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Neuregulins Mediate Calcium-induced Glucose Transport during Muscle Contraction*

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Neuregulin, a growth factor involved in myogenesis, has rapid effects on muscle metabolism. In a manner analogous to insulin and exercise, neuregulins stimulate glucose transport through recruitment of glucose transporters to surface membranes in skeletal muscle. Like muscle contraction, neuregulins have additive effects with insulin on glucose uptake. Therefore, we examined whether neuregulins are involved in the mechanism by which muscle contraction regulates glucose transport. We show that caffeine-induced increases in cytosolic Ca^{2+} mediate a metalloproteinase-dependent release of neuregulins, which stimulates tyrosine phosphorylation of ErbB4 receptors. Activation of ErbB4 is necessary for Ca^{2+}-derived effects on glucose transport. Furthermore, blockage of ErbB4 abruptly impairs contraction-induced glucose uptake in slow twitch muscle fibers, and to a lesser extent, in fast twitch muscle fibers. In conclusion, we provide evidence that contraction-induced activation of neuregulin receptors is necessary for the stimulation of glucose transport and a key element of energetic metabolism during muscle contraction.

Skeletal muscle is the main tissue responsible for insulin-stimulated glucose utilization in absorptive states (1). The rate-limiting step for muscle glucose utilization is glucose transport, which can be rapidly induced by translocation of GLUT4 glucose transporters from intracellular vesicles to the plasma membrane. Contraction and insulin act independently through distinct signaling pathways to induce GLUT4 translocation. Most interestingly, in insulin-resistant states, such as type 2 diabetes, the effects of contraction on glucose uptake are unchanged (2–4). Thus, efforts have been made to understand the molecular mechanisms involved in contraction-stimulated glucose uptake.

Muscle contraction induces glucose transport by a mechanism involving local factors within the myocyte (3). It has been suggested that two main effectors mediate contraction-induced glucose transport, namely activation of AMP-activated protein kinase (AMPK),[3] a metabolic fuel gauge regulated by cellular energy charge, and transient increases in cytosolic Ca^{2+} within myofibers, as a consequence of plasma membrane and T-tubule depolarization (3, 5). Little is known of the mechanism by which AMPK or Ca^{2+} leads to glucose uptake, although activation of Ca^{2+}/calmodulin-dependent kinase II (CAMKII) has been implicated in Ca^{2+}-induced glucose uptake (6, 7).

Contraction also triggers the activation of neuregulin receptors (8). Neuregulins are growth factors that belong to the epidermal growth factor (EGF) family, and their action is essential for cardiac and skeletal muscle development and function (9). Neuregulins have traditionally been considered as nerve-derived factors that control acetylcholine receptor synthesis at the neuromuscular junction. In addition, there is growing evidence that they are essential muscle autocrine effectors (see Ref. 10 for review). Neuregulins activate ErbB receptors, ErbB2, ErbB3, and ErbB4, which belong to the type I tyrosine kinase receptor family (11, 12). Binding of neuregulins to ErbB3 and ErbB4 receptors induces receptor dimerization and activation of the tyrosine kinase domain. Although ErbB2 does not bind ligand, it is the preferred receptor for heterodimerization (12).

Previous observations in L6E9 myotubes and incubated skeletal muscle indicated that, similarly to contraction, neuregulins induce glucose transport by translocation of GLUT4 glucose transporters from an intracellular storage compartment to the plasma membrane in an additive manner to the insulin (13, 14). Since neuregulin receptors are activated during contraction (8),

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[3] The abbreviations used are: AMPK, AMP-activated protein kinase; HRG/Hrg, recombinant heregulin-β_1-(177–244); CAMKII, Ca^{2+}/calmodulin-dependent kinase II; EGF, epidermal growth factor; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; ACC, acetyl coenzyme A carboxylase; PI3K, phosphatidylinositol-3 kinase; EDL, extensor digitorum longus; PBS, phosphate-buffered saline.

we hypothesized that neuregulins could be involved in the regulation of glucose transport by muscle contraction. In the present study, we show that neuregulins are released by a Ca^{2+}-dependent metalloproteinase activity, which causes ErbB receptor phosphorylation. We also provide evidence that neuregulin receptor activation is needed to increase glucose transport in response to muscle contraction.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Purified porcine insulin was a kind gift from Eli Lilly Co. A recombinant heuregulin 1 isoform containing the bioactive EGF domain, heregulin-β_1-(177–244) (HRG), was donated by Genentech, Inc. (South San Francisco, CA). Caffeine, dantrolene, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), and anti-α-actin monoclonal antibody were purchased from Sigma. KN93, GM6001, and TAPI-2 were purchased from Calbiochem. ErbB4 (Ab3) and ErbB3 (Ab5) ligand-binding domain blocking monoclonal antibodies and anti-neuregulin antibodies against the extracellular domain (Ab1) or EGF domain (Ab2) were purchased from Neomarkers (Fremont, CA). Anti-phosphotyrosine monoclonal antibody and anti-insulin receptor β-chain were purchased from BD Transduction Laboratories (San Jose, CA). Anti-ErbB2 (C-18) and anti-ErbB3 (C-17) polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal antibodies against phospho-CAMKII (Thr-286), phospho-AMPKα (Thr-172), and phospho-ACC (Ser-79 site) were purchased from Cell Signaling (Beverly, MA). Anti-ErbB4 polyclonal (for Western blot) and monoclonal (for immunoprecipitation) antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A polyclonal antibody against GLUT4 (OSCRX, raised against the 15 C-terminal amino acid residues) was produced in our laboratory.

Studies in Incubated Skeletal Muscle—Male Wistar rats (100–150 g) were anesthetized with pentobarbital (5–7 mg/100 g of body weight). Extensor digitorum longus (EDL) and soleus muscles were extracted and stripped longitudinally. Muscles were then allowed to recover for 45 min in flasks containing 2 ml of incubation medium (Krebs-Henseleit bicarbonate buffer, including 5 mM HEPES, 0.1% bovine serum albumin, 5 mM glucose, and 15 mM mannitol) continuously oxygenated with 95% O_2, 5% CO_2 in a shaking water bath (35 °C). After recovery, muscles were submitted to treatments specified in the figure legends.

Ex Vivo Muscle Contraction—Strips of soleus muscles were placed in a controlled-temperature incubation chamber and immersed in 5 ml of incubation medium with or without recombinant neuregulins (HRG, 5 nM). Muscles were subjected to contraction as described previously (13) and then either frozen immediately to obtain total lysates or further incubated to assess glucose transport activity or cell surface GLUT4 content.

