Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B₂ receptor in dog skeletal muscle and rat L6 myoblasts

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Abstract

Previously we demonstrated that bradykinin infusion could increase glucose uptake into dog peripheral tissues, and that bradykinin could potentiate insulin-induced glucose uptake through glucose transporter 4 (GLUT4) translocation in dog adipocytes. However, skeletal muscle is the predominant tissue for insulin-mediated glucose disposal. The aim of this study was to determine how bradykinin affected insulin-stimulated glucose uptake in dog skeletal muscle and myotubes transformed from rat L6 myoblasts. The bradykinin receptor binding studies revealed that dog skeletal muscle and rat L6 myoblasts possessed significant numbers of bradykinin receptors ($K_d = 88$ and 76 pmol/l, $B_{max} = 82.5$ and 20 fmol/mg protein respectively). An RT-PCR (reverse transcriptase-polymerase chain reaction) amplification showed mRNA specific for bradykinin B₂ receptor in both cells. Bradykinin significantly increased 2-deoxyglucose uptake in isolated muscle and L6 myoblasts in the presence of insulin (10^{-7} mol/l) in a dose-dependent manner, but not in the absence of insulin. Bradykinin also enhanced insulin-stimulated GLUT4 translocation, and insulin-induced phosphorylation of insulin receptor β subunit and insulin receptor substrate-1 (IRS-1) without affecting the binding affinities or numbers of cell surface insulin receptors in both cells. It is concluded that bradykinin could potentiate the insulin-induced glucose uptake through GLUT4 translocation in dog skeletal muscle and rat L6 myoblasts. This effect could be explained by the potency of bradykinin to upregulate the insulin receptor tyrosine kinase activity which stimulates phosphorylation of IRS-1, followed by an increase in GLUT4 translocation.

European Journal of Endocrinology 138 344–352

Introduction

Bradykinin is a nonapeptide involved in multiple biological processes: vasodilatation, increase in capillary permeability, smooth muscle relaxation/contraction, and inflammation (1). Moreover, bradykinin seems to be involved in the modulation of glucose metabolism in peripheral tissues (2–4). It has been reported that angiotensin-converting enzyme inhibitors with a sulfhydryl group could improve insulin sensitivity *in vivo* through the actions of bradykinin (2, 5). We demonstrated that bradykinin infusion increased glucose uptake into dog peripheral tissues and that infusion of a bradykinin antagonist abolished this effect on insulin sensitivity (2). We have already demonstrated the presence of bradykinin B₂ receptors in dog adipocytes; bradykinin could increase insulin-stimulated phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) which, in turn, could potentiate the translocation

of glucose transporter 4 (GLUT4), resulting finally in increased glucose uptake (6). Taken together these results show that bradykinin increases glucose uptake, at least in adipocytes.

Skeletal muscle is the main tissue involved in insulin-induced stimulation of glucose uptake (7–9). However, the existence of the bradykinin receptor in muscle cells and the mechanism by which this occurs have not yet been elucidated, although in isolated rat diaphragm muscle, bradykinin increases cytosolic free Ca^{2+} (10) and decreases glucose 1,6-bisphosphate levels (11), providing functional evidence for the presence of bradykinin receptors in skeletal muscle.

Recently, immunohistochemical studies demonstrated the presence of bradykinin B₂ receptors on the surface of striated muscle cells (12), and Rabito *et al.* demonstrated in both displacement and saturation studies of bradykinin binding assays that bradykinin B₂ receptors might be expressed in skeletal muscle (13).

The aims of our study are to determine whether bradykinin B₂ receptors are expressed in dog skeletal muscle and rat L6 myoblasts, and to establish how bradykinin affects glucose uptake with insulin and stimulates the insulin signaling pathway, including phosphorylation of the insulin receptor and its substrate IRS-1, and translocation of GLUT4 in these cells.

