Bradykinin Directly Triggers GLUT4 Translocation Via an Insulin-Independent Pathway

Kazuhiro Kishi, Naoko Muromoto, Yutaka Nakaya, Ikuko Miyata, Akifumi Hagi, Hideki Hayashi, and Yousuke Ebina

Physical exercise induces translocation of GLUT4 from an intracellular pool to the cell surface in skeletal muscles and increases glucose uptake via an insulin-independent pathway. However, the molecular mechanism remains to be identified. Some studies have suggested that bradykinin is locally released from contracting muscles and may be responsible for GLUT4 translocation and the increase of glucose transport in skeletal muscles. To determine whether bradykinin directly triggers GLUT4 translocation, we established L6 myotubes, 3T3-L1 adipocytes, and Chinese hamster ovary cells stably expressing c-*myc* epitope-tagged GLUT4 (GLUT4*myc*) and bradykinin B_2 receptors. We found that bradykinin directly triggered GLUT4*myc* translocation and increased the rate of glucose uptake in a dose-dependent manner in these cells. The translocation with bradykinin occurred even after pretreatment with an islet-activating protein, wortmannin, and phorbol 12,13-dibutyrate. The signaling pathway does not seem to be mediated by G_i, phosphatidylinositol 3-kinase, or protein kinase C. It is insulin-independent and via trimeric G-protein G_q. Bradykinin is probably one of the factors responsible for exercise-stimulated glucose uptake in skeletal muscles. *Diabetes* 47:550–558, 1998

GLUT4 is expressed exclusively in adipocytes and skeletal and heart muscles (1,2), and translocation of GLUT4 from an intracellular pool to the plasma membrane is a major mechanism of the insulin-stimulated glucose uptake in these tissues (1–4). A similar recruiting mechanism of GLUT4 is presented for the enhanced glucose uptake during physical exercise (5,6) and under conditions of hypoxia (7). However, exercise-induced GLUT4 translocation is thought to be mediated via an insulin-independent pathway (8–10), but related mechanisms are unknown.

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BK_2R, bradykinin B_2 receptor; BSA, bovine serum albumin; CHO, Chinese hamster ovary; IAP, islet-activating protein; IR, insulin receptor; IRS-1, insulin receptor substrate-1; KRHB, Krebs-Ringer-HEPES buffer; PBS, phosphate-buffered saline; PDBu, phorbol 12,13-dibutyrate; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

To examine the molecular mechanisms of GLUT4 translocation, we developed a highly sensitive and quantitative method to measure directly c-*myc* epitope-tagged GLUT4 (GLUT4*myc*) on the cell surface (11). Using this system, we found that phosphatidylinositol (PI) 3-kinase (p85/p110 heterodimer type) plays a key role in GLUT4 translocations that are triggered by insulin and also by platelet-derived growth factor and epidermal growth factor (12–14). We reported that G_q-coupled α_{1b}-adrenergic receptors transmit the signal for GLUT4 translocation via an insulin-independent pathway, and that G_q-coupled receptors are one target of the GLUT4 translocation induced by guanosine 5′-*O*-(3-thiotriphosphate) (15). The adrenergic stimulations of GLUT4 translocation and glucose uptake via G_q may possibly contribute to the fuel supply required for thermogenesis in brown adipocytes and for enhanced contractility in cardiomyocytes.

In skeletal muscles in vivo, bradykinin receptors are expressed (16,17) and coupled with G_q (18–20). Bradykinin is released from muscle cells during physical exercise (21–23). Therefore, the possibility that bradykinin might be responsible for the exercise-stimulated GLUT4 translocation in skeletal muscles requires attention (24). We now provide evidence that bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway in L6 myotubes, 3T3-L1 adipocytes, and Chinese hamster ovary (CHO) cells expressing G_q-coupled bradykinin B_2 receptors (BK_2Rs). The bradykinin-stimulated GLUT4 translocation may explain the exercise-induced glucose uptake in muscle cells.

Bradykinin is a nonapeptide hormone that mediates physiological effects, such as pain, inflammation, vascular permeability, hypotension, edema formation, smooth muscle contraction, and glucose utilization (25–27). Bradykinin receptors are mainly classified pharmacologically into two subtypes, i.e., B_1 and B_2 (28), and molecular cloning revealed their existence (29–32). A bradykinin B_3 receptor has been proposed (33,34). Most of the known actions of bradykinin are mediated via B_2 receptors. BK_2Rs couple to mainly heterotrimeric G-protein, the G_q subfamily; G_i and G_s subfamilies are also reported to couple bradykinin receptors in some cell types (18–20). BK_2Rs are present on the surface of skeletal muscle tissues (16,17).

L6 myotubes have been widely used to investigate the mechanism of glucose transport in skeletal muscles (35,36). Insulin stimulates GLUT4 translocation and increases the rate of glucose uptake in L6 myotubes, as in skeletal muscles (37,38). Therefore, bradykinin-induced GLUT4 translocation is possibly one mechanism of exercise-stimulated glucose transport in skeletal muscles.

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RESEARCH DESIGN AND METHODS

Cells and materials. The parent cell lines used in this study were L6-GLUT4*myc*, an L6 cell line (provided by Dr. Amira Klip, the Hospital for Sick Children, Toronto, Ontario, Canada) expressing GLUT4*myc*, constructed by inserting a human c-*myc* epitope (14 amino acids) into the first ectodomain of GLUT4 (11); 3T3-L1-GLUT4*myc*, a 3T3-L1 fibroblast line expressing GLUT4*myc*; and CHO-GLUT4*myc*, a CHO cell line expressing GLUT4*myc*. The L6-GLUT4*myc* myoblasts were cultured to the stages of myotubes, as described previously (37). The 3T3-L1-GLUT4*myc* fibroblasts were induced to differentiate into adipocytes, as described previously (11). All other reagents were of analytical grade.

Establishment of stable cell lines expressing BK_2Rs. The mouse BK_2R (32) and the human BK_2R (31) were subcloned into a mammalian expression vector, pCXN (39). These plasmids were cotransfected into L6-GLUT4*myc* cells, 3T3-L1-GLUT4*myc* cells, and CHO-GLUT4*myc* cells with pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Several independent clones expressing BK_2Rs were established and designated as follows: L6-GLUT4*myc*-BK_2R cells were L6-GLUT4*myc* cells stably expressing the mouse BK_2Rs; 3T3-L1-GLUT4*myc*-BK_2R cells were 3T3-L1-GLUT4*myc* cells stably expressing the mouse BK_2Rs; and CHO-GLUT4*myc*-BK_2R cells were CHO-GLUT4*myc* cells stably expressing the human BK_2Rs.