In Vivo Muscle Contraction—Male Wistar rats (100–150 g) were anesthetized using 4% isoflurane and maintained at 2%. EDL and soleus muscles from each leg were injected with 50 μl of phosphate-buffered saline (PBS, as vector), anti-ErbB3 blocking antibody (3 μg), anti-ErbB4 blocking antibody (3 μg), or HRG (0.8 pmol, final concentration 5 nM). After 20 min, the right leg was made to contract *in situ* by sciatic nerve electric stimulation (trains of 500 ms of supramaximal voltage, 7 V, were delivered at a rate of 1/s; each pulse in the train lasted 0.1 ms, and pulses were delivered at 100 Hz) for 2 × 5-min bouts separated by a 1 min rest (14). The left leg was used as a control. During contraction, the tendons of plantaris and gastrocnemius muscles were intact, and the ankle was left free. Thereafter, muscle strips were immediately extracted and incubated for 2-deoxyglucose transport or frozen to obtain proteic lysates for assessment of the metabolic parameters.

Glucose Uptake Measurements—After treatments, muscles were rinsed for 10 min (*ex vivo* experiments) or 20 min (*in vivo* experiments) in oxygenated Krebs-Henseleit bicarbonate buffer with 5 mM HEPES, 0.1% bovine serum albumin, and 20 mM mannitol. Muscles were then transferred to vials containing 2-deoxy-[2,6,^{3}H]glucose (1 mM, 2.5 μCi/ml) and [^{14}C]mannitol (19 mM, 0.7 μCi/ml) and incubated for 20 min. Muscles were frozen, weighed, and digested in 1 ml of 0.5 N NaOH. Digested muscles were processed as described previously (15) for 2-deoxyglucose uptake determination.

Plasma Membrane GLUT4 Levels Assessed by Photolabeling—Cell surface GLUT 4 content was determined by photolabeling using a method derived from Koumanov *et al.* (16). Briefly, after treatment, muscles were rinsed in Krebs-Henseleit bicarbonate buffer, 20 mM mannitol, for 8 min. Muscles were placed in 35-mm polystyrene culture dishes containing 1 ml of the rinse medium containing 0.2 mM of a cell-impermeable biotinylated photoaffinity compound that labels glucose transporters (Bio-LC-ATB-BGPA, 4,4′-O-(2-(2-(2-(2-(2-(6-(Biotinylamino) hexanoyl) amino) ethoxy)-ethoxy)ethoxy)-4-(1-azi-2,2,2, trifluoroethyl)benzoyl)amino-1,3-propanediyl-bis-D-glucose), donated by Dr. Geoffrey D. Holman, University of Bath, UK. Muscles were incubated for 8 min at 18 °C in the dark to slow down membrane traffic. The dish was gently shaken every 2–3 min. Thereafter, muscles were transferred to another dish with 1 ml of rinse medium containing the photolabeling reagent and then irradiated for 6 min in a Rayonet photochemical reactor, using 300 nm lamps, to fix the reagent to GLUT4 transporters. Muscles were trimmed of tendons, frozen, and stored at −80 °C. The total lysate (300 μg) from each muscle was immunoprecipitated with 30 μl of streptavidin-agarose beads (Pierce), and GLUT4 levels were detected by Western blot analysis.

Total Muscle Lysates, Immunoprecipitation, and Western Blot—All these procedures were performed essentially as described previously (15, 17). For total lysates, a frozen muscle strip was collected in an Eppendorf tube placed on ice and pulverized with an Eppendorf homogenizer. Then, 200 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, containing freshly added protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin), was added, and muscles were further homogenized. The homogenates were diluted to a final volume of 25 μl/mg of muscle by the addition of lysis buffer. Homogenates were mixed in an orbital shaker from 30 min to 1 h at 4 °C and then centrifuged at maximal speed in an Eppendorf centrifuge (~13,000 rpm) for 10 min at 4 °C. The pellet was discarded, and supernatant was collected and stored at −80 °C until used. Protein levels were

measured by the Bradford method. Samples were immunoprecipitated to identify ErbB2, ErbB3, ErbB4, and insulin receptor phosphorylation levels by conjugating 30 μl of protein G-Sepharose beads with 2–5 μg of the corresponding polyclonal antibody (except for ErbB4, which was monoclonal) for 1 h at 4 °C and then washing twice in lysis buffer and incubating with 0.5–1 mg of protein lysate overnight with constant shaking at 4 °C. After brief centrifugation, the supernatant was discarded. The pellet was washed several times in the lysis buffer and boiled with 50 μl of Laemmli sample buffer (LBS) for Western blot analysis using a monoclonal anti-phosphotyrosine antibody or polyclonal ErbB4 antibody to determine phosphorylated or total ErbB4 content. For Western blot assays, protein samples containing LSB were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes as described previously (15). Total muscle lysates (200 μg for p-CAMKII, p-AMPK, and p-ACC; 10 μg for GLUT4; and 5 μg for β-actin) were used for immunoblot detection.

Measurement of Neuregulin Release—Incubation medium (1 ml) was complemented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin), incubated with 5 μg of a biotinylated antibody against the extracellular domain of neuregulins (Ab1) for 2 h, and then immunoprecipitated with streptavidin beads. Western blot analysis was performed with an antibody against the conserved EGF region of neuregulins (Ab2).

Measurement of Glycogen, ATP, Phosphocreatine, and Lactate Muscle Levels—Measurements were performed as described previously (18).

Statistical Analysis—All values are presented as means ± S.E. Differences between two groups were assessed using unpaired two-tailed *t*-tests. Analysis of variance, assessed by Bonferonni's multiple comparison test, was used to compare more than two groups.