Materials and methods

Chemicals

[2,3-prolyl-3,4-³H(N)]Bradykinin, 2-[1,2-³H(N)]deoxyglucose, [1-³H(N)]L-glucose, [α -³²P]dCTP, [¹²⁵I-Tyr-A14]insulin and ¹²⁵I-labeled protein-A were supplied by NEN-Dupont (Wilmington, DE, USA). Anti-GLUT4 antibody, anti-phosphotyrosine antibody, anti-insulin receptor β subunit monoclonal antibody (Ab-3), and anti-rat carboxy-terminal IRS-1 antibody were obtained from ZYMED Laboratories Inc. (South San Francisco, CA, USA), Transformation Research Inc. (Framingham, MA, USA), Oncogene Science (Manhasset, NY, USA) and Upstate Biotechnology Inc. (New York, NY, USA) respectively. All other reagents and chemicals, unless otherwise stated, were obtained from Sigma (St Louis, MO, USA).

Cell preparations

Dog skeletal muscle The muscles were obtained from fasting normal male dogs weighing between 10 and 15 kg. Thigh skeletal muscle fragments (6 g) were immediately dissected after the induction of general anesthesia by pentobarbital sodium, removing fat and connective tissues. The dissected muscles were kept in Krebs-Henseleit bicarbonate buffer (pH 7.4) at 4 °C until use. All surgical and experimental procedures were conducted only after prior approval from the Animal Care and Use Committee of Kumamoto University School of Medicine.

For the bradykinin receptor and insulin receptor binding studies, partially purified plasma membrane (PM) was prepared. The dissected muscle was weighed and homogenized in a sucrose-HEPES buffer (in mmol/l: pH 7.4, 250 sucrose, 20 HEPES), and centrifuged at 100 000 *g* for 20 min at 4 °C. All of the following steps were performed at 4 °C, unless otherwise indicated. The pellet was resuspended in sucrose-HEPES buffer to obtain a protein concentration of 1–2 mg/ml (partially purified PM) using a Micro BCA protein assay system (Pierce, Rockford, IL, USA).

For studies of deoxyglucose uptake, GLUT4 translocation, and phosphorylation of the insulin receptor and IRS-1, purified PM fraction and low density microsomal fraction (LDM) were prepared using the following steps. The dissected muscle was weighed and homogenized in a sucrose-HEPES buffer and centrifuged at 34 000 *g* for 20 min at 4 °C. Purified PM was prepared by resuspending the pellet in a sucrose-HEPES buffer, followed by

centrifugation for 1 h at 227 000 *g*. The pellet was resuspended in 34% sucrose-20 mmol/l HEPES buffer and was fractionated using the sucrose gradient technique with centrifugation for 16.5 h at 68 000 *g*. The PM fraction was finally centrifuged at 227 000 *g* for 60 min. LDM from skeletal muscle was prepared from the supernatant of the initial centrifugation at 34 000 *g*. The fraction for LDM was also collected using the sucrose gradient technique with centrifugation for 60 min at 135 000 *g* and was finally centrifuged at 227 000 *g* for 60 min. Each resulting pellet from purified PM and LDM was resuspended in sucrose-HEPES buffer to make a final protein concentration of 1–2 mg/ml. The fragments of purified PM in the final preparation sample spontaneously seal to form vesicles in which it is possible to measure the glucose uptake (14).

Rat L6 myoblasts Rat L6 myoblasts were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum on 0.1% gelatin-coated plates. After the cells became subconfluent, the medium was changed to one containing 2% bovine serum for 5 days in order to transform from L6 myoblasts to mature myotubes.

PM and LDM from L6 myoblasts were isolated as described by Simpson *et al.* with slight modification (15). The cells were homogenized in a buffer (in mmol/l: pH 6.8, 300 sucrose, 25 TES buffer, 1 1,10-phenanthroline with 140 mg/ml bacitracin) and centrifuged at 2500 *g* for 10 min, and the supernatant was saved for preparation of PM and LDM. The supernatant was centrifuged at 70 000 *g* for 20 min yielding a pellet of PM and a supernatant of the cytosol fraction including LDM. The pellet was resuspended in incubation buffer to obtain a membrane protein at a concentration of 2–3 mg/ml.

For all the following experiments, PM and LDM were prepared as described above, unless otherwise indicated.

Receptor binding studies

Bradykinin receptor binding assay Partially purified PM (0.2 mg) from skeletal muscle and PM from L6 myoblasts were incubated with various concentrations (2–1000 pmol/l) [³H]bradykinin for 2 h in polyethylene tubes in a total volume of 0.5 ml. After equilibrium was reached, the binding reactions were terminated by rapid filtration over Whatman GF/B glass-fiber filters. The test tubes and filters were washed three times with 2–3 ml ice-cold 25 mmol/l TES buffer, pH 6.8, and the filters were then counted in a scintillation counter (13).

