Insulin Enhances the Bradykinin Response in L8 Rat Skeletal Myoblasts

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Inhibitors of ACE/kininase II enhance insulin sensitivity, an action that is mediated in part by bradykinin (BK). We investigated whether insulin interacts with the BK receptor signaling to modulate the inositol 1,4,5-trisphosphate (IP₃) response to BK in L8 rat skeletal myoblasts. Stimulation of the cultures with BK (10 nmol/l) for 15 s increased IP₃ from a basal level of 75.2 ± 7.6 to 200.2 ± 15.7 pmol/mg protein. Treatment of the cultures with 1, 2, and 20 nmol/l of insulin for 90 min before adding BK increased IP₃ formation by the same BK dose to 328.2 ± 19, 434.5 ± 18, and 460.8 ± 21.3 pmol/mg protein, respectively. When wortmannin was administered to inhibit phosphatidylinositol (PI) 3-kinases at lower concentration (1 nmol/l), it increased IP₃ formation stimulated by BK only when insulin was present. At a higher concentration (100 nmol/l), wortmannin significantly enhanced BK-induced IP₃ formation in the absence of insulin. Genistein and tyrphostin A-23, tyrosine kinase inhibitors, completely reversed the elevated IP₃ formation by BK and insulin. The IP₃ response to 10 nmol/l BK was 223.3 ± 11.8 pmol/mg protein in the absence of insulin and 402.2 ± 12.0 pmol/mg protein in the presence of 2 nmol/l insulin. However, when exposing the cultures to 1 nmol/l genistein or tyrphostin A-23, the IP₃ response to BK in the presence of insulin decreased to 211.8 ± 46.7 and 187.7 ± 19.9 pmol/mg protein. Tyrphostin A-1, the inactive analog, was ineffective. Exposing the cells to 1 mmol/l 3,4,5-trimethoxybenzoic acid 8-[diethylamino]octyl ester, an intracellular Ca²⁺ antagonist, did not change the potentiation by insulin. But, exposing them to 0.1 mmol/l n-[6-aminohexyl]-5-chloro-1-naphthalene-sulfonamide, a calmodulin antagonist, resulted in enhanced IP₃ response to BK alone to 292.2 ± 18.5 pmol/mg protein and to BK in the presence of 1, 2, and 20 nmol/l insulin to 488 ± 22.2, 625.5 ± 11.6, and 665.2 ± 15.9 pmol/mg protein, respectively. In conclusion, insulin potentiates BK-induced IP₃ production in L8 rat skeletal myoblasts, and this action of insulin involves a tyrosine kinase. Inhibition of PI 3-kinases potentiated BK-induced IP₃ formation in the presence of insulin. Calmodulin blocked the action of insulin. These results support a modulatory effect of insulin on the BK signaling system via a tyrosine kinase in L8 rat skeletal myoblasts that results in increased IP₃ formation. Because BK release from skeletal muscle increases during contractions, this action of insulin is likely to play a role in the modulation of the excitation-contraction coupling process of the skeletal muscle.

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ACE/kininase II inhibitors improve insulin sensitivity (1–7). This action of ACE inhibitors is mediated in part by bradykinin (BK), a peptide known to enhance GLUT4 translocation (8–10). Skeletal muscle, making up 25–45% of the body weight in humans, represents the largest target for insulin. In this tissue, insulin binds to insulin receptors on the cell membrane and promotes phosphorylation of the β-subunit of the receptor, a process that, in turn, results in phosphorylation of the intracellular insulin receptor substrate (IRS)-1 on tyrosine residues and activation of phosphatidylinositol (PI) 3-kinase (11). Activation of PI 3-kinase is an important step in the translocation of GLUT4 in skeletal muscle (11).

Several components of the kallikrein-kinin system have been identified in skeletal muscle. A tissue kallikrein, an enzyme responsible for the formation of kinins, has been isolated and purified from and quantitated in rat skeletal muscle (12). BK is released from skeletal muscle during brief static contractions of relatively high intensity (13). We recently reported that BK B₂ receptors are present in skeletal muscle and that stimulation of these receptors results in activation of a phosphoinositide-specific phospholipase C (PLC) and a transient increase in the formation of inositol 1,4,5-trisphosphate (IP₃) (14,15). In skeletal muscle, IP₃ may not only participate in the physiological regulation of excitation-contraction coupling (16–18), but could also play a role in the pathophysiology of muscle diseases such as malignant hyperthermia (19–21) and exertional rhabdomyolysis (22,23).