Cell surface anti–c-*myc* antibody binding assay (GLUT4*myc* translocation assay). Cells in 24-well plates were incubated in 500 µl of Krebs-Ringer-HEPES buffer (KRHB) (11) for 20 min at 37°C and then with indicated concentrations of ligands for indicated periods at 37°C. After fixation with 2% paraformaldehyde, cells were washed three times with phosphate-buffered saline (PBS) and then incubated with 0.1N glycine/PBS for 15 min. After blocking with KRHB for 30 min at room temperature, cells were incubated with 300 µl of anti–c-*myc* antibody (monoclonal antibody 9E10; 1:1,000 dilution) (provided by ATCC, Rockville, MD) for 2 h, washed with PBS, and incubated for 1 h with 300 µl of horseradish peroxidase-conjugated anti-mouse IgG (1:2,000 dilution; Chemicon, El Segundon, CA) at room temperature. The wells were then washed five times with PBS. GLUT4*myc* translocation was determined and analyzed by enhanced chemiluminescence (Amersham, Amersham, U.K.) and Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan).

2-Deoxyglucose uptake measurement. Cells in 24-well plates were treated with indicated concentrations of ligands for indicated periods at 37°C. 2-Deoxyglucose uptake was measured as described previously (11).

Downregulation of protein kinase C (PKC) with phorbol 12,13-dibutyrate (PDBu) and pretreatment with islet-activating protein (IAP) or wortmannin. Cells were pretreated with or without PDBu (100 ng/ml) for 24 h at 37°C in medium for downregulating PKC and with or without IAP (100 ng/ml; Funakoshi) for 24 h at 37°C in medium that abolishes G_i-coupled pathway(s). To inactivate PI 3-kinases, the cells were pretreated with or without the indicated concentrations of wortmannin for 20 min at 37°C.

Glycogen synthesis assay. 3T3-L1-GLUT4*myc*-BK_2R adipocytes or L6-GLUT4*myc*-BK_2R myotubes (six-well plate) were incubated in glycogen assay buffer (25 mmol/l Tris, pH 7.5, 140 mmol/l NaCl, 1.7 mmol/l KCl, 0.9 mmol/l $CaCl_2$, 1.47 mmol/l K_2HPO_4, 0.8 mmol/l $MgSO_4$, 0.2% bovine serum albumin [BSA]) for 20 min at 37°C, then treated with insulin, bradykinin, or insulin plus bradykinin for 10 min at 37°C, and the reaction was initiated by the addition of [^{3}H]glucose (3 µCi/sample) and 4 mmol/l glucose. After 1 h of incubation, the reaction was terminated by washing three times with ice-cold KRHB (BSA-free), and the cells were then solubilized in 20% KOH. The radiolabeled glucose incorporation into glycogen was measured as described previously (40).

Lipogenesis assay. Lipogenesis assay was performed as described previously (41) but with some modifications. In brief, 3T3-L1-GLUT4*myc*-BK_2R adipocytes (6-well plate) were incubated in KRHB for 20 min at 37°C, then treated with insulin, bradykinin, or insulin plus bradykinin for 10 min at 37°C, and the reaction was initiated by the addition of [^{3}H]glucose (3 µCi/sample) and 4 mmol/l glucose. After a 1-h incubation, the reaction was terminated by washing three times in ice-cold KRHB (BSA-free). The radiolabeled glucose into lipid was assessed by scraping cells into 1 ml of PBS and shaking with 5 ml of toluene-based scintillant. After leaving it to settle overnight, the radioactivity that partitioned into the organic phase was determined by scintillation counting.

RESULTS

Insulin-stimulated GLUT4 translocation in L6 myotubes. L6 myoblasts originated from day-old rat skeletal muscle (36). The myoblasts differentiate spontaneously after confluency into multinucleated myotubes when cultured in low concentrations of serum. With the progression of L6 cell fusion, expression of endogenous GLUT4 as well as of myogenic factors increases (37). To examine the molecular mechanisms of GLUT4 translocation in skeletal muscle

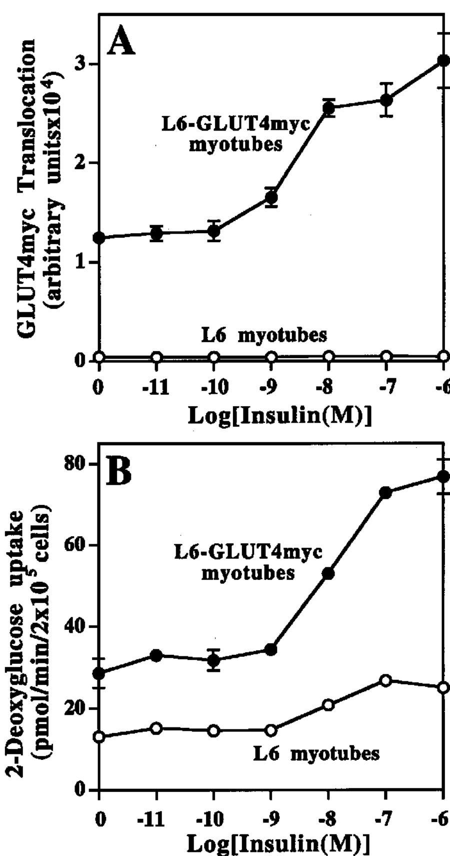

FIG. 1. Dose-dependent GLUT4*myc* translocation (*A*) and glucose uptake (*B*) in response to insulin in L6-GLUT4*myc* myotubes. L6 myoblasts were differentiated into L6 myotubes by incubating with low serum, as described under METHODS, and used for GLUT4 translocation assays. The parent cells (L6 myotubes) (○) and those expressing GLUT4*myc* (L6-GLUT4*myc* myotubes) (●) were incubated with various concentrations of insulin for 10 min at 37°C. The GLUT4*myc* translocation (*A*) and glucose uptake (*B*) were measured as described under METHODS. Values represent means ± SE of three separate experiments done in triplicate.

cells, we established L6 myoblasts stably expressing c-*myc* epitope-tagged GLUT4 (GLUT4*myc*). Insulin triggered GLUT4*myc* translocation in a dose-dependent manner in the L6 myotubes expressing GLUT4*myc* (L6-GLUT4*myc* myotubes) (Fig. 1*A*). As shown in Fig. 1*B*, insulin treatment also increased the rate of glucose uptake in almost the same dose-dependent manner as GLUT4*myc* translocation in the L6-GLUT4*myc* myotubes. The increment of glucose uptake in L6-GLUT4*myc* myotubes was greater than that of L6 myotubes, and the insulin dose-dependencies between the two cell lines were much the same. Therefore, the GLUT4*myc* expressed in L6 myotubes is functional to take up glucose and behaves like endogenous GLUT4 in L6 myotubes. The extent of insulin-stimulated GLUT4 translocation and glucose uptake in L6 myotubes is less than that in 3T3-L1 adipocytes (11,15).