To calculate the specific binding in each of the cells, non-specific binding described as the amount of [³H]bradykinin bound in the presence of 3 μ mol/l unlabeled bradykinin was subtracted from the total count. The data from binding assays calculated from six determinations were analyzed using Scatchard analysis

(16). Binding site is expressed per milligram membrane protein.

Insulin receptor binding studies Insulin binding studies of skeletal muscle were performed as described by Freidenberg *et al.* (17). The partially purified PM from skeletal muscle was prepared after preincubation with or without bradykinin (10^{-8} mol/l). After adsorption to wheat germ agglutinin (WGA) columns, the partially purified PM were eluted from WGA columns in a buffer (in mmol/l: pH 7.4, 120 NaCl, 1 KCl, 1 CaCl_2 , 1 MgSO_4 , 2 orthovanadate, 2 phenylmethylsulfonyl fluoride, 50 NaF, 10 sodium pyrophosphate, 5 EDTA, 25 HEPES with 300 KIU/ml aprotinin, 10% glycerol, and 0.05% Triton) containing 0.3 mmol/l N-acetyl-glucosamine. The WGA eluate (50 μ l) was incubated with [^{125}I -Tyr-A 14]insulin (30 pmol/l) and with increasing concentrations of unlabeled insulin (0.04–20 nmol/l) for 18 h. The amount of receptor-bound insulin was determined by the polyethylene glycol precipitation method (18).

Insulin receptor binding studies of L6 myoblasts were also performed according to the method of Kobayashi *et al.* (19). L6 myoblasts were pretreated without or with bradykinin for 30 min at 37 °C and were then incubated with [^{125}I -Tyr-A 14]insulin (30 pmol/l) and various concentrations (0.04–20 nmol/l) of unlabeled insulin for 60 min at 37 °C. After the incubation, cells were washed with phosphate-buffered saline and solubilized with 0.1 mol/l NaOH.

The radioactivities of both experiments were measured by gamma counter. The data from six determinations were analyzed using Scatchard analysis (16).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

To detect the presence of bradykinin B_2 receptor mRNA in dog skeletal muscle and rat L6 myoblasts, total RNA was isolated from these cells using the guanidinium thiocyanate-CsCl technique (20). Total RNA (3 μ g) from each of the cells was reverse-transcribed into cDNA with avian Moloney virus reverse transcriptase (Seikagaku Corp. Tokyo, Japan) in RT buffer (in mmol/l: pH 8.3, 50 Tris, 50 KCl, 8 MgCl_2 and 5 dithiothreitol) using oligo dT primer, and the first-strand cDNA was amplified by PCR as described previously (6). The primers used for the PCR reaction were designed from nucleotide sequences which were highly conserved between mouse (21) and human (22) B_2 receptor cDNAs (sense primer: 5'-GGGCCCCACTCTTAACGGGA-3'; antisense primer: 5'-GACGTCCTGACCCGTCCCT-3'). To determine the sequence, each PCR product was purified and subcloned into the pUC 19 plasmid and sequenced.

2-Deoxyglucose uptake

Purified PM vesicles from skeletal muscle were prepared after preincubation with varying concentrations of cold

bradykinin (0, 10^{-10} – 10^{-7} mol/l) in the presence or absence of insulin (10^{-7} mol/l). During this treatment, insulin-stimulated translocation of intracellular GLUT4 to plasma membrane occurred. [^3H]2-Deoxyglucose uptake into purified PM vesicles was measured under conditions of equilibrium exchange ([glucose] in = [glucose] out) using a rapid filtration technique as described by Napoli *et al.* (23) with slight modification. Briefly, the uptake was initiated by combining 20 μ l purified PM and 80 μ l Krebs-Ringer solution containing 0.1 mmol/l [^3H]2-deoxyglucose and was stopped after 3 min by adding 1 ml stop solution (in mmol/l: pH 7.8, 20 NaCl, 5 KCl, 1.2 MgCl_2 , 20 HEPES) containing 0.2 mmol/l phloretin. The vesicles were then transferred to a nitrocellulose filter under vacuum. The filter was washed with 6 ml stop solution and analyzed by scintillation counter.