RESULTS

Muscle Contraction Induces ErbB Receptors Phosphorylation by a Ca^{2+}-dependent Pathway—To explore the involvement of neuregulins in regulation of glucose transport by muscle contraction, we first examined the effects of *ex vivo* electrically stimulated muscle contraction on tyrosine phosphorylation of neuregulin receptors. Contraction stimulated phosphorylation of ErbB4 and ErbB2 but not ErbB3 receptors (Fig. 1*A*). This is consistent with the finding that an *in vitro* exposure of skeletal muscle to saturating exogenous neuregulins induced tyrosine phosphorylation of ErbB4 and ErbB2 but only induced weak phosphorylation of ErbB3 (Fig. 1, *A* and *B*) (15). These results indicated that ErbB2 and ErbB4 are the main neuregulin receptors in adult skeletal muscle. The addition of neuregulins to resting muscles did not increase phosphorylation of AMPK or CAMKII (Fig. 1*A*), indicating that these kinases are not downstream targets of neuregulin action. Therefore, we tested whether induction of ErbB receptor phosphorylation during contraction was caused by AMPK activation or by increases in cytosolic Ca^{2+}. Incubation of soleus muscle with AICAR, an AMPK activator, did not induce ErbB receptor phosphorylation, whereas caffeine treatment at a concentration that induces Ca^{2+} release to subcontractile levels (6, 7) mimicked contraction effects on ErbB receptor phosphorylation (Fig. 1*B*). No effect was observed on ErbB3 phosphorylation in response to caffeine unless the ErbB4 ligand-binding domain was blocked with specific antibodies (data not shown). To test whether the effects of caffeine on ErbB phosphorylation were specific to Ca^{2+} release from the sarcoplasmic reticulum, we pretreated isolated soleus muscles with dantrolene to block Ca^{2+} release through the ryanodine receptors. Dantrolene abrogated caffeine-induced ErbB4 phosphorylation, which was restored by the addition of exogenous neuregulins (Fig. 1*C*), indicating that dantrolene did not affect ErbB4 phosphorylation in an unspecific manner. The Ca^{2+} effects on ErbB4 phosphorylation were independent of CAMKII activity since incubation of muscle with KN93, a specific CAMKII inhibitor, did not prevent caffeine-induced ErbB4 phosphorylation (Fig. 1*C*). Furthermore, when analyzing muscle incubation medium during caffeine treatment, we detected a bioactive extracellular fragment of neuregulin (40–45 kDa). Caffeine-induced neuregulin release was prevented by dantrolene, but not by KN93 (Fig. 1*C*), indicating that an increase of cytosolic Ca^{2+}, but not CAMKII activation, was involved in this process.

Since membrane metalloproteinases are the main neuregulin-shedding enzymes (19, 20) and their activity is modulated by Ca^{2+} levels (20), the above results prompted us to hypothesize that increases in cytosolic Ca^{2+} could lead to a metalloproteinase-dependent shedding of neuregulins, which would induce ErbB4 phosphorylation. GM6001, a wide range metalloproteinase inhibitor, abolished caffeine-induced ErbB4 phosphorylation and this effect was restored by the addition of exogenous neuregulins (Fig. 1*D*), indicating that GM6001 did not affect ErbB4 phosphorylation in an unspecific manner. ADAM17 (also known as tumor necrosis factor-α-converting enzyme) is a main neuregulin metalloproteinase (19), but its specific inhibitor, TAPI-2, only partially blocked caffeine-induced ErbB4 phosphorylation (Fig. 1*D*). Caffeine-induced neuregulin release was also inhibited in muscles incubated with GM6001, although only partially blocked by TAPI-2 (Fig. 1*D*). Our results cannot exclude the possibility that tumor necrosis factor-α-converting enzyme could be mainly responsible for calcium-induced neureugulin release since the partial effects observed when using TAPI-2 could be a consequence of compensatory activities elicited by redundant metalloproteinases (20). To further demonstrate that only neuregulins, and no other product of Ca^{2+}-dependent metalloproteinase action, were responsible for ErbB4 phosphorylation, we incubated soleus muscles with an ErbB4 blocking antibody or a neuregulin antibody that recognizes the conserved bioactive EGF domain. In both situations, caffeine-induced ErbB4 phosphorylation was totally abolished. The addition of exogenous neureugulins did not restore ErbB4 phosphorylation when ErbB4 ligand-binding domain was blocked and only slightly restored phosphorylation when sequestering neuregulins by the use of specific antibodies (Fig. 1*E*), probably because high concentrations of exogenous neuregulins were added. In no case was neuregulin release compromised (Fig. 1*E*). Collectively, these results indicate that contraction induces ErbB4 phosphorylation through Ca^{2+}-dependent metalloproteinase activity that leads to neuregulin shedding.

