Insulin and Isoproterenol Differentially Regulate Mitogen-Activated Protein Kinase—Dependent Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter Activity in Skeletal Muscle

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Recent studies have demonstrated that p44/42 MAPK extracellular signal–regulated kinase (ERK)1 and -2–dependent Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (NKCC) activity may contribute to total potassium uptake by skeletal muscle. To study the precise mechanisms regulating NKCC activity, rat soleus and plantaris muscles were stimulated ex vivo by insulin or isoproterenol (ISO). Both hormones stimulated total uptake of the potassium congener \(^{86}\)Rb by 25–70%. However, only ISO stimulated the NKCC-mediated \(^{86}\)Rb uptake. Insulin inhibited the ISO-stimulated NKCC activity, and this counteraction was sensitive to the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 in the predominantly slow-twitch soleus muscle. Pretreatment of the soleus muscle with the phosphatidylinositol (PI) 3-kinase inhibitors wortmannin and LY294002 or with SB203580 uncovered an insulin-stimulated NKCC activity and also increased the insulin-stimulated phosphorylation of ERK. In the predominantly fast-twitch plantaris muscle, insulin-stimulated NKCC activity became apparent only after inhibition of PI 3-kinase activity, accompanied by an increase in ERK phosphorylation. PI 3-kinase inhibitors also abolished insulin-stimulated p38 MAPK phosphorylation in the plantaris muscle and Akt phosphorylation in both muscles. These data demonstrated that insulin inhibits NKCC-mediated transport in skeletal muscle through PI 3-kinase–sensitive and SB203580-sensitive mechanisms. Furthermore, differential activation of signaling cascade elements after hormonal stimulation may contribute to fiber-type specificity in the control of potassium transport by skeletal muscle. *Diabetes* 51:615–623, 2002

Skeletal muscle is generally considered to be the predominant tissue responsible for glucose disposal and regulation of plasma potassium (1–3). In patients with type 1 and type 2 diabetes, skeletal muscle has been shown to have a blunted insulin-mediated glucose transport and insufficient potassium uptake (4–6). Because potassium participates in glycogen synthesis (7), it is possible that potassium entering the cell might also be a significant physiological modulator of glucose uptake. Despite this important role in glucose metabolism, the mechanisms regulating muscle potassium transport have not been established.

Both insulin and catecholamines are important hormonal mediators of potassium uptake by skeletal muscle and other tissues, and Na-K ATPase is thought to be a main effector (8,9). Several animal and clinical studies aimed at unraveling the mechanism of altered potassium uptake in diabetes have shown depression of sodium pump activity (10,11). However, other transport mechanisms in skeletal muscle also appear to be present. Recently, the role of the Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (NKCC) in potassium transport by skeletal muscle has been studied (12). Our previous data indicated that NKCC activity can be stimulated by catecholamines or electrical stimulation to contribute as much as 35% of the potassium uptake (13). However, we did not find that insulin significantly stimulated NKCC activity in isolated rat skeletal muscle (13).

The basic mechanisms for the regulation of total and NKCC-mediated potassium uptake by catecholamines and insulin are not fully understood. The extracellular signal–regulated kinase (ERK1/ERK2 or p44/42 MAPK) and p38 mitogen-activated protein kinase (MAPK) are components of MAPK signaling cascades that have been implicated in the regulation of cellular potassium uptake (14,15). We and others have shown that α- and β-adrenergic stimulation of NKCC in striated muscle is dependent on ERK activation (13,14). It is of interest that insulin receptor stimulation is recognized as an activator of the ERK pathway, but that insulin has no effect on NKCC activity. Activation of the p38 MAPK pathway has been characterized in response to stressors (such as hyperosmolarity), insulin, and, recently, β-adrenergic stimulation (16–19). However, hyperosmolarity and catecholamines are also known to stimulate inwardly directed NKCC, whereas insulin is not able to activate the cotransporter in skeletal muscle (20–22).