2-Deoxyglucose uptake in L6 myoblasts was measured according to Olefsky with some modification (24). After preincubation with various concentrations of bradykinin (0, 10^{-10} – 10^{-7} mol/l) and insulin (0, 10^{-10} – 10^{-7} mol/l) for 60 min at 24 °C, [^3H]2-deoxyglucose (0.1 mmol/l) uptake was measured for the last 3 min of the incubation period in Krebs-Ringer solution, pH 7.4, containing 1% BSA for 3 min at 24 °C. The assay was terminated by transferring 200 μ l aliquots from the assay mixture to plastic microtubes containing 100 μ l silicone oil. The amount of glucose trapped in the extracellular space of the cell layers was determined using [^3H]l-glucose and used to correct the data of glucose uptake.

GLUT4 translocation

Purified PM and LDM from skeletal muscle and L6 myoblasts were prepared after stimulation with or without bradykinin in the presence or absence of insulin as described in the section '2-Deoxyglucose uptake'. Each protein (40 μ g) from purified PM and LDM obtained from skeletal muscle and L6 myoblasts was applied to the SDS-PAGE with 10% acrylamide gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was immunoblotted with anti-GLUT4 antibody and then visualized using ^{125}I -labeled protein-A. GLUT4 contents were obtained by scanning bands corresponding to the approximate 55 kDa GLUT4 using a Bio-Image Analyzer (FUJIX, Tokyo, Japan), and expressed as arbitrary units.

Phosphorylation of insulin receptor β subunit and IRS-1

Purified PM and LDM (40 μ g) obtained from skeletal muscle and L6 myoblasts treated as described in 'GLUT4 translocation' were immunoprecipitated with either anti-insulin receptor antibody or anti-IRS-1 antibody (1:1000) for 2 h respectively. Immunoprecipitates were collected by adding 30 μ l protein A-sepharose 4B,

Figure 1 Bradykinin receptor binding assays in dog skeletal muscle and rat L6 myoblasts. Equilibrium binding of [³H]bradykinin (left panels) and Scatchard plots of equilibrium binding (right panels) to dog skeletal muscle (A) and rat L6 myoblasts (B). Membrane proteins from skeletal muscle and L6 myoblasts were incubated for 2 h at 4 °C with the indicated concentrations of [³H]bradykinin with or without 3 μmol/l unlabeled bradykinin. The difference between total (Δ) and non-specific (◆) binding is considered to be specifically bound [³H]bradykinin (●). Results are expressed as the mean of six experiments. In the Scatchard plot of equilibrium binding, Free refers to the difference between added and bound [³H]bradykinin.

washed three times with washing buffer (in mmol/l; pH 7.6, 20 Tris-HCl, 137 NaCl) and dissolved in SDS sample buffer. SDS-PAGE was performed with 6% acrylamide gels. Immunoblot analysis was performed with anti-phosphotyrosine antibody as described previously (6). Scanning bands corresponding to the approximate 95 kDa insulin receptor β subunit or 180 kDa IRS-1 were obtained as described above.

Calculation and statistical analysis

For the purpose of comparison, the results were expressed as arbitrary units. A scanning band corresponding to 70 nCi ¹²⁵I-protein A measured by Bio-Image Analyzer was assigned the value of 1 arbitrary unit and all other samples were expressed relative to that value in the studies of GLUT4 translocation, phosphorylation of insulin receptor β subunit and IRS-1. Data were expressed as means ± S.D. Student's *t*-test was used to assess the statistical significance.

Results

Bradykinin receptor binding assay

To examine the presence of the bradykinin receptor in dog skeletal muscle and rat L6 myoblasts, bradykinin receptor binding assays were performed. Specific [³H]bradykinin binding in these cells increased with increasing amounts of [³H]bradykinin (Fig. 1). Scatchard transformation of these data yielded apparent *K_d* values of 88 pmol/l in dog skeletal muscle and 76 pmol/l in rat L6 myoblasts, and *B_{max}* values of 2.5 and 20 fmol/mg protein respectively.

These results suggested the presence of comparable numbers of bradykinin receptors with a single homogeneous population of receptor binding sites for bradykinin on dog skeletal muscle and rat L6 myoblasts.