Bradykinin-stimulated GLUT4 translocation in L6 myotubes, 3T3-L1 adipocytes, and CHO cells. Bradykinin is locally released from contracting skeletal muscles and is a candidate molecule involved in exercise-

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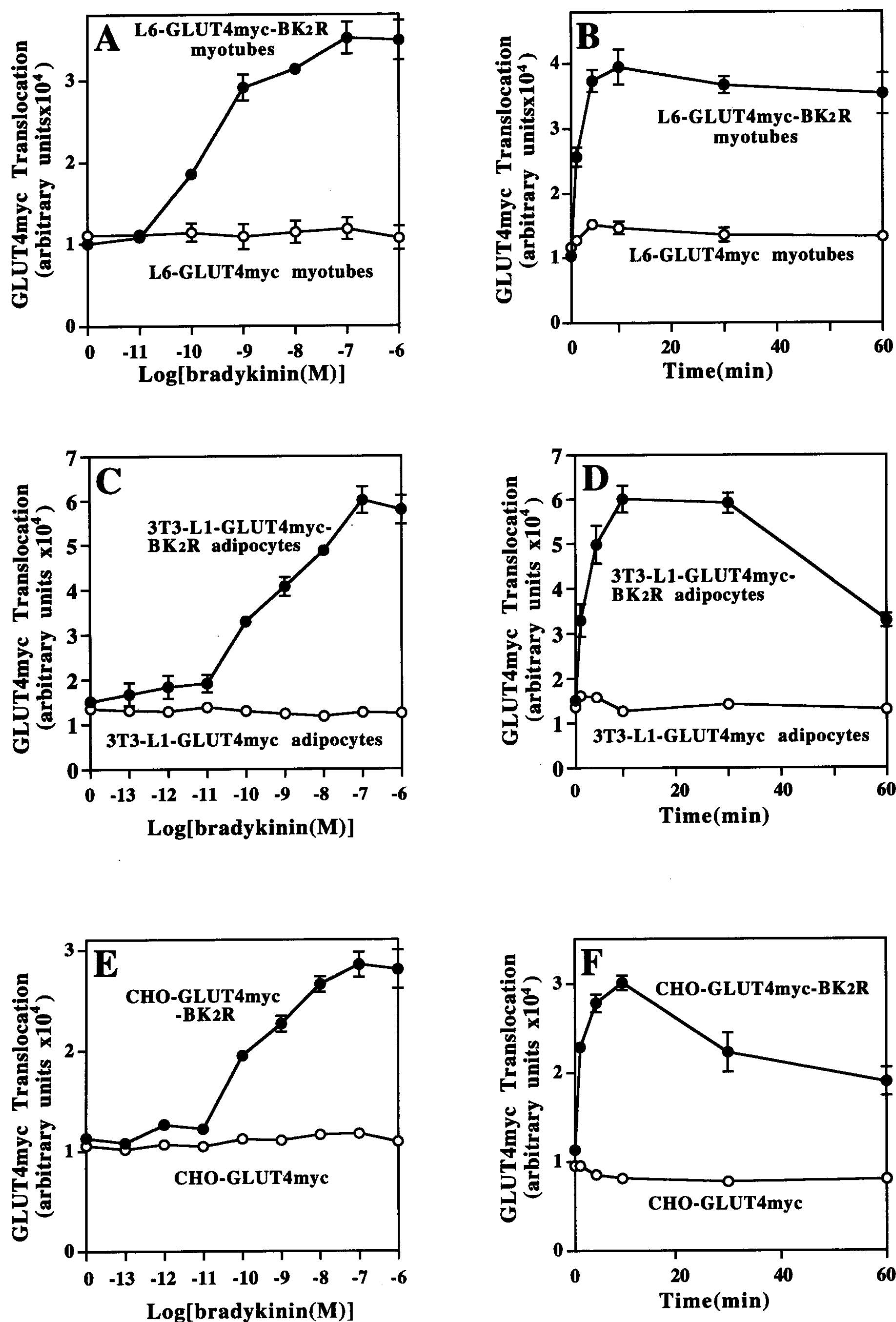

FIG. 2. Dose- and time-dependent GLUT4*myc* translocation in response to bradykinin in L6-GLUT4*myc*-BK_2R myotubes, 3T3-L1-GLUT4*myc*-BK_2R adipocytes, and CHO-GLUT4*myc*-BK_2R cells. *A* and *B*: The parent cells (L6-GLUT4*myc* myotubes) (○) and those expressing BK_2Rs (L6-GLUT4*myc*-BK_2R myotubes) (●) were incubated with various concentrations of bradykinin for 10 min at 37°C (*A*) or with 10^{-7} mol/l bradykinin for the indicated periods (*B*). The GLUT4*myc* translocation was measured as described under METHODS. *C* and *D*: The parent cells (3T3-L1-GLUT4*myc* adipocytes) (○) and those expressing BK_2Rs (3T3-L1-GLUT4*myc*-BK_2R) (●) were stimulated with various concentrations of bradykinin for 10 min at 37°C (*C*) or with 10^{-7} mol/l bradykinin for the indicated periods (*D*). The GLUT4*myc* translocation was measured. *E* and *F*: The parent cells (CHO-GLUT4*myc* cells) (○) and those expressing BK_2Rs (CHO-GLUT4*myc*-BK_2R) (●) were stimulated with various concentrations of bradykinin for 10 min at 37°C (*E*) or with 10^{-7} mol/l bradykinin for the indicated periods (*F*). The GLUT4*myc* translocation was measured. Values represent means ± SE of three separate experiments done in triplicate.