Therefore, the current study was undertaken to delineate the MAPK signaling pathways that stimulate total and NKCC-mediated potassium uptake by skeletal muscle in response to insulin and the β-adrenergic receptor agonist...
membranes were incubated overnight at 4°C in 25 mM Tris-HCl buffer at 95% relative humidity (Pierce, Rockford, IL). Then, 250 μl of the homogenate was mixed with a 4-0 surgical silk suture to a certain ratio of trichloroacetic acid to a certain level of glucose. The bumetanide-sensitive 86Rb rate constant was used as an index of NKCC activity (13). The bumetanide-sensitive portion of 86Rb uptake was expressed as a rate constant, as previously described (13).

Muscle incubation and 86Rb/K influx constant calculation. Each muscle was attached with a 4-0 surgical silk suture to glass wands for rapid transfer among solutions. The muscles were preincubated for 15 min at 30°C in preincubation media (oxygenated Krebs-Ringer containing bumetanide [10−5 mol/l] or vehicle [DMSO] for the contralateral muscle, and kinase inhibitor as necessary). For hormonal stimulation, after preincubation muscles were taken directly to incubation medium (oxygenated Krebs-Ringer containing 1 μCi/ml 86Rb, either bumetanide or vehicle, and the appropriate kinase inhibitor or vehicle) at 30°C, which contained 100 μM insulin and/or 30 μM ISO. Incubation was for 10 min. Muscles were immediately washed with ice-cold 0.9% saline solution. After being washed, the muscles were blotted, weighed, and homogenized in 2 ml of 0.3 mol/l trichloroacetic acid.

86Rb uptake by the muscle was measured by Cerenkov counting. 86Rb uptake and phosphorylation of the ERK1/ERK2 and p38 MAPK and polyclonal anti-ERK2 and anti-p38 MAPK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal rabbit antibodies to phospho-Akt-Ser (473) and Akt were obtained from New England Biolabs (Beverly, MA).

Animal care and muscle preparation. Female SD rats (100–150 g) were rapidly anaesthetized with pentobarbital sodium (45 mg/kg ip) for tissue removal. Soleus and plantaris muscles were used for the experiments. Soleus (predominantly slow-twitch fibers, ~87% type I and 13% type II) and plantaris muscles (predominantly fast-twitch fibers, ~9% type I and 91% type II) (23) were used to disassemble the proximal and distal tendons. The muscles were placed in oxygenated Krebs-Ringer buffer at 25°C in preparation for further treatment. The Animal Care and Use Committee of the University of Tennessee approved all procedures.

Materials. 86RbCl was from obtained from Du Pont-NEN (Boston, MA). Insulin, ISO, and bumetanide were obtained from Sigma (St. Louis, MO). The kinase inhibitors SB203580, wortmannin, and LY294002 were obtained from CalBiochem (La Jolla, CA). Monoclonal phospho-specific mouse antibodies to ERK and p38 MAPK and polyclonal anti-ERK2 and anti-p38 MAPK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal rabbit antibodies to phospho-Akt-Ser (473) and Akt were obtained from New England Biolabs.

Analysis of ERK1/ERK2 and p38 MAPK phosphorylation. The muscles were preincubated and incubated as described, except for inclusion of 86Rb. After incubation with kinase inhibitors and stimulation by insulin and iso, the muscles were placed in ice-cold lysis buffer (10 mmol/l Tris-HCl [pH 7.4], 5 mmol/l EDTA, 50 mmol/l NaCl, 30 mmol/l Na4P2O7, 50 mmol/l NaF, 100 μmol/l Na3VO4, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml pepstatin A), homogenized with a Teflon pestle, and centrifuged at 4°C for 5 min at 5,000g. The protein concentration of the supernatant was measured by the bicinchoninic acid assay (Pierce, Rockford, IL). Then, 25 μg protein was denatured in SDS-PAGE buffer at 95°C for 5 min and electrophoresed on a 10% SDS-PAGE gel. The gels were electroblotted onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C in blocking buffer (1.5% bovine serum albumin in Tris-buffered saline) and probed overnight with primary antibodies. Immunoreactive bands were visualized with chemiluminescence (ECL Plus; Amersham, Piscataway, NJ). Bands were quantitated by video densitometry.