RT-PCR analysis

To detect the presence of B₂ receptor mRNA in dog skeletal muscle and rat L6 myoblasts, RT-PCR was performed. The RT-PCR-amplified PCR products from these cells were approximately 1000 base pairs (bp) on the gels (Fig. 2). The sizes of these PCR products were almost identical to those expected from human and mouse B₂ receptor cDNA (1063 bp and 1053 bp respectively). Sequence analysis of these PCR products showed high homology with human and mouse B₂ receptor cDNA (87% and 82% for dog skeletal muscle, 83% and 85% for rat L6 myoblasts respectively), which indicated that these fragments were amplified from dog and rat B₂ receptor mRNAs respectively.

2-Deoxyglucose uptake

Effects of bradykinin and insulin on glucose uptake were evaluated in both cell types. As shown in Fig. 3A and B, 2-deoxyglucose uptake in the presence of insulin (10⁻⁷ mol/l) showed significantly higher values compared with those in the absence of insulin (*P* < 0.001) in both cell types. In the presence of insulin in both cell types, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ mol/l bradykinin increased 2-deoxyglucose uptake in a dose-dependent manner. The increases in 2-deoxyglucose uptake at 10⁻⁹, 10⁻⁸ and

Figure 2 Detection of bradykinin B₂ receptor mRNA by RT-PCR amplification in dog skeletal muscle and rat L6 myoblasts. Total RNA (3 µg) prepared from dog skeletal muscle or rat L6 myoblasts was reverse-transcribed into cDNA using oligo dT primer. PCR amplifications were carried out with a pair of primers designed from bradykinin B₂ receptor cDNA, and the PCR product from each of the cells was subjected to 1.0% agarose gel electrophoresis. The expected size of DNA fragment was observed in each of the cells (middle and right lanes).

10⁻⁷ mol/l bradykinin were statistically significant ($P < 0.001$) in these cells. However, in the absence of insulin, bradykinin had no effect on glucose uptake at any concentration in both cell types.

2-Deoxyglucose uptake with various concentrations of insulin (0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ mol/l) in the absence or presence of bradykinin (10⁻⁷ mol/l) in L6 myoblasts is shown in Fig. 3C. In the absence of bradykinin, 10⁻⁸ and 10⁻⁷ mol/l insulin significantly stimulated 2-deoxyglucose uptake. In the presence of 10⁻⁷ mol/l bradykinin, the effect of insulin on 2-deoxyglucose uptake was significantly enhanced, and 2-deoxyglucose uptake increased by approximately 2-, 2.5- and 2.7-fold in the presence of 10⁻⁹, 10⁻⁸ and 10⁻⁷ mol/l insulin respectively when compared with insulin alone.

Effect of bradykinin on GLUT4 translocation

The effect of bradykinin on insulin-stimulated GLUT4 translocation from LDM to PM in dog skeletal muscle and rat L6 myoblasts was analyzed by immunoblotting using anti-GLUT4 antibody. A 55 kDa band corresponding to GLUT4 was observed in both PM and LDM in both cell types (Fig. 4). As shown in the upper panels of Fig. 4A and B, treatment with insulin (10⁻⁷ mol/l) significantly increased the GLUT4 content in the PM by 52% in skeletal muscle and by 55% in L6 myoblasts ($P < 0.05$). In contrast, the GLUT4 content in the LDM significantly decreased by 45% in skeletal muscle and by 47% in L6 myoblasts as shown in the lower panels of Fig. 4A and B ($P < 0.05$). Treatment with bradykinin

Figure 3 Effects of bradykinin treatment on [³H]deoxyglucose uptake in dog skeletal muscle and rat L6 myoblasts. After preincubation with various concentrations of bradykinin (0, 10⁻¹¹, 10⁻¹⁰, 10⁻⁹ and 10⁻⁸ mol/l) in the presence (hatched bars) or absence (open bars) of insulin (10⁻⁷ mol/l), [³H]deoxyglucose uptake into purified plasma membrane (PM) vesicles from isolated dog skeletal muscle (A), and L6 myoblasts (B) was measured for the last 3 min of the incubation period, and then corrected for [³H]-glucose trapped in the extracellular space of the cell layers. [³H]Deoxyglucose uptake was also measured in L6 myoblasts in the presence (solid bars) or absence (open bars) of bradykinin (10⁻⁷ mol/l) after preincubation with various concentrations of insulin (0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ mol/l) (C). Results are expressed as the mean $\pm$ s.d. ($n=6$). In (A) and (B), * $P < 0.001$ vs value without insulin, § $P < 0.001$ vs value with insulin alone. In (C), * $P < 0.001$ vs value without bradykinin, § $P < 0.001$ vs value with insulin alone, ¶ $P < 0.001$ vs value without insulin.

