. Each value indicates the mean value from 4 individual determinations and the standard error.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 4)</th>
<th>Bradykinin (n = 4)</th>
</tr>
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<tbody>
<tr>
<td><strong>Number of binding sites</strong> (fmoles/mg protein)</td>
<td>55.7 ± 2.1</td>
<td>44.4 ± 3.4$^*$</td>
</tr>
<tr>
<td><strong>K$_D$ 29°C</strong> (× 10$^{-10}$ M)</td>
<td>7.1 ± 0.1</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td><strong>Weight of uterus</strong> (mg/g body weight)</td>
<td>2.0 ± 0.03</td>
<td>1.9 ± 0.03</td>
</tr>
<tr>
<td><strong>Protein</strong> (mg/g uterus weight)</td>
<td>6.8 ± 0.4</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td><strong>5'-Nucleotidase</strong> (10$^{-2}$ μmol/min/mg protein)</td>
<td>7.8 ± 0.5</td>
<td>7.3 ± 0.7</td>
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$^*$ indicates statistical significance at $p < 0.05$ by Student's $t$-test, compared to vehicle.
interaction.

We have described here a simple and reproducible method for the study of bradykinin receptors in smooth muscle. In such a study, the characteristic of the radiolabeled ligand selected is critical. We have used the iodinated bradykinin analogues, $^{125}$I-Tyr$^8$-bradykinin as bradykinin does not contain an amino acid suitable for radioactive labeling by the chloramine T or peroxidase methods. The parent analogue Tyr$^8$-bradykinin has approximately 15% of the activity of bradykinin assessed by the blood pressure dose-response curves in a rat bioassay system. Such activity was not altered by the iodination procedure. It was reported that $^{125}$I-Tyr$^8$-bradykinin had only 24% of the activity of bradykinin as measured by the biological response in vitro of the rat uterus (Odya et al. 1980). Odya et al. (1980) concluded that $^{125}$I-Tyr$^8$-bradykinin was an unsatisfactory ligand because of its susceptibility to kininase II activity (Chiu et al. 1975). However no evidence of breakdown of the radiolabeled ligand was found under our assay conditions. The absence of kininase II activity was also confirmed by the lack of effect on binding by specific kininase II inhibitor, captopril. Furthermore intact myometrium has been reported to have no kinin degrading properties (Stewart 1979). These results suggest that the binding site would be dissimilar to that of kininase II. There may be potential limitations in defining the full characteristics of the hormone-receptor interaction because of the reduced biological activity of Tyr$^8$-bradykinin compared to bradykinin itself. However no difference in the binding characteristics between $^{125}$I-Tyr$^8$-bradykinin and $^3$H-bradykinin which has no significant conformational change was found. In addition, Tyr$^8$-bradykinin and bradykinin behaved identically to the native hormone in the binding inhibition studies. These results support the validity of using $^{125}$I-Tyr$^8$-bradykinin to characterise bradykinin-receptor interaction in uterine smooth muscle.

Our finding of bradykinin binding sites in microsomal fractions of rat uterine smooth muscle with an apparent $K_d$ of $5.0-6.6 \times 10^{-10}$ M is consistent with data obtained by Reissmann et al. (1977) using an $^3$H-bradykinin analogue to study binding to plasma membrane from rat uterus. The association of binding sites with 5'-nucleotidase activity in the preparation suggests that the bradykinin receptor is localized to the plasma membrane of the myometrial cells in common with several other peptide hormones (Cuatrecasas 1974; Chang et al. 1975).

Concentration dependent binding data has suggested the presence of more than one class of binding sites. The dissociation constant of the high affinity binding sites estimated from Scatchard plot analysis, gives a value within the dose-range which produces biological responses (Stewart 1979). Hence the bradykinin binding sites could act as the cellular receptors for this agonist. The high affinity binding sites with the apparent dissociation constant of $6.6 \times 10^{-10}$ M were detectable at initial concentrations less than $1.2 \times 10^{-9}$ M. This is close to the range of agonist activity in rat uterus, the most sensitive target organ and also
in the range of circulating concentrations of this hormone (Talamo and Good-
friend 1979). For these reasons, the high affinity binding sites probably represent
the binding sites responsible for mediating agonist activity in this tissue. The
low affinity binding sites which appeared to be unsaturable could not be related
to any biological effect in this tissue.

Binding inhibition studies suggest that the C-terminal Arg may be critical for
binding to the receptor since absence of this residue precludes both binding and
biological activity (Stewart 1979). The addition of Lys does not effect biological
activity and was not shown to impair binding whereas the Met-Lys or Tyr-
bradykinin showed impaired binding and biological activity. Similarly, internal
substitution in Tyr\textsuperscript{5}-bradykinin was associated with a lower affinity of binding
than bradykinin. Thus, these two tyrosine analogues may not be optimal as
radiolabeled ligands in the study of bradykinin binding in this preparation.

The reduced number of bradykinin binding sites seen after prolonged brady-
kinin infusion suggest that elevated endogenous levels of bradykinin play a role
in the bradykinin receptor regulation. This phenomenon is unlikely to be due to
receptor occupancy since variations in the occupancy of binding sites by the
endogenous hormone should result in a change in the apparent dissociation
constant without modification in the total number of receptors. Furthermore
since no changes in wet weight of uterus and protein content and 5'-nucleotidase
of uterine membrane were observed, the decrease in the number of binding sites
can be considered to be attributed to the variations in receptor concentration of
the preparation. These findings are consistent with autoregulation theory of
receptors described by Raff (1976).

Thus, receptors mediating the effect of bradykinin on uterine smooth muscle
are situated on the plasma membrane similar to receptors for other peptide
hormones. Regulation of these receptors is in part under the control of en-
dogenous bradykinin levels. Receptor modification could thus play an indepen-
dent role in the modulation of bradykinin actions specifically in situations where
kinins may be increased.

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