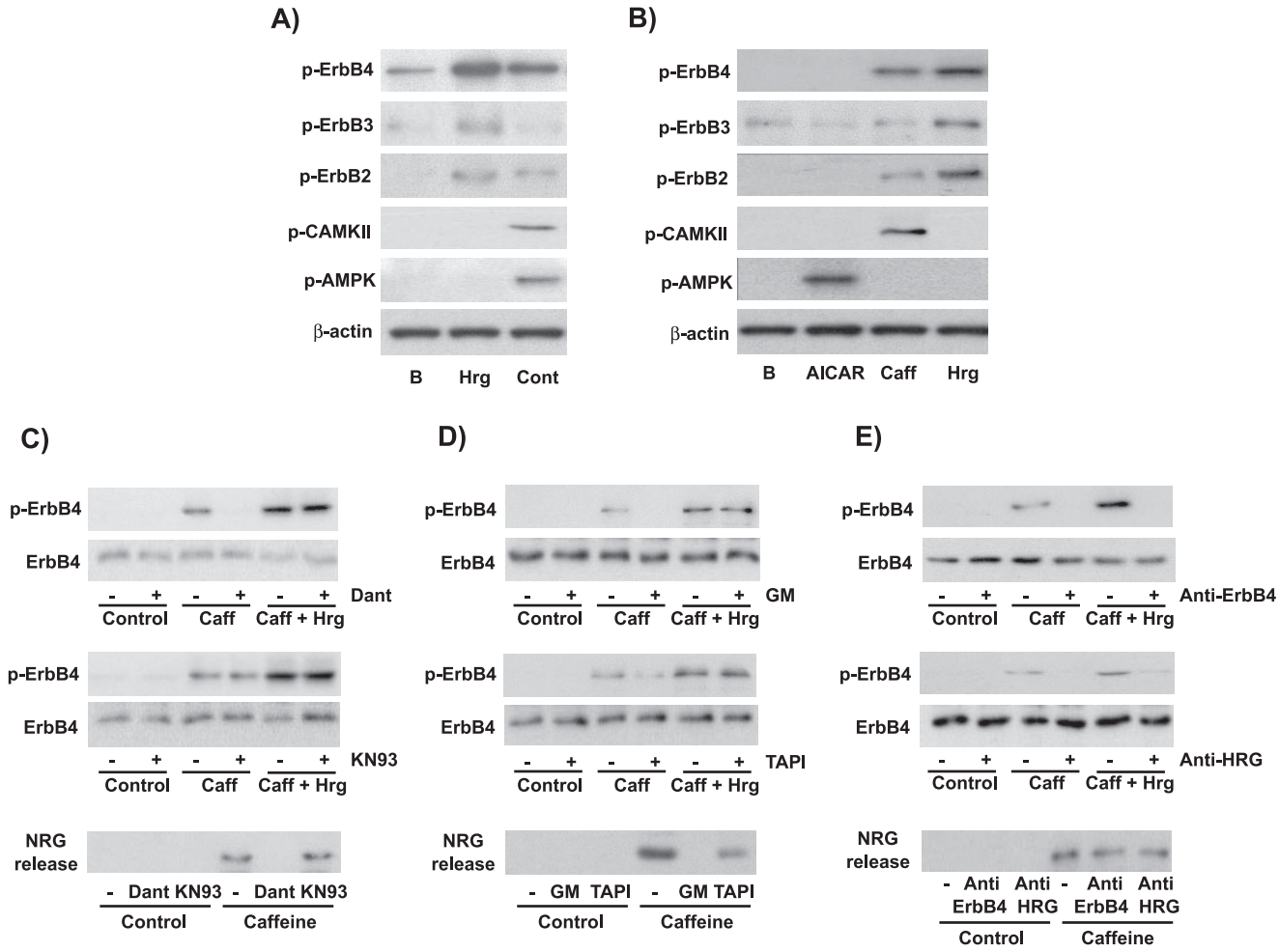

FIGURE 1. **Muscle contraction or caffeine induces ErbB4 and ErbB2 phosphorylation by stimulating neuregulin release.** *A–E*, strips of soleus muscle were treated with: recombinant neuregulins (HRG, 5 nM, 15 min) or submitted to *ex vivo* contraction (*Cont*) for 10 min (*A*); AICAR (2 mM, 30 min), caffeine (*Caff*, 3.5 mM, 15 min), neuregulins (Hrg, 5 nM, 15 min) (*B*); dantrolene (*Dant*, 5 μM), KN93 (10 μM), GM6001 (*GM*, 10 μM), TAPI-2 (*TAPI*, 50 μM), anti-ErbB4 blocking antibody (20 μg/ml), or anti-heregulins (*Ab2*, 50 μg/ml) for 30 min before treatment with caffeine and/or neuregulins (*C–E*). Phosphorylation (*p*) of ErbB receptors, CAMKII and AMPK, was analyzed in muscle lysates. Immunoprecipitated endogenous neuregulins (*NRG*) from muscle incubation medium were detected by immunoblot (*C–E, lower panels*). Each panel is a representative image from $n = 4–9$ different muscles extracts.

Activation of ErbB4 Is Required for the Ca^{2+}-dependent Stimulation of Glucose Transport—We next explored whether ErbB4 phosphorylation had a role in the induction of glucose transport during contraction. First, we evaluated the impact of ErbB4 ligand-binding domain blockage on glucose transport induced by increases in cytosolic Ca^{2+} or AMPK activation. ErbB4 blockage impaired neuregulin- and caffeine-induced glucose transport in incubated soleus muscle (Fig. 2*A*). Insulin action was unaffected under ErbB4 blockage, indicating that antibodies against ErbB4 did not have unspecific effects on glucose transport (Fig. 2*A*). In contrast, treatment with specific antibodies that induce ErbB3 blockage did not affect neuregulin- or caffeine-induced glucose transport in soleus muscle (Fig. 2*B*). Therefore, ErbB4, but not ErbB3, is involved in the regulation of Ca^{2+}-dependent stimulation of glucose uptake in soleus muscle.

Consistent with previous reports (6, 21–23), AICAR increased glucose uptake in EDL muscle but not in soleus (data not shown). Thus, we tested the impact of ErbB4 blockage on AICAR-induced glucose uptake. Although ErbB4 blocking antibodies impaired neuregulin- and caffeine-induced glucose uptake, AICAR action remained unaffected (Fig. 2*C*). In addition, caffeine-induced CAMKII phosphorylation was unaffected by ErbB4 blockage in soleus or EDL muscles (Fig. 2, *A* and *C*), indicating that the impairment in glucose uptake by ErbB4 blockage was unrelated to impaired Ca^{2+} release.

We next tested whether inhibitors that impair caffeine-induced ErbB4 phosphorylation also affect caffeine-induced glucose transport. As reported elsewhere (6, 7), we show that dantrolene inhibited caffeine-induced glucose uptake, but this effect was not restored by adding exogenous neuregulins (Fig. 3*A*), indicating that Ca^{2+}-derived effectors, other than neuregulins, are necessary for the induction of glucose uptake. GM6001 also blocked caffeine-induced glucose uptake, and the addition of exogenous neuregulins prevented the GM6001 effect on glucose uptake (Fig. 3*B*). These data indicate that GM6001 inhibition of caffeine-induced glucose uptake relies exclusively on the impairment of neuregulin release. Interestingly, upon the addition of exogenous neuregulins, the caffeine effect on glucose uptake improved ~3-fold. Thus, the enhancement of ErbB4 signaling during caffeine action by the exogenous addition of neuregulins greatly improves glucose uptake. Similar results for glucose uptake were obtained when exoge-