Figure 4 Effect of bradykinin on GLUT4 translocation in dog skeletal muscle (A) and rat L6 myoblasts (B). Representative results of the immunoblot analysis with anti-GLUT4 antibody and summary graph of GLUT4 contents in the PM (upper panels) and LDM fraction (lower panels) are shown. PM and LDM were prepared from dog skeletal muscle and L6 myoblasts as described in Materials and methods. Protein from each fraction (40 μ g) was subjected to immunoblot analysis with anti-GLUT4 polyclonal antibody. GLUT4 contents were quantitated using an Image Analyzer, and expressed as arbitrary units (see Materials and methods). Results are expressed as the mean $\pm$ s.d. ($n=6$). * $P<0.05$ vs value without insulin and bradykinin, § $P<0.05$ vs value with insulin alone.

alone (10^{-9} and 10^{-8} mol/l) did not change the GLUT4 content in the PM or LDM in these cells (data at 10^{-8} mol/l not shown). However, addition of bradykinin (10^{-8} mol/l) to insulin significantly increased GLUT4 content in the PM (by 45% and 48%, $P<0.05$) and decreased GLUT4 content in the LDM (by 45% and 47%, $P<0.05$) in skeletal muscle and L6 myoblasts respectively, when compared with insulin treatment alone. Although it was not statistically significant, 10^{-9} mol/l bradykinin in addition to insulin also increased GLUT4 content by 17% and 19% in the PM and decreased GLUT4 content by 25% and 27% in the LDM in skeletal muscle and L6 myoblasts respectively. These results suggested that bradykinin potentiated the insulin-induced translocation of GLUT4 from LDM to PM in dog skeletal muscle and L6 myoblasts.

Effect of bradykinin on insulin binding

To analyze the effect of bradykinin on the insulin binding affinities and numbers of insulin receptors in

dog skeletal muscle and rat L6 myoblasts, insulin binding assays were performed in the presence or absence of bradykinin. As summarized in Table 1, Scatchard analysis revealed typical curvilinear plots for insulin receptors with affinity constants of high (K_1) and low (K_2) affinity sites, and binding sites for high (R_1) and low (R_2) affinity sites in skeletal muscle and L6 myoblasts. As the results show, bradykinin treatment had no significant effect on either insulin binding affinities or numbers of insulin receptors in both cells.

Effect of bradykinin on phosphorylation of insulin receptor β subunit and IRS-1

To analyze the effect of bradykinin on tyrosine phosphorylation of insulin receptor and IRS-1, phosphorylation of each protein was analyzed by immunoblotting using anti-phosphotyrosine antibody. As shown in the upper panels of Fig. 5A and B, in the absence of insulin, tyrosine phosphorylation of insulin receptor β subunit in dog skeletal muscle and rat L6 myoblasts was

Table 1 Scatchard analysis of insulin binding assay in dog skeletal muscle and rat L6 myoblasts. K_1 and K_2 refer to the dissociation constants of insulin for the high- and low-affinity sites respectively. R_1 and R_2 refer to B_{max} (total binding capacity) of the high- and low-affinity sites respectively. Values are means $\pm$ s.d.

	K_1 (pmol/l)	K_2 (pmol/l)	R_1	R_2
			(sites/mg protein)	(sites/mg protein)
Skeletal muscle	2.6 ± 0.3	$4.5 \pm 0.8 \times 10^3$	$1.7 \pm 0.3 \times 10^5$	$5.1 \pm 1.3 \times 10^5$
Skeletal muscle + BK	2.5 ± 0.4	$4.6 \pm 0.9 \times 10^3$	$1.6 \pm 0.3 \times 10^5$	$5.0 \pm 0.8 \times 10^5$
			(sites/cell)	(sites/cell)
L6 myoblasts	1.4 ± 0.2	$3.9 \pm 0.3 \times 10^2$	$1.3 \pm 0.2 \times 10^5$	$3.7 \pm 0.8 \times 10^5$
L6 myoblasts + BK	1.5 ± 0.3	$4.1 \pm 0.9 \times 10^2$	$1.1 \pm 0.3 \times 10^5$	$3.8 \pm 0.9 \times 10^5$

BK, bradykinin.