induced GLUT4 translocation and glucose uptake in skeletal muscles (21–23,42). Therefore, we asked whether bradykinin treatment directly triggers GLUT4 translocation and stimulates glucose uptake in three types of cells, i.e., L6 myotubes, 3T3-L1 adipocytes, and CHO cells. Skeletal muscle cells in vivo have endogenous bradykinin receptors, but we found that cultured L6 myotubes, 3T3-L1 adipocytes, and CHO cells have few or no bradykinin receptors. Therefore, we sequentially transfected bradykinin receptors after GLUT4*myc* transfection into these cells. As shown in Fig. 2, L6-GLUT4*myc* myotubes stably expressing BK_2Rs showed bradykinin-stimulated GLUT4*myc* translocation in a dose- and time-dependent manner ($ED_{50} = 4.7 \times 10^{-10}$ mol/l), while the parent L6-GLUT4*myc* myotubes did not respond to any applied concentrations of bradykinin (Fig. 2*A* and *B*). Bradykinin treatment also increased the rate of glucose uptake in L6-GLUT4*myc*-BK_2Rs ($ED_{50} = 4.1 \times 10^{-10}$ mol/l) in proportion to the GLUT4*myc* translocation (Figs. 2*A* and 3*A*). Almost the same dose- and time-dependent GLUT4*myc* translocation and glucose uptake in response to bradykinin were observed in 3T3-L1-GLUT4*myc* adipocytes expressing BK_2Rs (3T3-L1-GLUT4*myc*-BK_2R; GLUT4*myc* translocation $ED_{50} = 4 \times 10^{-10}$ mol/l, glucose uptake $ED_{50} = 1.5 \times 10^{-10}$ mol/l) and CHO-GLUT4*myc* cells expressing BK_2Rs (CHO-GLUT4*myc*-BK_2R; GLUT4*myc* translocation $ED_{50} = 1.3 \times 10^{-10}$ mol/l, glucose uptake $ED_{50} = 1.3 \times 10^{-10}$ mol/l) but were not observed in either parent 3T3-L1-GLUT4*myc* adipocytes or CHO-GLUT4*myc* cells (Figs. 2*C*–*F* and 3*B* and *C*). GLUT4*myc*

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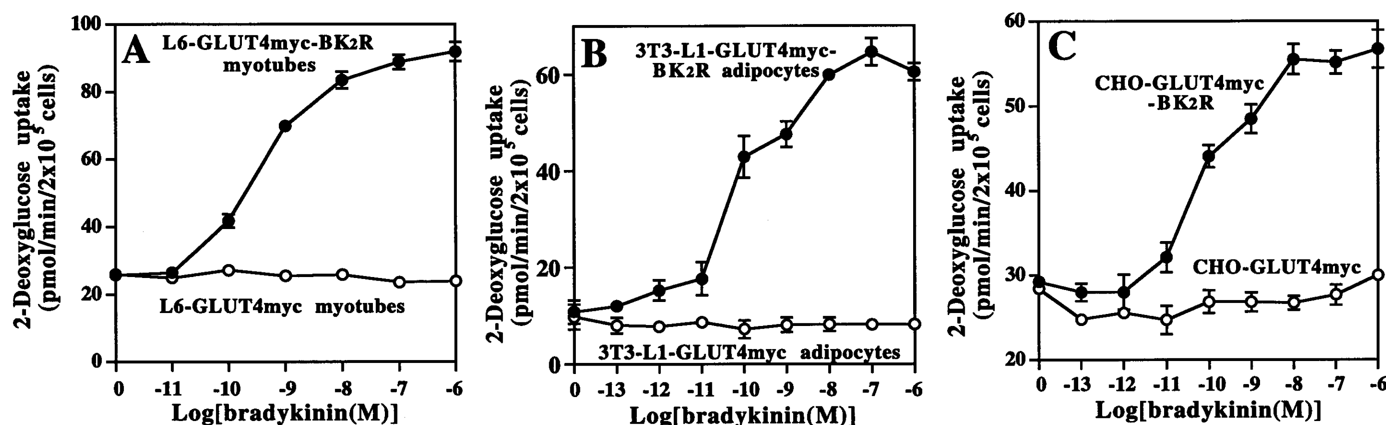

FIG. 3. Dose-dependent glucose uptake in response to bradykinin in L6-GLUT4*myc*-BK_2R myotubes, 3T3-L1-GLUT4*myc*-BK_2R adipocytes, and CHO-GLUT4*myc*-BK_2R cells. *A*: The parent cells (L6-GLUT4*myc* myotubes) (○) and those expressing BK_2Rs (L6-GLUT4*myc*-BK_2R myotubes) (●) were incubated with various concentrations of bradykinin for 10 min at 37°C. The 2-deoxyglucose uptake was measured as described under METHODS. *B*: The parent cells (3T3-L1-GLUT4*myc* adipocytes) (○) and those expressing BK_2Rs (3T3-L1-GLUT4*myc*-BK_2R) (●) were stimulated with various concentrations of bradykinin for 10 min at 37°C. The 2-deoxyglucose uptake was measured. *C*: The parent cells (CHO-GLUT4*myc* cells) (○) and those expressing BK_2Rs (CHO-GLUT4*myc*-BK_2R) (●) were stimulated with various concentrations of bradykinin for 10 min at 37°C. The 2-deoxyglucose uptake was measured. Values represent means ± SE of three separate experiments done in triplicate.

translocation and glucose uptake stimulated with bradykinin plus insulin treatment are partially additive compared with the case of single treatments of bradykinin or insulin in L6-GLUT4*myc*-BK_2R myotubes (Fig. 4*B*) and in 3T3-L1-GLUT4*myc*-BK_2R adipocytes (data not shown).

To examine molecular mechanisms related to bradykinin-induced GLUT4 translocation, effects of wortmannin on bradykinin-stimulated GLUT4 translocation in L6-GLUT4*myc*-BK_2R myotubes and 3T3-L1-GLUT4*myc*-BK_2R adipocytes were investigated. Wortmannin completely inhibited the insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes and myocytes by abolishing PI 3-kinase activity (8,12,43). As shown in Figs. 4*A* and 5*A*, wortmannin at 10^{-7} mol/l inhibited the insulin-stimulated GLUT4*myc* translocation in both cell lines. However, the bradykinin-induced GLUT4*myc* translocations were little affected even after pretreatment of up to 10^{-6} mol/l wortmannin. Almost the same data were obtained in the case of CHO-GLUT4*myc*-BK_2R cells (data not shown). Therefore, the pathway of bradykinin-stimulated GLUT4*myc* translocation is apparently independent of wortmannin-sensitive PI 3-kinases and independent of insulin signaling.