The aim of the present study was to investigate whether insulin interacts with the signaling from the BK receptor to modulate BK-induced IP₃ formation in L8 rat skeletal myoblasts. Because several peptides, including BK (24) and insulin (25), induce tyrosine phosphorylation of proteins in cultured cells and intact animals, we investigated whether a tyrosine kinase participates in the regulation of BK-induced IP₃ formation by insulin. Because PI 3-kinase is one of the key signal transducers in insulin-stimulated glucose uptake, we also studied whether insulin-stimulated PI 3-kinase mediates...
the effect of insulin on BK-induced IP3 formation. In addition, we also examined whether intracellular calcium or calmodulin modulate this combined action of insulin and BK.

**RESEARCH DESIGN AND METHODS**

**Cell culture and incubations.** Monolayers of L8 rat skeletal myoblasts were grown for 4 days in a mixture of M199 and Dulbecco's modified Eagle's medium in a 1:4 ratio, containing antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin) and supplemented with 20% horse serum. Cells were grown on 0.1% gelatin-coated 25 cm² flasks (Costar, Cambridge, MA) in an atmosphere of 5% CO2/95% air at 37°C. At 24 h before the experiments, the nearly confluent cells were incubated in serum-depleted medium.

Cultures were incubated with one of the following reagents: wortmannin (an inhibitor of PI 3-kinases), 3,4,5-trimethoxybenzoic acid 8-[diethylamino]octyl ester (TMB-8) (a calcium antagonist), n-(6-aminohexyl)-5-chloro-2-naphthalene-sulfonamide (W-7) (a calmodulin antagonist), or the vehicle for 120 min and with insulin or the vehicle for the last 90 min before adding BK. Incubations with genistein, tyrphostin A-23 (two tyrosine kinase inhibitors), or the vehicle were done for 90 min before insulin (2 nmol/ml). The cultures were then incubated for 120 min at 37°C. At 1 nmol/l concentrations, the cultures were incubated with 1, 2, and 20 nmol/l insulin for 90 min before adding BK.

**IP3 measurement.** After the serum-depleted L8 cells were incubated with BK for 15 s, the reaction was stopped by the addition of 0.2 ml ice-cold 100% trichloroacetic acid (TCA) for each 1 ml of culture medium. The acid extract was homogenized at 0–4°C, incubated for 15 min on ice, centrifuged for 30 min at 2,000 × g, and the pellet was discarded. TCA was removed from the extracts by adding 2 ml of a mixture of 3 volumes of 1,1,2-trichloro-1,2,2-trifluoroethane plus 1 volume of tri-octylamine for each 1 ml of TCA extract.

IP3 content in the aqueous top layer was determined by using a radioreceptor assay kit from NEN Research Products–Du Pont (Boston, MA).

**Protein measurements.** Protein was determined by the method of Bradford with bovine serum albumin as standard (26).

**Drugs and solutions.** L8 rat skeletal myoblasts were obtained from the American Type Culture Collection (Rockville, MD). TMB-8, W-7, and wortmannin (from Penicillium fumiculosum) were purchased from Calbiochem (San Diego, CA) and dissolved in 0.5% DMSO and kept protected from light. The final concentration of this solvent in the culture flasks was <0.006%. Wortmannin was dissolved in 0.1% dimethyl sulfoxide and was added to the culture medium in <10 µl volume. All other drugs were dissolved in water and added to the culture medium in <100 µl volume.

**Statistical analysis.** Results are expressed as means ± SE. Each experiment was carried out in duplicate. Every experiment was repeated six times with different cell preparations. The significance of the differences was determined with analysis of variance. When a significant F value was obtained, comparisons of mean values were done with the Student’s t-test for paired and unpaired samples, the Bonferroni test, and the Student-Newman-Keuls multiple comparison test. A P < 0.05 was considered statistically significant.