RESULTS

Muscle total 86Rb uptake. Both ISO and insulin significantly increased skeletal muscle 86Rb uptake by 35–70 and 24–29%, respectively (Fig. 1A). The most prominent effect was detected in the predominantly slow-twitch soleus muscle; stimulated K-transport was greater by 45–70% compared with the predominantly fast-twitch plantaris muscle. Further, ISO-stimulated 86Rb influx was considerably greater in both soleus and plantaris muscles compared with insulin-stimulated muscles (P < 0.01). Surprisingly, the stimulation of total 86Rb influx by the combination of ISO and insulin was not different from insulin stimulation alone (P > 0.05). NKCC-mediated 86Rb uptake. To determine whether the ISO- and insulin-mediated increase in 86Rb influx might involve activation of NKCC, isolated muscles were incubated in the presence of bumetanide, a specific inhibitor of NKCC activity (24). Curiously, incubation of unstimulated muscle with bumetanide slightly enhanced the rate of 86Rb transport relative to vehicle-treated muscle (Fig. 1B). There was a significant increase in the bumetanide-sensi-

Statistics. Comparisons within and among treatments for the rate constant data were made by ANOVA. Differences between treatments were considered significant at P < 0.05. Data are reported as means ± SE. For differences that were not significant, the power of these tests was typically >0.85.
tive portion of $^{86}$Rb influx in ISO-stimulated muscle, contributing as much as 40% of the stimulated $^{86}$Rb influx in the soleus muscle and 45% of the stimulated influx in the plantaris muscle (Fig. 1B). In contrast, insulin did not stimulate bumetanide-sensitive transport in either muscle, in agreement with our previous report (13). Of particular note, insulin abrogated the ISO-stimulated, bumetanide-sensitive portion of $^{86}$Rb uptake regardless of muscle fiber type (Fig. 1B).

**Effect of kinase inhibitors on stimulated $^{86}$Rb uptake.** Previously, we showed that ERK activation is required for NKCC upregulation (13). Because PI 3-kinase and p38 MAPK are known to modulate ERK activity and are stimulated by ISO and insulin, we evaluated changes in total and NKCC-mediated $^{86}$Rb uptake in response to pharmacological inhibitors of these kinases (17,25–29).

PI 3-kinase inhibitor wortmannin reduced the ISO-stimulated total $^{86}$Rb influx in the slow-twitch soleus muscle, in part through a 50% inhibition of NKCC-mediated transport ($P < 0.01$) (Fig. 2). Transport by the fast-twitch plantaris muscle was not affected. Another PI 3-kinase inhibitor, LY294002, also reduced both total and bumetanide-sensitive $^{86}$Rb transport in the soleus muscle in a fashion similar to that of wortmannin (Fig. 2). These data indicated that NKCC activation by ISO is partially PI 3-kinase-dependent only in slow-twitch oxidative muscle.

We next investigated whether p38 MAPK activation was involved in ISO-stimulated NKCC activity. The p38 MAPK inhibitor SB203580 (30) reduced the ISO-stimulated total $^{86}$Rb uptake by 20% ($P < 0.01$) in the soleus muscle but did not affect activation of NKCC in slow- or fast-twitch oxidative muscle. We next investigated whether p38 MAPK activation was involved in ISO-stimulated NKCC activity. The p38 MAPK inhibitor SB203580 (30) reduced the ISO-stimulated total $^{86}$Rb uptake by 20% ($P < 0.01$) in the soleus muscle but did not affect activation of NKCC in slow- or fast-twitch skeletal muscle (Fig. 2).

Wortmannin abolished the insulin-mediated stimulation of total $^{86}$Rb influx by the soleus muscle (Fig. 3A). Remarkably, wortmannin and LY294002 increased NKCC-mediated $^{86}$Rb transport by the soleus and plantaris muscles in response to insulin (Fig. 3B). This stimulation was ~50% the ISO-stimulated value in the soleus muscle and equiva-
 lent to the ISO-stimulated value in the plantaris muscle. Treatment with the SB203580 reduced the insulin-stimulated total $^{86}$Rb transport by 10% in the soleus muscle ($P < 0.05$) and prevented activation of $^{86}$Rb influx in the plantaris muscle. Interestingly, NKCC activation in the soleus muscle was significantly increased by insulin in the presence of SB203580 to an extent similar to that of $\beta$-adrenergic stimulation (Fig. 3B). In the absence of stimulation by insulin, there were no significant effects of SB203580 or the PI 3-kinase inhibitors on either total or NKCC-mediated $^{86}$Rb influx (not shown). These findings indicated that PI 3-kinase and p38 MAPK activity regulate NKCC function in response to insulin through a potent inhibitory mechanism that is fiber-type specific. Therefore, we hypothesized that activation of these pathways could be implicated in the downregulation of the NKCC detected by the combination of ISO and insulin.