Most of the bradykinin effects in vivo are considered to be mediated through G_q-coupling to the receptor. However, G_i-coupling, which is sensitive to IAP, transmits the bradykinin-mediated signals in some cell lines (19,20). GLUT4*myc* translocations of L6 myotubes and the 3T3-L1 adipocytes were not affected significantly by treatment with 100 ng/ml IAP (Figs. 4*B* and 5*B*), which abolished G_i-coupling to the receptor (44,45). Mitogen-activating protein kinase activity in CHO cells expressing G_i-coupled receptors stimulated by the ligand was inhibited by pretreatment of IAP (100 ng/ml; 24 h) (H.H., Y.E., unpublished observations). The G_q-coupling activates phosphoinositide-specific phospholipase C β to hydrolyze phosphatidylinositol 4,5-bisphosphate (46,47), and the breakdown products, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, activate a Ca^{2+} channel and PKC, respectively (48,49). Therefore, we next asked whether the bradykinin-mediated GLUT4*myc* translocation is due to the consequent activation of PKC.

Phorbol 12-myristate 13-acetate (PMA) induces GLUT4 translocation by activating PKC in L6 myotubes and 3T3-L1 adipocytes (Figs. 4*B* and 5*B*). The PMA-stimulated GLUT4*myc* translocations in L6-GLUT4*myc*-BK_2R myotubes (1.8-fold increase) and 3T3-L1-GLUT4*myc*-BK_2R adipocytes (3.0-fold increase) were abolished with PDBu pretreatment by downregulating PKC (Figs. 4*B* and 5*B*). However, the same PDBu pretreatment had practically no effects on bradykinin-stimulated GLUT4*myc* translocation in both cell lines. Even after the simultaneous treatments of IAP, PDBu, and wortmannin, bradykinin triggered GLUT4*myc* translocation and stimulated glucose uptake in the two cell lines (Figs. 4*B* and *C* and 5*B* and *C*). The IAP-, PDBu-, and wortmannin-insensitive GLUT4*myc* translocation in response to bradykinin was also observed in CHO-GLUT4*myc*-BK_2R cells (data not shown). Pretreatment with these reagents reduced GLUT4*myc* translocation and glucose uptake stimulated by bradykinin (Figs. 4*B* and *C* and 5*B* and *C*), possibly because the translocation was a little inhibited by wortmannin (Figs. 4*A* and 5*A*).

Bradykinin induces transient elevations of the cytoplasmic-free calcium concentration $[Ca^{2+}]_i$ in a number of cell types (50,51). Depletion of $[Ca^{2+}]_i$ by 1,2-bis-(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester inhibited the bradykinin-stimulated GLUT4*myc* translocation in the L6-GLUT4*myc*-BK_2R myotubes, 3T3-L1-GLUT4*myc*-BK_2R adipocytes, and CHO-GLUT4*myc*-BK_2R cells (data not shown). Thus, a certain level of $[Ca^{2+}]_i$ was required for bradykinin-stimulated GLUT4*myc* translocation. But Ca^{2+}-ionophores did not trigger GLUT4*myc* translocation in the three cell lines (15). Therefore, only a $[Ca^{2+}]_i$ increase is insufficient to trigger GLUT4*myc* translocation.

Bradykinin-stimulated glycogen synthesis and lipogenesis. Insulin enhances glycogen synthesis and lipogen-

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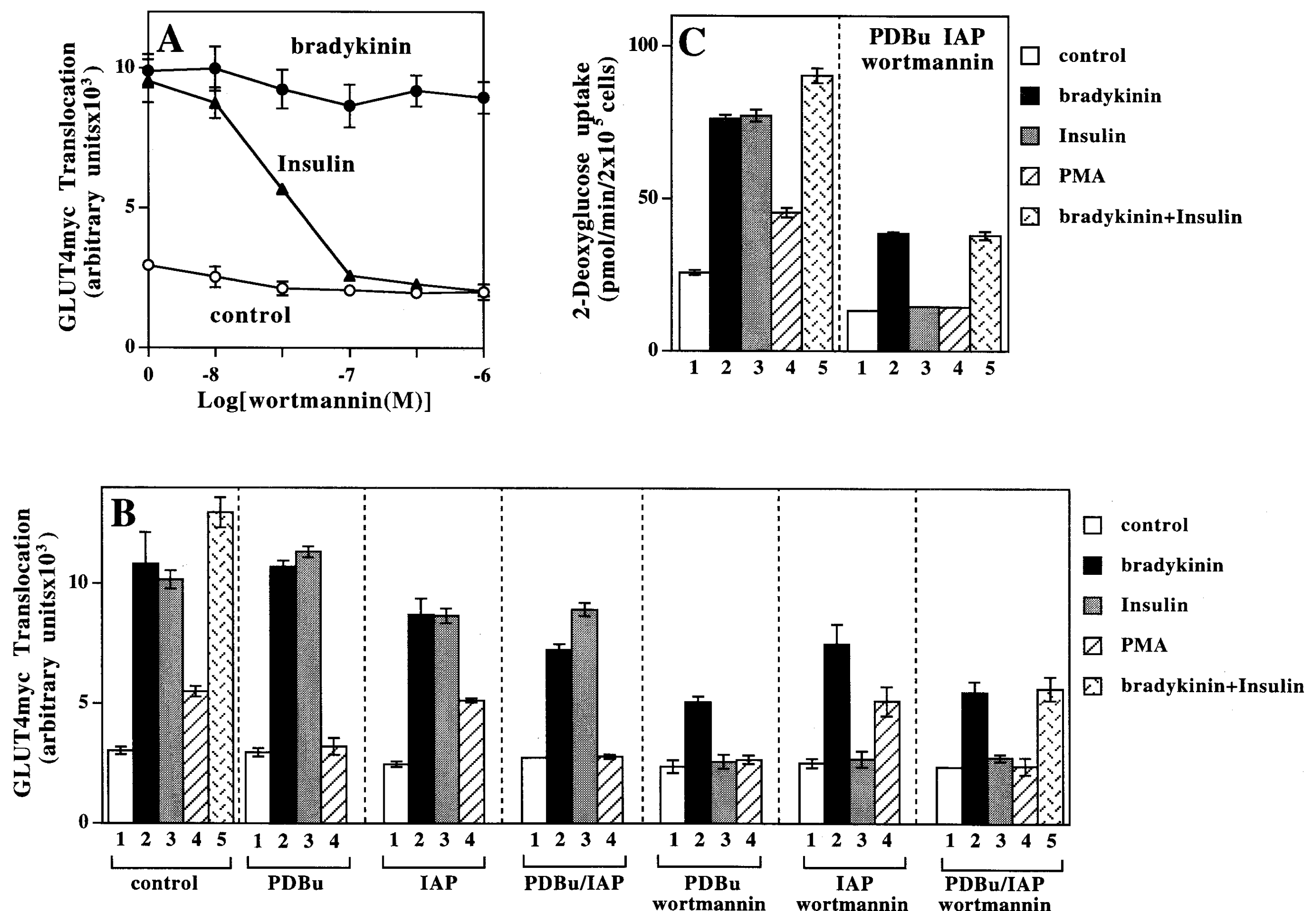