**RESULTS**

Stimulation of L8 rat skeletal myoblasts with BK (10 nmol/l) for 15 s increased IP3 formation from a basal level of 75.2 ± 7.6 to 434.5 ± 18, and 460.8 ± 21.3 pmol/mg protein, respectively (Fig. 1). Treatment of the cultures with 1, 2, and 20 nmol/l of insulin for 90 min before adding BK increased the IP3 response to the same BK dose to 328.2 ± 19, 434.5 ± 18, and 460.8 ± 21.3 pmol/mg protein, respectively (Fig. 1). Insulin alone did not increase IP3 formation; this required stimulation of the BK receptor by the ligand.

We investigated the effect of wortmannin, a PI 3-kinase antagonist, on BK-induced IP3 production in combination with various concentrations of insulin. Wortmannin at 10 nmol/l had no effect on IP3 production during basal conditions or after BK was added. However, it significantly increased IP3 formation in response to BK in the presence of insulin (Figs. 2 and 3). At a higher concentration (100 nmol/l), wortmannin significantly enhanced the BK-induced IP3 formation by itself, without insulin (Fig. 3).

To study the effect of tyrosine kinase inhibition on the potentiation of BK-induced IP3 formation by insulin, genistein or tyrphostin A23 were added to the cultures 10 min before the addition of buffer or insulin (2 nmol/ml). The cultures were then incubated for 120 min at 37°C. At 1 nmol/l concentration, both genistein and tyrphostin A23, highly selective tyrosine kinase inhibitors, completely reversed the potentiation of IP3 formation by insulin (Figs. 4 and 5). The IP3 response to 10 nmol/l BK was 223.3 ± 11.8 pmol/mg protein in the absence of insulin and 402.2 ± 12.0 pmol/mg protein in the presence of 2 nmol/l insulin. However, when the cultures were exposed to 1 nmol/l genistein or tyrphostin A23, the IP3 response to BK in the presence of insulin was 211.8 ± 46.7 and 187.7 ± 19.9 pmol/mg protein, values not significantly different from the response to BK alone. On the other hand, at a higher concentration (1 µmol/l), genistein or tyrphostin A23 appear to have nonspecific effects, since both potentiated the...
basal IP3 release and the BK-induced IP3 formation without affecting the action of insulin (Figs. 4 and 5). Tyrphostin A1, the inactive analog, had no significant effect on the level of IP3 in response to BK plus insulin. In the presence of 1 nmol/l tyrphostin A1, BK increased IP3 formation from 95.2 ± 5.6 to 236.8 ± 9.9 pmol/mg protein, and insulin (2 nmol/l) potentiated the effect of BK to 401.8 ± 10.6 pmol/mg protein. Increasing the concentration of tyrphostin A1 to 1 µmol/l yielded similar results. BK increased IP3 formation from 144.2 ± 12 to 352.7 ± 17.8 pmol/mg protein, and insulin (2 nmol/l) potentiated the effect of BK to 425 ± 12.7 pmol/mg protein.

BK acts through Ca2+-dependent mechanisms, and many Ca2+-mediated events require the formation of Ca2+-calmodulin complexes (27). Likewise, Ca2+ and calmodulin participate in the molecular mechanism whereby binding of insulin to its receptor is coupled to changes in cellular metabolism (28,29). To determine whether intracellular Ca2+ and calmodulin are involved in the potentiation of BK-induced IP3 formation by insulin, we studied the effect of TMB-8, an intracellular Ca2+ antagonist, and W-7, a calmodulin blocker. Treatment of the cultures with TMB-8 (1 µmol/l) did not affect the IP3 response to BK alone or in combination with insulin. In the presence of this inhibitor, IP3 formation increased from 75.2 ± 8 to 244.8 ± 18 pmol/mg protein after BK alone and to 371.5 ± 16.5, 477 ± 18, and 488 ± 16 pmol/mg protein after BK in combination with 1, 2, and 20 nmol/l insulin, respectively. In contrast, W-7 (0.1 µmol/l), which by itself had no effect on basal or BK-induced IP3 formation, significantly increased the IP3 response to BK in the presence of 1, 2, and 20 nmol/l insulin to 488 ± 22.2, 625.5 ± 11.6, and 665.2 ± 15.9 pmol/mg protein, respectively (Fig. 6).