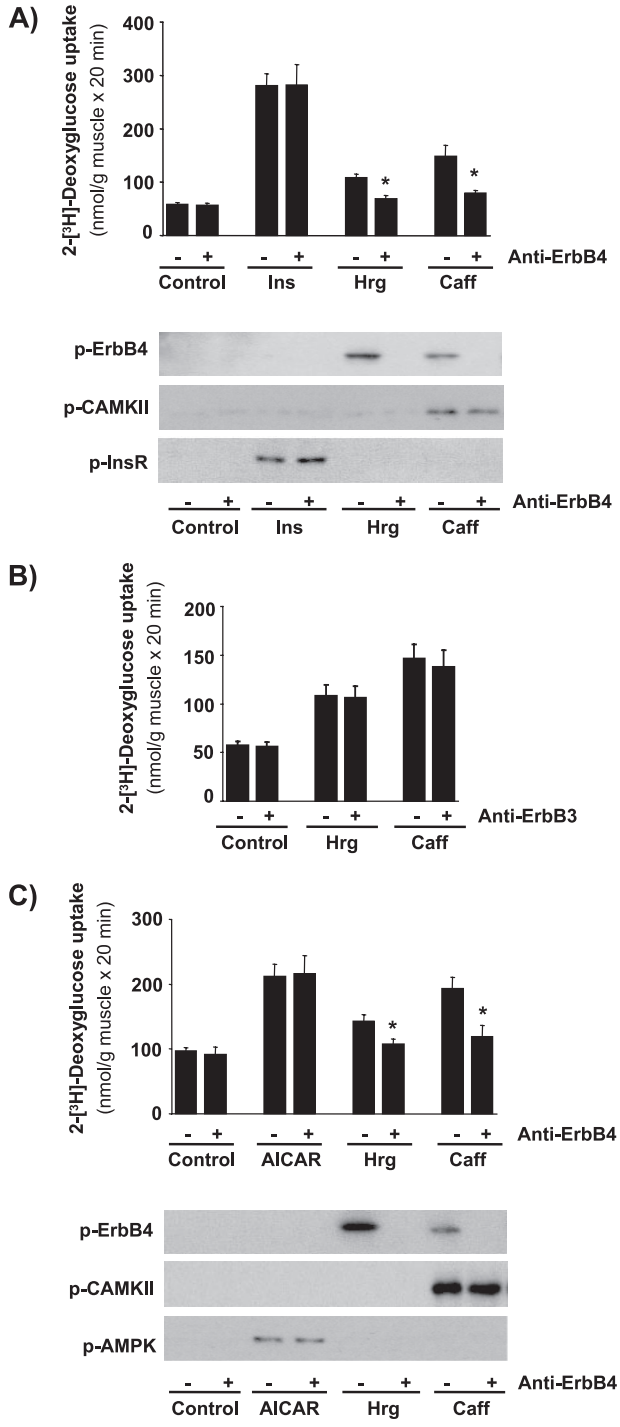

FIGURE 2. **Neuregulins mediate caffeine (*Caff*)-induced glucose uptake.** Strips of soleus (*A* and *B*) or EDL (*C*) muscles were treated with anti-ErbB4 blocking antibody (20 μg/ml) (*A* and *C*) or anti-ErbB3 blocking antibody (10 μg/ml) (*B*) 30 min before treatments with insulin (*Ins*, 100 nM, 30 min), AICAR (2 mM, 30 min), caffeine (3.5 mM, 15 min), or neuregulins (Hrg, 5 nM, 90 min). ErbB4, CAMKII, AMPK, and insulin receptor phosphorylation (*p*) was analyzed in total muscle lysates. 2-Deoxyglucose transport was determined as described under "Experimental Procedures." Results are expressed as the mean ± S.E. Glucose uptake, and representative images were obtained for $n = 3–6$ incubated muscles. * indicates statistical significance *versus* corresponding anti-ErbB4 non-treated group at $p < 0.05$. *Error bars* indicate standard error values.

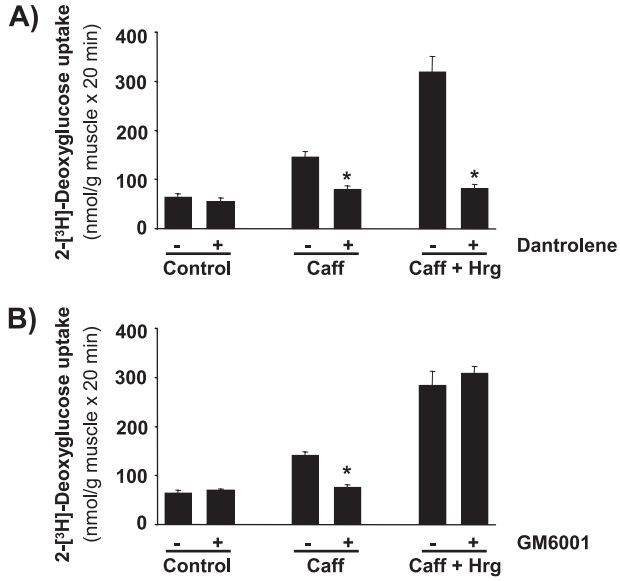

FIGURE 3. **Effects of different agents affecting ErbB4 phosphorylation on caffeine (*Caff*)-induced glucose uptake.** *A* and *B*, strips of soleus muscle were treated with dantrolene 10 μM (*A*) or GM6001 10 μM (*B*) 30 min before treatments with caffeine (3.5 mM, 15 min) or caffeine and neuregulins (5 nM). When caffeine and neuregulins were added together, muscles were treated for 15 min since the combined effects of both agents already reached their highest effect at this time (not shown). 2-Deoxyglucose uptake transport was assayed, and results are shown as mean ± S.E. for $n = 4–6$ muscles per group. * indicates statistical significant difference *versus* the corresponding dantrolene or GM6001 untreated group at $p < 0.01$. *Error bars* indicate standard error values.

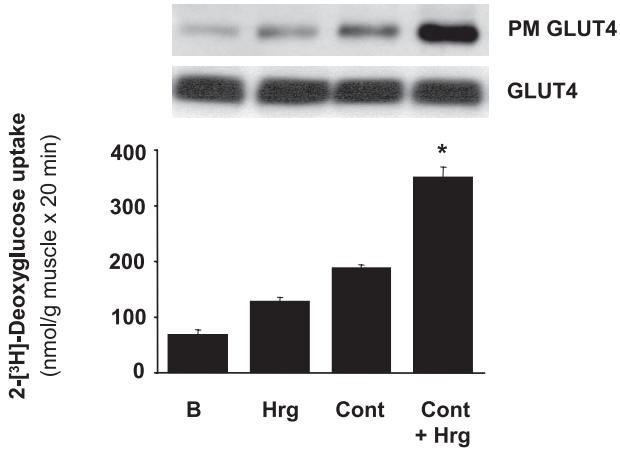

FIGURE 4. **Neuregulin addition increases *ex vivo* contraction effects on glucose transport and GLUT4 recruitment at the plasma membrane.** Strips of soleus muscles were incubated with neuregulins (Hrg, 5 nM, 90 min) and/or subjected to *ex vivo* contraction (*Cont*) for 10 min. 2-Deoxyglucose uptake and cell surface GLUT4 was expressed as mean ± S.E., whereas total and plasma membrane (*PM*) GLUT4 content is shown as a representative image, from $n = 5$ muscles per group. * indicates statistical significant difference for contraction + HRG *versus* contraction and HRG alone at $p < 0.001$. *Error bars* indicate standard error values.

nous neuregulins were added during *ex vivo* contraction. These glucose transport responses correlated with improved GLUT4 recruitment to plasma membranes (Fig. 4).