FIG. 4. Effects of wortmannin, IAP, and PDBu on the GLUT4*myc* translocation and glucose uptake in L6-GLUT4*myc*-BK$_2$R myotubes. *A*: L6-GLUT4*myc*-BK$_2$R myotubes were stimulated with 10^{-7} mol/l bradykinin (●), 3 × 10^{-7} mol/l insulin (▲), or buffer alone (○) for 10 min at 37°C after pretreatment with the indicated concentrations of wortmannin for 20 min at 37°C. GLUT4*myc* translocations are shown. *B*: L6-GLUT4*myc*-BK$_2$R myotubes were treated with 10^{-7} mol/l bradykinin (column 2), 3 × 10^{-7} mol/l insulin (column 3), 10^{-6} mol/l PMA (column 4), 10^{-7} mol/l bradykinin, 3 × 10^{-7} mol/l insulin (column 5), or buffer alone (column 1) for 10 min at 37°C after pretreatment with the indicated reagents (PDBu: 100 ng/ml, 24 h; IAP: 100 ng/ml, 24 h; wortmannin: 10^{-7} mol/l, 20 min) at 37°C. The GLUT4*myc* translocations are shown. *C*: L6-GLUT4*myc*-BK$_2$R myotubes were treated with 10^{-7} mol/l bradykinin (column 2), 3 × 10^{-7} mol/l insulin (column 3), 10^{-6} mol/l PMA (column 4), 10^{-7} mol/l bradykinin, 3 × 10^{-7} mol/l insulin (column 5), or buffer alone (column 1) for 10 min at 37°C after pretreatment with 100 ng/ml PDBu and 100 ng/ml IAP for 24 h and 10^{-7} mol/l wortmannin for 20 min (right) or medium alone (left) at 37°C. The 2-deoxyglucose uptakes are shown. Values represent means ± SE for three separate experiments done in triplicate.

esis in different cells. We examined whether the glucose taken up by bradykinin treatment is used for the synthesis of glycogen (and lipid) in 3T3-L1 adipocytes and L6 myotubes. Bradykinin stimulated glycogen synthesis in 3T3-L1-GLUT4*myc*-BK$_2$R adipocytes by 3.3-fold, while insulin stimulated it by 17.7-fold (Fig. 6*A*). In L6-GLUT4*myc*-BK$_2$R myotubes, bradykinin stimulated glycogen synthesis 1.9-fold, while insulin stimulated it 5.8-fold (Fig. 6*B*). After pretreatment with wortmannin, the insulin-stimulated glycogen synthesis was completely inhibited, but bradykinin-stimulated glycogen synthesis was not affected in the two cell lines (data not shown). Therefore, bradykinin was thought to stimulate glycogen synthesis via an insulin-independent pathway. In contrast to glucose uptake (Figs. 4 and 5), however, bradykinin had no additive effects to insulin on glycogen synthesis (Fig. 6*A* and *B*); rather, it inhibited insulin-stimulated glycogen synthesis in 3T3-L1 adipocytes (Fig. 6*A*). Insulin also increased the rate of lipogenesis in 3T3-L1 adipocytes. 3T3-L1-GLUT4*myc*-BK$_2$R adipocytes with bradykinin treatment produced a 2-fold increase in the conversion of radiolabeled glucose into lipid, while adipocytes with insulin produced a 13-fold increase (Fig. 6*C*). Bradykinin attenuated to some extent the insulin-induced lipogenesis in adipocytes (Fig. 6*C*), as seen in the case of glycogen synthesis (Fig. 6*A*).

DISCUSSION

Bradykinin-stimulated GLUT4 translocation. Physiological experiments have shown that acute physical exercise increases the rate of glucose uptake into skeletal muscles in vivo. This increase has been thought to be via an insulin-independent pathway, but the exact molecular mechanism is unknown. Dieze and Wicklmayr (42) reported that bradykinin may be involved in exercise-stimulated glucose transport. They also reported that bradykinin is liberated by working skeletal muscles in healthy human volunteers (23)