Figure 5 Effects of bradykinin on phosphorylation of insulin receptor β -subunit and IRS-1 in dog skeletal muscle and rat L6 myoblasts. Protein fractions from dog skeletal muscle (A) and L6 myoblasts (B) were immunoprecipitated with anti-insulin receptor antibody (upper panels) or anti-IRS-1 antibody (lower panels) and then immunoblotting was performed with anti-phosphotyrosine antibody. The phosphorylation levels of insulin receptor β subunit and IRS-1 were examined, as described in Materials and methods. Results are expressed as the mean $\pm$ S.D. ($n=6$). * $P<0.05$ vs value with insulin alone.

not recognized at 0 and 10^{-9} mol/l bradykinin. On the other hand, phosphorylation of insulin receptor β subunit was detected by stimulation with 10^{-7} mol/l insulin in both cell types. In the presence of insulin, bradykinin (10^{-9} and 10^{-8} mol/l) significantly increased the phosphorylation of insulin receptor β subunit by 43 and 84% ($P<0.05$) in skeletal muscle and by 45 and 86% ($P<0.05$) in L6 myoblasts respectively compared with insulin alone. The same results were observed in the phosphorylation of IRS-1 in these cells (lower panels of Fig. 5A and B). In the absence of insulin, phosphorylation of IRS-1 in these cells was not recognized at 0 and 10^{-9} mol/l bradykinin. On the other hand, phosphorylation of IRS-1 was observed by stimulation with 10^{-7} mol/l insulin in both cell types. In the presence of insulin, bradykinin (10^{-9} and 10^{-8} mol/l) significantly increased phosphorylation of IRS-1 by 72 and 172% ($P<0.05$) in skeletal muscle and by 79 and 188% ($P<0.05$) in L6 myoblasts respectively.

Discussion

In this study, first we have demonstrated the presence of the bradykinin receptor in dog skeletal muscle by the RT-PCR method and a bradykinin receptor binding assay. Because the membrane preparations from dog skeletal muscle might have been contaminated with membranes from cells other than skeletal muscle, we also examined bradykinin receptors in a pure muscle system, using the L6 rat myoblast cell line. Although the dog bradykinin B_2 receptor cDNA has not yet been cloned, we expected to detect B_2 receptor mRNA in dog skeletal muscle and L6 myoblasts by RT-PCR using primers designed from highly conserved sequences

between human and mouse B_2 receptor cDNA (21, 22), since human and mouse cDNA have been revealed to be highly homologous with each other. As the results show, RT-PCR exhibited an apparent single band on the gel in each cell type. The size of the band from each cell type was almost identical to that predicted from human and mouse B_2 receptor cDNA. These PCR products do not seem to be those from B_1 receptor mRNA because they have less than 60% homology with reported B_1 receptor cDNA (25). Compared with our previous demonstration of PCR product from dog adipocyte B_2 receptor, the sequence of the PCR product from dog skeletal muscle in this study was identical with that of the dog adipocyte B_2 receptor (6).

In the bradykinin receptor binding assay, Scatchard transformation of the data yielded significant numbers of B_2 receptors in dog skeletal muscle and rat L6 myoblasts. The values of K_d are within the range of those determined for bradykinin binding in a number of cell types (26–30). Moreover, a K_d and a B_{max} from our previous data in dog adipocytes ($K_d=83$ pmol/l and $B_{max}=2.8$ fmol/ 10^5 cells) are almost the same as those in dog skeletal muscle. This evidence confirmed for us the presence of comparable numbers of functional B_2 receptors in dog skeletal muscle and rat L6 myoblasts.