Muscle Contraction Requires ErbB4 Activity for Glucose Uptake—To evaluate the overall impact of ErbB4 signaling on contracting skeletal muscle, we injected saturating concentrations of ErbB4 blocking antibody (3 μg/muscle) into soleus and EDL muscles before inducing contraction by electrical stimulation of the sciatic nerve. As a control, muscles were injected with PBS (vehicle used to dilute the antibodies) or with anti-ErbB3 blocking antibodies. The addition of an ErbB4 blocking antibody inhibited contraction-induced ErbB4 phosphoryla-

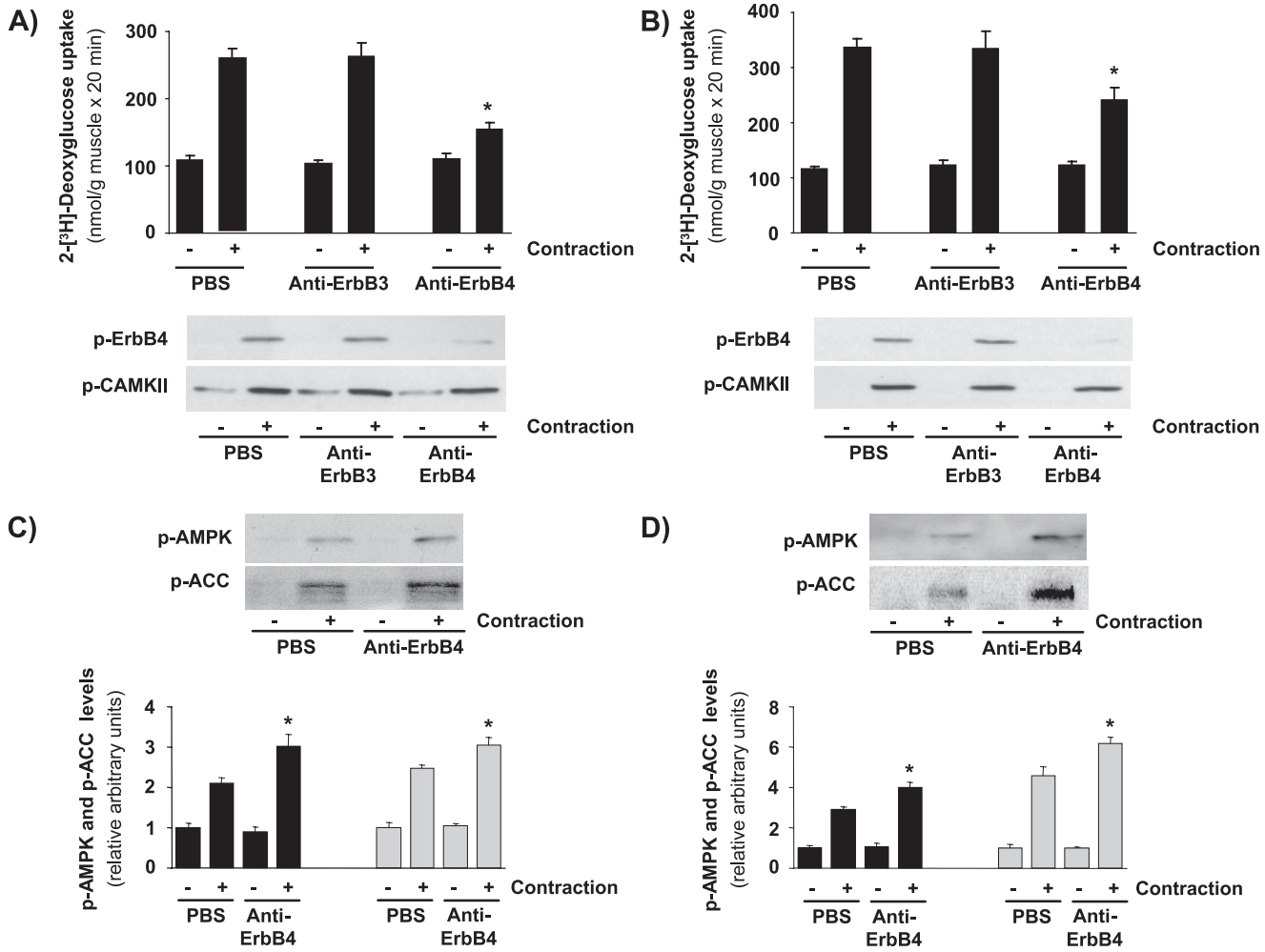

FIGURE 5. **ErbB4 blockage abrogates *in vivo* muscle contraction-induced glucose transport.** Soleus (*A* and *C*) and EDL (*B* and *D*) muscles were injected with PBS or PBS containing anti-ErbB3 or anti-ErbB4 blocking antibodies (*Anti-ErbB3* or *anti-ErbB4*, 3 μg in 50 μl). Muscles were forced to contract by sciatic nerve electrical stimulation. *p*, phosphorylation. *A* and *B*, 2-deoxyglucose uptake was determined. Results are expressed as mean ± S.E. Representative images were obtained from $n = 6–16$ muscles/group. *C* and *D*, representative images (*top*) and total quantifications (*bottom*: *black bars* are p-AMPK, and *gray bars* are p-ACC), expressed as mean ± S.E., were obtained for $n = 6–8$ muscles. * indicates statistical significant difference *versus* corresponding PBS-injected group at $p < 0.05$. *Error bars* indicate standard error values.

tion 86% in soleus and 91% in EDL muscles with a concomitant 71 and 36% impairment in contraction-induced glucose uptake, respectively. In contrast, contraction effect on glucose uptake was unaltered by either PBS or the ErbB3 blocking antibodies (Fig. 5, *A* and *B*). In all cases, phosphorylation of CAMKII was unaffected by ErbB4 blockage. Moreover, AMPK phosphorylation during contraction increased when ErbB4 was blocked, and AMPK phosphorylation correlated with an increase in its activity, as measured by phosphorylation of acetyl coenzyme A carboxylase (ACC) (Fig. 5, *C* and *D*). Accordingly, blockage of ErbB4 led to a greater decrease of glycogen, ATP, and phosphocreatine level during contraction, paralleled by an increase in intracellular lactate level (Table 1). This indicates that muscle energy metabolism is altered under these circumstances. Neuregulin effects on glucose uptake are not secondary to an altered muscle metabolism, and under resting conditions, injection of exogenous neuregulins (5 nM) to soleus and EDL muscles induced glucose uptake without altering the levels of energy metabolites (Table 2). Exogenous neuregulins did not improve *in vivo* sciatic-stimulated contraction effects on glucose uptake, probably because *in vivo* contraction elicited a maximal increase in ErbB4 phosphorylation (Fig. 6), whereas caffeine or *ex vivo* contraction of incubated skeletal muscle only led to a submaximal increase in phosphorylation.