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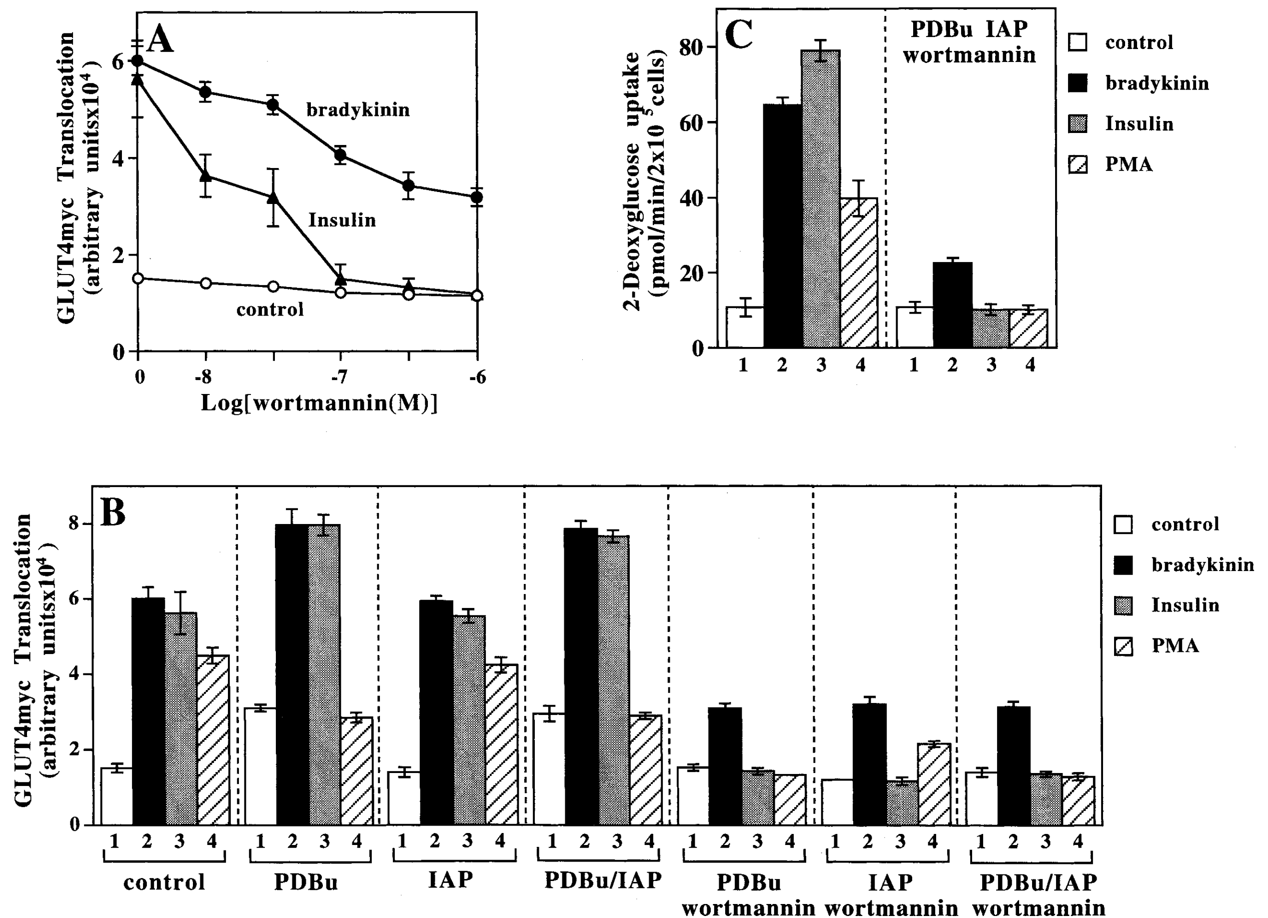

FIG. 5. Effects of wortmannin, IAP, and PDBu on the GLUT4*myc* translocation and glucose uptake in 3T3-L1-GLUT4*myc*-BK_2R adipocytes. *A*: 3T3-L1-GLUT4*myc*-BK_2R adipocytes were stimulated with 10^{-7} mol/l bradykinin (●), 3 × 10^{-7} mol/l insulin (▲), or buffer alone (○) for 10 min at 37°C after pretreatment with the indicated concentrations of wortmannin for 20 min at 37°C. GLUT4*myc* translocations are shown. *B*: 3T3-L1-GLUT4*myc*-BK_2R adipocytes were treated with 10^{-7} mol/l bradykinin (column 2), 3 × 10^{-7} mol/l insulin (column 3), 10^{-6} mol/l PMA (column 4), or buffer alone (column 1) for 10 min at 37°C after pretreatment with the indicated reagents (PDBu: 100 ng/ml, 24 h; IAP: 100 ng/ml, 24 h; wortmannin: 10^{-7} mol/l, 20 min) at 37°C. The GLUT4*myc* translocations are shown. *C*: 3T3-L1-GLUT4*myc*-BK_2R adipocytes were treated with 10^{-7} mol/l bradykinin (column 2), 3 × 10^{-7} mol/l insulin (column 3), 10^{-6} mol/l PMA (column 4), or buffer alone (column 1) for 10 min at 37°C after pretreatment with 100 ng/ml PDBu and 100 ng/ml IAP for 24 h and 10^{-7} mol/l wortmannin for 20 min (right) or medium alone (left) at 37°C. The 2-deoxyglucose uptakes are shown. Values represent means ± SE for three separate experiments done in triplicate.

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but not in patients with NIDDM (52). ACE activates angiotensin I and degrades bradykinin. ACE inhibitors are prescribed for subjects with hypertension, and it has become evident that they not only are effective for this pathology but also improve contraction-stimulated glucose uptake in skeletal muscle of obese rats (53). These results suggest that bradykinin is one of the candidate mediators of exercise-stimulated glucose transport in skeletal muscle. However, the role of bradykinin in exercise-induced glucose uptake is open to argument (54–56).

In this study, we have shown that bradykinin directly triggers GLUT4 translocation and stimulates glucose uptake in three types of cultured cells, including L6 myotubes. Therefore, we conclude that bradykinin is one factor related to exercise-stimulated GLUT4 translocation and glucose transport in skeletal muscles (24). It is desirable to examine the effect of bradykinin in whole body or skeletal muscle after exercise. Subcellular fractionation techniques are widely used to examine the translocation of GLUT4 to the plasma membrane of skeletal muscle (57,58), but it is difficult to accurately and quantitatively measure the translocation of GLUT4. Therefore, to demonstrate the effect of bradykinin on GLUT4 translocation in muscle, we used cultured L6 myotubes, which expressed GLUT4*myc* in addition to 3T3-L1 adipocytes and CHO cells.

The concentrations of endogenous bradykinin in human blood and interstitial fluid are reported to fluctuate between 10^{-13} and 10^{-10} mol/l with radioimmunoassay (59). Because the half-life of bradykinin in blood is <30 s (26), it seems to be difficult to exactly measure the concentration of local circulating bradykinin before and after exercise in vivo. There is a possibility that the concentration of bradykinin previously reported might be underestimated.

Isami et al. (60) have shown that bradykinin does not induce GLUT4 translocation in dog adipocytes but enhances insulin-induced GLUT4 translocation, possibly through an increase in insulin receptor (IR) tyrosine kinase activity or insulin receptor substrate-1 (IRS-1) tyrosine kinase activity.