We repeated the above experiments by stimulating muscle $^{86}$Rb influx with a combination of ISO and insulin. Treatment with SB203580 resulted in a marked increase in NKCC activity in the fast-twitch plantaris muscle (Fig. 4B). This effect contributed to a significant elevation of total $^{86}$Rb influx to the same degree as with ISO alone (Fig. 1). We found that neither wortmannin or LY294002 had any effect on NKCC-mediated $^{86}$Rb influx in either the soleus or plantaris muscles (Fig. 4B). These results indicated that inhibition of p38 MAPK precludes downregulation of NKCC activity by insulin in fast-twitch skeletal muscle.

**Isoproterenol-stimulated ERK and p38 MAPK phosphorylation.** ERK activation is necessary for NKCC activation by ISO (13). Also, it was recently shown that p38 MAPK activity in striated muscle is altered upon catecholaminergic stimulation (19). To further determine the intracellular signaling pathways responsible for $\beta$-adrenergic signal transduction in skeletal muscle, ERK and p38 MAPK phosphorylation were assessed by immunoblot analysis.

Isoproterenol significantly increased ERK phosphorylation in the soleus and plantaris muscles (Fig. 5). The magnitude of ISO-stimulated ERK phosphorylation was greater in the slow-twitch soleus muscle than in the fast-twitch plantaris muscle. Fiber-type specificity in agonist-mediated ERK phosphorylation might be attributable to differences in the total amount of ERK. Video densitometry of Western blots with equal loading of samples showed that expression of ERK protein was 38% greater in the soleus muscle than in plantaris muscle ($P < 0.05$; data not shown). To determine the mechanism of ISO-mediated ERK phosphorylation, muscles were incubated with PI 3-kinase inhibitors before and during ISO stimulation. Consistent with our results in the $^{86}$Rb uptake assay, wortmannin and LY294002 significantly diminished ERK phosphorylation by 35% ($P < 0.05$) in the soleus muscle but had no effect on the plantaris muscle (Fig. 5). Short exposure to hormone or inhibitors had no effect on total ERK protein expression (not shown), indicating that alterations in ERK phosphorylation were a direct result of intracellular signaling.

In the next set of experiments, we found that ISO led to a twofold increase ($P < 0.05$) in p38 MAPK phosphorylation in the plantaris muscle, but not in the soleus muscle (Fig. 6). The p38 MAPK upregulation in plantaris muscle was not PI 3-kinase–sensitive because neither wortman-
the upregulation of NKCC-mediated $^{86}$Rb uptake in a fiber-type-specific manner. Because ERK phosphorylation is required for NKCC stimulation (13), we examined insulin-stimulated ERK activation in PI 3-kinase and p38 MAPK-inhibited muscles. In muscle treated with insulin in the presence of wortmannin, ERK phosphorylation was quantified by densitometry and the phospho/total ratio was calculated. Data are expressed relative to the basal level of phosphorylation (taken as 1.0). Data were obtained from four to six different muscles and are given as means ± SE. $^*P < 0.05$ compared with nonstimulated (basal) state; $^†P < 0.05$ compared with ISO-stimulated state.