DISCUSSION

Skeletal muscle contraction increases glucose transport via a complex mechanism involving at least two major pathways, one feed-forward pathway derived from transient increases in cytosolic Ca^{2+} released from the sarcoplasmic reticulum and another feedback pathway that is activated by the energetic demands of the working muscle via AMPK. This study provides new insights into the mechanism by which contraction regulates glucose uptake and implicates neuregulins in this process. This novel role of neuregulin action may explain why neuregulin receptor expression is not confined to the neuromuscular junction in skeletal muscle, as was first described (24), but also enriched in the transverse tubular system (25, 26), in which significant amounts of GLUT4 are recruited during contraction (27, 28).

TABLE 1

Effects of ErbB4 blockage on intramuscular glycogen, ATP, phosphocreatine, and lactate levels in contracting muscles

Results are expressed as μmol/g of muscle; mean ± S.E. for $n = 7–14$ muscles/group.

	PBS	Anti-ErbB3	Anti-ErbB4
Soleus			
Glycogen			
NC	66.3 ± 1.5	65.2 ± 2.6	68.1 ± 2.9
C	46.9 ± 1.7	48.9 ± 1.9	33.6 ± 1.4[a]
ATP			
NC	21.4 ± 0.7	21.6 ± 0.8	21.2 ± 0.6
C	16.4 ± 0.5	16.7 ± 1.0	11.8 ± 0.9[a]
Phosphocreatine			
NC	63.8 ± 2.1	64.7 ± 1.4	62.6 ± 1.4
C	48.8 ± 1.4	48.8 ± 1.8	30.7 ± 2.3[a]
Lactate			
NC	7.2 ± 0.3	7.0 ± 0.3	7.7 ± 0.6
C	10.6 ± 0.5	10.0 ± 0.3	13.6 ± 1.3[a]
EDL			
Glycogen			
NC	97.4 ± 3.6	99.6 ± 3.2	100.8 ± 4.0
C	59.3 ± 3.3	58.1 ± 3.8	41.3 ± 4.1[a]
ATP			
NC	26.9 ± 0.5	25.4 ± 0.6	27.9 ± 1.0
C	17.1 ± 0.7	16.4 ± 0.5	13.0 ± 1.6[a]
Phosphocreatine			
NC	81.0 ± 1.8	79.9 ± 1.3	80.5 ± 1.9
C	47.0 ± 1.6	47.1 ± 2.4	28.3 ± 2.6[a]
Lactate			
NC	9.4 ± 0.6	9.8 ± 0.4	9.3 ± 0.8
C	13.8 ± 0.8	13.4 ± 0.4	19.9 ± 1.8[a]

[a] Indicates statistical difference at $p < 0.05$ *versus* the corresponding contractile (C) or non-contractile (NC) groups.

TABLE 2

Effects of neuregulins microinjection (HRG, 50 μl, 0.8 pmol, 20 min) on glucose uptake and intramuscular glycogen, ATP, phosphocreatine, and lactate levels in resting conditions

Results are expressed as μmol/g of muscle, except glucose uptake (nmol/g of muscle × 20 min); mean ± S.E. for $n = 6–8$ muscles/group.

	PBS	HRG	*p* value
Soleus			
Glucose uptake	106.9 ± 5.8	187.7 ± 15.8	0.0022
Glycogen	66.4 ± 2.9	67.3 ± 4.8	0.8494
ATP	20.1 ± 0.5	22.1 ± 0.8	0.0573
Phosphocreatine	63.3 ± 2.5	60.2 ± 1.7	0.3194
Lactate	7.1 ± 0.5	7.8 ± 0.7	0.4498
EDL			
Glucose uptake	111.6 ± 4.7	143.1 ± 10.5	0.0281
Glycogen	97.7 ± 5.0	96.1 ± 1.7	0.7745
ATP	27.0 ± 0.7	25.9 ± 2.2	0.5671
Phosphocreatine	82.7 ± 1.9	81.9 ± 2.2	0.7705
Lactate	8.9 ± 0.7	9.4 ± 0.9	0.6098

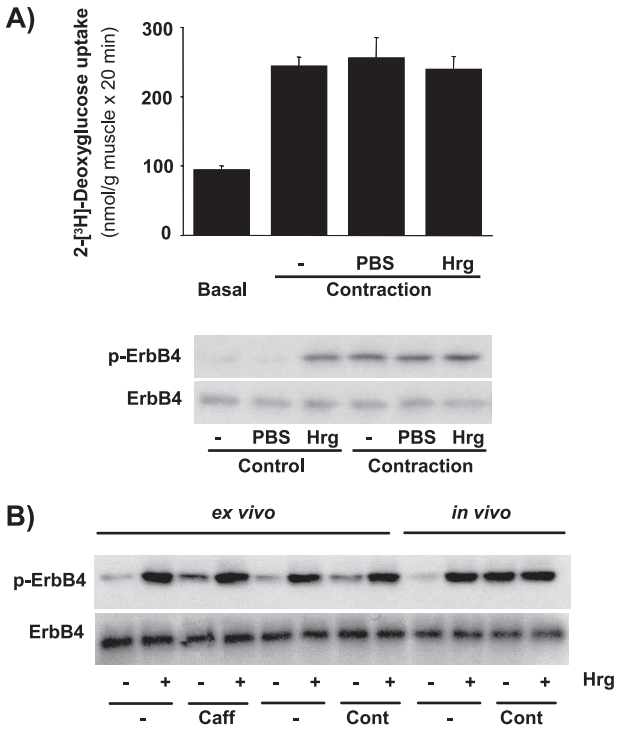

FIGURE 6. **Neuregulin addition does not enhance *in vivo* contraction effects on glucose uptake and ErbB4 phosphorylation (*p*).** *A*, comparative results obtained in soleus muscles from sham operated rats (−) *versus* rats injected with PBS or HRG, under rested or sciatic nerve-stimulated contraction, are shown. 2-Deoxyglucose uptake was determined. Results are expressed as mean ± S.E. Glucose uptake and representative images were obtained for $n = 6$ muscles. *B*, 750 μg or total lysates from strips of soleus muscle incubated *in vitro* with neuregulins (Hrg; 15 min, 5 nM), caffeine (*Caff*, 15 min, 3.5 mM), or *ex vivo* contraction (*Cont*) for 10 min or total lysates from *in vivo* contraction, 20 min after injection of 50 μl of PBS (as basal) or neuregulins (Hrg, 0.8 pmol) in rested or sciatic nerve-stimulated (10 min) electric stimulation, were immunoprecipitated and tested for ErbB4 tyrosine phosphorylation. A representative image from $n = 4$ muscles is shown. *Error bars* indicate standard error values.