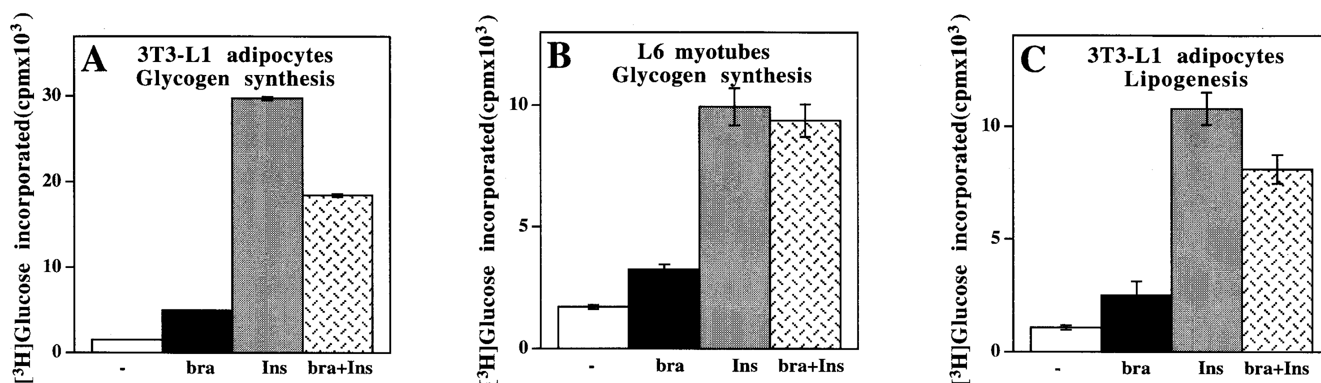

FIG. 6. Glycogen synthesis and lipogenesis in 3T3-L1-GLUT4*myc*-BK_2R adipocytes and L6-GLUT4*myc*-BK_2R myotubes. *A* and *B*: 3T3-L1-GLUT4*myc*-BK_2R adipocytes (*A*) and L6-GLUT4*myc*-BK_2R myotubes (*B*) were incubated with 10^{-7} mol/l bradykinin (■), 3×10^{-7} mol/l insulin (▩), 10^{-7} mol/l bradykinin plus 3×10^{-7} mol/l insulin (▨), or buffer alone (□) for 10 min at 37°C. The reaction was initiated by the addition of 4 mmol/l [^{3}H]glucose, and the radiolabeled glucose incorporation into glycogen for 1 h was determined as described under METHODS. *C*: 3T3-L1-GLUT4*myc*-BK_2R adipocytes were incubated with 10^{-7} mol/l bradykinin (■), 3×10^{-7} mol/l insulin (▩), 10^{-7} mol/l bradykinin plus 3×10^{-7} mol/l insulin (▨), or buffer alone (□) for 10 min at 37°C. The reaction was initiated by the addition of 4 mmol/l [^{3}H]glucose, and the radiolabeled glucose incorporation into lipid for 1 h was determined. Values represent means ± SE of three separate experiments done in triplicate.

In our study, as shown above, bradykinin directly triggers GLUT4 translocation in L6 myotubes, 3T3-L1 adipocytes, and CHO cells expressing GLUT4*myc* and BK_2Rs. Bradykinin treatment enhanced GLUT4*myc* translocation induced by insulin, but it did not increase the activity of IR tyrosine kinase or IRS-1 tyrosine kinase in CHO-GLUT4*myc*-BK_2R cells (data not shown). Whether bradykinin directly triggers GLUT4 translocation may be dependent on the number of BK_2Rs on the cell surface. According to Isami et al. (60), dog adipocytes possessed 1.7×10^4 bradykinin binding sites/cell. L6-GLUT4*myc*-BK_2R myotubes, 3T3-L1-GLUT4*myc*-BK_2R adipocytes, and CHO-GLUT4*myc*-BK_2R cells that we constructed had 1.1×10^5, 3.4×10^5, and 1.8×10^5 bradykinin binding sites per cell, respectively.

The major glucose transporter expressed in skeletal muscles is GLUT4, but a small amount of GLUT1 is also expressed in these muscles (61,62). GLUT1 is widely expressed in various cells and is mainly located in the plasma membrane, although a small part resides in intracellular compartments. Using our system, we detected the insulin-dependent translocations of GLUT1*myc* in CHO cells and 3T3-L1 adipocytes, but the extent was much less than that of GLUT4 (63). When we examined the effect of bradykinin on GLUT1 translocation in CHO-GLUT1*myc* cells expressing BK_2Rs, bradykinin triggered GLUT1 translocation in the cells (K.K., Y.E., unpublished observations). Rett et al. (64) reported that bradykinin induced GLUT1 and GLUT4 translocation in cardiac muscle. Therefore, GLUT1 translocation stimulated by locally secreted bradykinin might contribute to exercise-induced glucose transport in skeletal muscles.

Bradykinin-stimulated glycogen synthesis and lipogenesis. Another factor to consider is how the glucose taken up with bradykinin treatment is metabolized in cells. Glycogen synthesis is regulated mainly by incorporated glucose and by enzyme activities for glycogen synthesis, because three kinds of transgenic mice expressing GLUT1 (65), GLUT4 (66,67), or glycogen synthase (68) showed significantly increased glycogen synthesis compared with controls. The bradykinin-stimulated glycogen synthesis is much less than the insulin-stimulated in both 3T3-L1 adipocytes and L6 myotubes (Fig. 6*A* and *B*), although the increase in GLUT4 translocation and the glucose uptake stimulated by bradykinin and that by insulin are similar (Figs. 4*B* and *C* and 5*B* and *C*). This would suggest that the bradykinin-stimulated glycogen synthesis is due to the enhanced glucose uptake, although the possibility that bradykinin may affect enzymes regulating glycogen synthesis would need to be considered. Considering that bradykinin attenuated the insulin-stimulated glycogen synthesis and lipogenesis in 3T3-L1 adipocytes (Fig. 6*A* and *C*), bradykinin might inhibit activities of these enzymes stimulated by insulin. Insulin treatment stimulates both glucose uptake and enzyme activities for glycogen and lipid syntheses. The glucose incorporated by insulin is mainly stored as glycogen and lipid. In contrast, a large part of glucose incorporated by bradykinin is presumably used as fuel for muscle contraction and not as a resource for storage of glycogen and lipid.

In conclusion, bradykinin directly triggers GLUT4 translocation and stimulates glucose uptake via an insulin- and PKC-independent pathway in three species of cultured cells. Bradykinin is possibly one factor responsible for the exercise-stimulated glucose transport in skeletal muscle.

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