In the present study we demonstrated that bradykinin stimulated glucose uptake in dog skeletal muscle and rat L6 myoblasts as well as in isolated adipocytes as we have previously described (6). Since the physiological concentrations of tissue bradykinin are reported to be in the range of 0.2 to 3.5×10^{-10} mol/l, higher bradykinin concentrations than the circulating blood bradykinin concentration (10^{-12} to 10^{-11} mol/l) were used in the

present *in vitro* study (2, 31). Taken together with our previous results which showed that bradykinin infusion could increase glucose uptake into dog peripheral tissues during a hyperinsulinemic euglycemic clamp (2), it was concluded that a primary effect of bradykinin was, at least in part, involved in glucose uptake into peripheral tissues (including skeletal muscle and adipocytes) *in vivo*.

Secondly, we evaluated the direct effect of bradykinin on the translocation of GLUT4 from LDM to PM. As the results show, GLUT4 translocations in both cells were not affected by bradykinin alone, but they were increased by bradykinin in the presence of insulin. From these results, it seems that bradykinin stimulates glucose uptake through GLUT4 translocation only in the presence of insulin.

Although bradykinin did not alter the affinities and numbers of the cell surface insulin receptors in both cells, bradykinin enhanced insulin-stimulated phosphorylation of insulin receptor β subunit in a dose-dependent manner. In addition, insulin-stimulated phosphorylation of IRS-1 was also enhanced by bradykinin treatment in both cells. As the increase in IRS-1 phosphorylation paralleled that of insulin receptor phosphorylation, this phenomenon seems to be the effect of enhanced activity of insulin receptor tyrosine kinase. To elucidate the mechanism by which bradykinin increases insulin receptor tyrosine kinase, we previously studied the time-course of the insulin-stimulated phosphorylation of insulin receptor β subunit with or without treatment with bradykinin in dog adipocytes. From the results, it was suggested that bradykinin might inhibit, in part, the dephosphorylation of insulin receptor β subunit. Furthermore, in our preliminary experiments in L6 myoblasts, insulin significantly activated phosphatase activity in a biphasic fashion, with two peaks at 5 min and 30 min, while bradykinin alone had no effect on phosphatase activity. On the other hand, bradykinin increased the first peak of the insulin-stimulated phosphatase activity and blunted the second peak (data not shown), which suggested the involvement of the phosphatases in the cross-talk between insulin and bradykinin signalings.

Bradykinin B_2 receptor, a member of the superfamily of G-protein-coupled receptors, stimulates the inositol triphosphate pathway via phospholipase C_β (PLC_β) and/or the arachidonic pathway via phospholipase A_2 (PLA_2) (32). When PLC_β is activated, there is hydrolysis of phosphatidylinositol 4,5-bisphosphate and diacylglycerol (33). Whereas the former mobilizes intracellular Ca^{2+} , the latter activates protein kinase C (PKC). Since previous studies have shown that the activation of PKC resulted in decreased insulin receptor tyrosine kinase (34, 35), enhanced tyrosine phosphorylation of insulin receptor observed with bradykinin stimulation in our study could be due to pathways other than PKC. Recently, Leeb and Song reported that bradykinin rapidly stimulated tyrosine phosphorylation of focal adhesion kinase (FAK) through bradykinin B_2 receptor-

coupled heterotrimeric G-protein in Swiss 3T3 cells, independent of PKC activity, and suggested that bradykinin-stimulated tyrosine phosphorylation might be related to inositol phosphate formation (36). In addition to insulin and insulin-like growth factor-I, which act via receptors with tyrosine kinase, several cytokines, interferons, and growth hormones also stimulate phosphorylation of IRS-1 and IRS-2 through receptors that lack intrinsic kinase activity, probably by the recruitment of cytoplasmic tyrosine kinases such as the Janus kinase (JAK) family (37–39). On the other hand, recent evidence indicates that ligands signaling through G-protein-coupled receptors may mimic some effects classically observed after activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, $PP125^{FAK}$, JAK2, and phosphotyrosine phosphatases (40, 41). Although PLC_β and PLA_2 signals are known to cause the major action of bradykinin, it is possible that many other pathways exist in the bradykinin signaling pathway. To clarify the mechanism by which bradykinin receptor and insulin receptor signal transduction systems could cross-talk with each other, further analysis will be necessary.

It is concluded that in dog skeletal muscle and rat L6 myoblasts, as well as in dog adipocytes, bradykinin can increase insulin-stimulated phosphorylation of insulin receptor β subunit and IRS-1 which, in turn, potentiates the translocation of GLUT4, finally resulting in increased glucose uptake.

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Received 16 June 1997

Accepted 13 November 1997