Although two types of receptor, ErbB3 and ErbB4, bind neuregulins, several lines of evidence suggest that only ErbB4 mediates glucose uptake during contraction. First, *in vitro* induced contraction stimulated ErbB4, but not ErbB3, tyrosine phosphorylation levels. Second, saturating concentrations of neuregulins have little effect on ErbB3 phosphorylation levels when compared with the effects on ErbB4 in incubated soleus muscle (15). Third, when ErbB4 receptors were blocked by the use of specific antibodies, caffeine slightly induced ErbB3 receptor phosphorylation, but under these circumstances, caffeine did not induce glucose uptake in soleus muscle. Fourth, blockage of ErbB3 receptors did not impair the stimulation of glucose uptake in any of the conditions tested.

In this study, we demonstrate that neuregulins are necessary, but not sufficient, effectors for Ca^{2+} signaling to induce glucose uptake in skeletal muscle. Based on the use of specific inhibitors (3, 6, 7), CAMKII also appears to be needed for the Ca^{2+}-dependent regulation of glucose uptake. However, inhibition of CAMKII does not affect ErbB4 phosphorylation. Thus, inhibition of either of these pathways does not influence activation of the other but does compromise the Ca^{2+}-induced glucose uptake. Furthermore, our results show that AMPK-induced glucose uptake is independent of neuregulin action. AMPK does not induce phosphorylation of ErbB receptors, and its action on glucose uptake is unaltered by ErbB4 blockage. These results further support the view that Ca^{2+}/neuregulins and AMPK stimulate glucose transport through independent signaling pathways (Fig. 7).

In light of these observations, the results obtained from *in vivo* blockage of ErbB4 are consistent with previous data indicating that contraction-induced glucose transport in slow twitch muscle fibers relies primarily on Ca^{2+}-dependent mechanisms since blockage of ErbB4 had more dramatic effects on glucose uptake in soleus than in EDL muscles. In fact, soleus muscle displays higher protein levels (~40%) of ErbB4 than EDL muscle in a total membrane fraction.[4] Activation of AMPK induces glucose uptake in fast twitch fibers and may compensate for the defects in Ca^{2+}-dependent pathways. Thus, the effect of ErbB4 blockage on contraction-induced glucose

[4] C. Cantó and A. Gumà, unpublished observation.

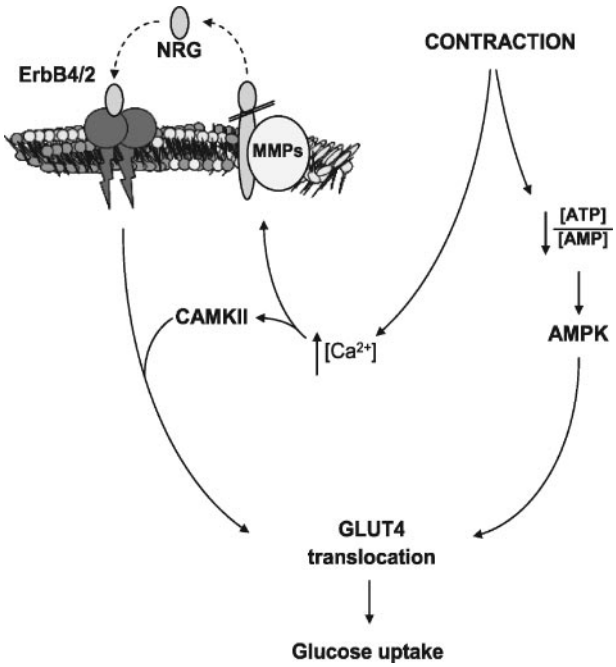

FIGURE 7. **Proposed scheme for the mechanisms underlying contraction-induced glucose uptake.** *NRG*, neuregulins; *MMPs*, membrane metalloproteinases.

uptake could be partially blunted in EDL muscles. However, we cannot exclude the possibility that the different effects of ErbB4 blockage on contraction-induced glucose uptake observed between soleus and EDL muscles was dependent on the mode of contraction elicited since the soleus muscle was subjected to concentric contractions and the EDL was subjected to eccentric contractions.

Our results implicating ErbB4 activity in mediating contraction-induced glucose uptake challenge a recent study in which chemical inhibitors were used to exclude tyrosine kinases from this process (29). However, in that study, tyrosine kinase inhibitors only partially affected insulin action at the timing and concentrations used; thus, other tyrosine kinase receptors such as ErbB4 and ErbB2 are likely to sustain enough activity to modulate glucose uptake. Moreover, in rat fast twitch epitrochlearis muscle, AMPK partially compensates for deficiencies in other signaling pathways leading to stimulation of glucose uptake.

Our observation showing that neuregulins mediate contraction-induced glucose uptake clarify previous data reporting the additive effects of neuregulins and insulin on glucose uptake in skeletal muscle and L6E9 myotubes (17). However, the effect of neuregulins on PI3K (15, 17) remains controversial. Although *ex vivo* contraction-induced glucose uptake seems to be mediated by a PI3K-independent pathway (30, 31), there is evidence that wortmannin, a PI3K-specific inhibitor, partially inhibits contraction-induced glucose uptake in perfused rat hindlimb (32, 33). As neuregulins poorly stimulate PI3K activity in muscle cells (15), the neuregulin dependence on PI3K activity is probably to be limited to situations in which there are low levels of cytosolic Ca^{2+}.

In conclusion, these studies provide evidence for the involvement of the growth factor neuregulin in the regulation of glucose uptake in skeletal muscle in response to contraction.

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