**FIG. 3.** Effect of wortmannin on BK-induced IP3 production. L8 rat skeletal myoblasts were incubated for 120 min in the presence of wortmannin (1, 10, and 100 nmol/l) with or without exposure to insulin (1 and 2 nmol/l) for the last 90 min at 37°C. IP3 production was measured after BK (10 nmol/l) stimulation for 15 s. Data are means ± SE; n = 6. **P < 0.01 vs. wortmannin 1 nmol/l. ‡‡ P < 0.01 vs. insulin 1 nmol/l. ##P < 0.01, ###P < 0.001.

**FIG. 4.** Effect of the tyrosine kinase inhibitor genistein on the potentiation of BK-induced IP3 formation by insulin. Serum-depleted L8 rat skeletal myoblasts were incubated for 130 min in the presence or absence of genistein (1 nmol/l and 1 µmol/l) with or without exposure to insulin (2 nmol/l) for the last 120 min at 37°C. IP3 production was measured after BK (10 nmol/l) stimulation for 15 s. Data are means ± SE; n = 6. ‡‡‡ P < 0.001 vs. buffer or insulin. ***P < 0.001 vs. either buffer or insulin and genistein 1 nmol/l. §§§P < 0.001 vs. either buffer or insulin and genistein 1 µmol/l. †††P < 0.001.

**FIG. 5.** Effect of the tyrosine kinase inhibitor tyrphostin A23 on the potentiation of BK-induced IP3 formation by insulin. Serum-depleted L8 rat skeletal myoblasts were incubated for 130 min in the presence of tyrphostin A23 (1 nmol/l and 1 µmol/l) with or without exposure to insulin (2 nmol/l) for the last 120 min at 37°C. IP3 production was measured after BK (10 nmol/l) stimulation for 15 s. Data are means ± SE; n = 6. ‡‡‡ P < 0.001 vs. either buffer or insulin. **P < 0.01, ***P < 0.001 vs. either buffer or insulin and tyrphostin A-23 1 nmol/l. §§§P < 0.001 vs. either buffer or insulin and tyrphostin A-23 1 µmol/l. †††P < 0.001.
in insulin, which had little effect on its own, potentiated IP₃ release by bombesin. On the other hand, in Xenopus oocytes, insulin itself induced early (15 s), intermediate (15–20 min), and late (2–3 h) increases in IP₃ formation (31).

Several enzymes are involved in phosphoinositide metabolism, including PLC, PI kinases, tyrosine kinases, and phosphatases. Activation of PLC mediated by G-proteins generates two second messengers: 1) diacylglycerol, which activates protein kinase C; and 2) IP₃, which can mobilize intracellular calcium (32). In addition, direct binding of the PLC-γ isozyme to some autophosphorylated protein tyrosine kinase receptors, such as the insulin receptor, can lead to the generation of the same two second messengers (33).

Transduction of the insulin signaling begins with the binding of insulin to the α-subunit of the receptor and activation of the protein tyrosine kinase associated with the β-subunit, which then phosphorylates itself at multiple tyrosine residues. When this occurs, IRS-1 and IRS-2 interact with the β-subunit and are then phosphorylated by the receptor at many tyrosine residues. This allows these substrates to interact with and recruit to the plasma membrane the enzyme PI 3-kinase, which plays an essential role in mediating nearly all the metabolic actions of insulin. In the present work, we found that the inhibition of PI 3-kinase with wortmannin, the fungal metabolite that irreversibly binds to the p110 catalytic subunit of PI 3-kinase and inhibits its serine kinase and lipid kinase activity (34), potentiated IP₃ formation after either BK alone (at 100 nmol/l wortmannin) or BK plus insulin (at 1 nmol/l wortmannin). Of the three forms of PI kinase (type I, II, and III), type I phosphorylates the D-3 position of the inositol ring, whereas types II and III phosphorylate the D-4 position (35). The three phosphoinositide products of the type I kinase [PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃] appear to be resistant to cleavage by PLC (36,37). Thus, when PI 3-kinase is inhibited, the pathway leading to the formation of phosphoinositides phosphorylated in position D-3 of the inositol ring is blocked and more substrate is available for IP₃ formation by PLC in response to BK (38).

In our study, insulin appears to potentiate the action of BK on IP₃ formation via activation of a tyrosine kinase, because this action of insulin was inhibited by genistein and tyrphostin. Tyrosine kinases modulate inositol phosphate formation and signaling. For example, BK and bombesin rapidly stimulate tyrosine phosphorylation of a 120-kDa group of proteins in Swiss 3T3 fibroblasts (39). In these cells, genistein appears to have a biphasic effect on the BK-promoted increase in inositol phosphate formation. At 10 μmol/l, genistein partially inhibited BK stimulation of inositol phosphate formation, whereas at 100 μmol/l genistein, the response to BK returned toward the control level. In ras-transformed NIH/3T3 fibroblasts, the signals from the B₂ BK receptor leading to Ca²⁺ influx follow two pathways that can be blocked with tyrosine kinase inhibitors (40). One pathway is via the ras-mitogen-activated protein kinase cascade and the other is via the PLC pathway that produces inositol 1,3,4,5-tetrakisphosphate (IP₄). Because the intracellular application of IP₄ did not affect Ca²⁺ influx, the tyrosine phosphorylation related to PLC/phosphoinositides is located downstream of IP₃ formation, a site unlikely to be the place of action of insulin to enhance IP₃ formation in response to BK. The fact that two structurally distinct tyrosine kinase inhibitors blocked the effect of insulin on BK-stimulated IP₃ formation, while an inactive analog of tyrphostins (tyrphostin A1) did not, strongly suggests that tyrosine kinases mediate this novel action of insulin.

Termination of the IP₃ signal occurs either by the IP₃ 3-kinase, which adds a phosphate to the inositol ring forming IP₄, or by the action of the polyphosphate 5-phosphatase, which removes a phosphate from the inositol ring producing inositol 1,4-bisphosphate (41). The activity of IP₃ 3-kinase is regulated by calcium-calmodulin (42–44), enzyme phosphorylation (45), and by altering mRNA expression (46). In this study, we assessed the contribution of intracellular Ca²⁺ and calmodulin to the potentiation of BK-induced IP₃ formation by insulin because of the existing evidence that both Ca²⁺ and calmodulin are spatially and functionally linked to the molecular events that occur upon the binding of insulin to its receptor (28,29). The lack of changes in the levels of IP₃ that are attained after insulin and BK in cultures that were treated with TMB-8, an intracellular Ca²⁺ antagonist (47), suggests that the action of insulin on BK-stimulated IP₃ formation does not depend on increases in intracellular Ca²⁺. However, when the cultures were treated with the calmodulin antagonist W-7, the action of insulin was significantly augmented. It is interesting to propose that calmodulin could be part of a negative feedback mechanism that controls the action of insulin on the IP₃ response to BK. On the other hand, inhibition by insulin of the metabolism of IP₃ by a polyphosphoinositol 5′-phosphatase is a possibility that needs to be explored, although insulin appears to rather stimulate polyphosphoinositol 5′-phosphatases (48).

In summary, although insulin does not increase IP₃ formation per se, it significantly potentiates the IP₃ production in response to BK. Inhibition of PI 3-kinase potentiated IP₃ formation in the presence of insulin. A tyrosine kinase with high affinity for the inhibitors genistein and tyrphostin appears to mediate the potentiation of BK-induced IP₃ formation by insulin. Calmodulin has a negative effect on the action of insulin on BK-induced IP₃ formation. Taken together, these results provide evidence for a modulatory action of insulin on the signal transduction initiated by BK in...
L8 rat skeletal myoblasts that results in potentiation of the BK action on phosphoinositide metabolism. This action of insulin is likely to play a role in the regulation of the excitation-contraction coupling process of skeletal muscle.

REFERENCES