FIG. 5. Phosphorylation of ERK1/ERK2 by ISO. Isolated soleus and plantaris muscles were treated with 30 μmol/l ISO for 10 min in the absence or presence of 15 μmol/l LY294002 (A) or 0.5 μmol/l wortmannin (B). Equal amounts of protein from whole cell lysates were immunoblotted with an antibody to phospho-ERK1/ERK2. Representative blots are shown. The immunoblots then were stripped and reprobed with anti-ERK2 antibody. The intensity of each band was quantified by densitometry and the phospho/total ratio was calculated. Data are expressed relative to the basal level of phosphorylation (taken as 1.0). Data were obtained from four to six different muscles and are given as means ± SE. $^*P < 0.05$ compared with nonstimulated (basal) state; $^†P < 0.05$ compared with ISO-stimulated state.

p38 MAPK by insulin could stimulate a negative feedback mechanism for ERK phosphorylation in the soleus muscle (Fig. 7A).

There is little evidence regarding upstream regulators of insulin-mediated p38 MAPK activation, though p38 MAPK is thought to have a significant effect on the regulation of muscle GLUT4 function (17,18). Stimulation of PI 3-kinase activity by insulin is a critical step in a myriad of signal cascades activated by this hormone (31). We hypothesized that PI 3-kinase inhibition might result in alteration of p38 MAPK activation. Insulin caused p38 MAPK phosphorylation in the fast-twitch plantaris muscle but not in the slow-twitch soleus muscle (Fig. 7B). As noted before, protein expression of p38 MAPK was not altered by the 10-min stimulation. Incubation of the soleus muscle with PI 3-kinase inhibitors did not change phospho-p38 MAPK.
immunoreactivity in response to insulin (Fig. 7B). However, when plantaris muscles were incubated with wortmannin and LY294002, the 1.5-fold increase in insulin-stimulated p38 MAPK phosphorylation was abrogated, clearly indicating that insulin mediates activation of p38 MAPK through a PI 3-kinase-dependent mechanism in the fast-twitch plantaris muscle (Fig. 7B). Recently, several investigators proposed that SB203580 was able to inhibit stimulus-induced phosphorylation of p38 MAPK, although the primary mechanism of action of the compound is to inhibit enzymatic activity of p38 MAPK (18,30). Exposure to the SB203580 did not alter either the basal or insulin-stimulated p38 MAPK phosphorylation in the soleus or plantaris muscles (Fig. 7B).

In this study we stimulated muscle with insulin in a postprandial concentration (100 μU/ml). Others have used much higher concentrations (40,43). We verified that this concentration stimulated the classical pathway of PI 3-kinase/Akt. As shown in Fig. 8, insulin treatment of soleus and plantaris muscles stimulated Akt Ser (473) phosphorylation. Akt activation was blocked by treatment with the PI 3-kinase inhibitors but not the p38 MAPK inhibitor.

**DISCUSSION**

Diabetes and insulin resistance are among the conditions known to reduce tissue potassium transport (4–6,10,11). In addition, β-blocker treatment inhibits stimulation of potassium uptake in skeletal muscle and reduces glucose tolerance (32). However, the cellular mechanisms that regulate potassium transport by hormones, insulin, and ISO are poorly understood. The major finding of this study was the counterregulatory mechanism of insulin on the β-adrenergic–stimulated kinase uptake in isolated rat skeletal muscle. The counterregulatory action of insulin appeared to operate through the inhibition of NKCC-mediated potassium uptake. Regulation of NKCC-mediated K-uptake by insulin or catecholamines has been previously documented, mostly in 3T3-L1 adipocytes, fibroblasts, vascular smooth muscle cells, and myocardium (14,15,22,23,33,34). This study is the first to evaluate hormone interaction in the regulation of NKCC activity in skeletal muscle. Although bumetanide is a specific inhibitor of NKCC activity at 10 μmol/l, and muscle demonstrates ion dependence of transport consistent with NKCC
activity (12), bumetanide also slightly increased an apparently compensatory transport in the basal, unstimulated muscle (Fig. 1B). We previously reported that insulin does not stimulate bumetanide-sensitive potassium uptake by skeletal muscle (13), and have now shown that insulin inhibits this transport mechanism. Further, the data demonstrated that the effect of insulin on NKCC-mediated potassium uptake was attributable to active control by the hormone on the signaling mechanisms that regulate potassium transport in skeletal muscle, as described below.

To evaluate the signaling pathways responsible for the counterregulatory action of insulin on the β-adrenergic–stimulated NKCC activity, we examined the role of PI 3-kinase and p38 MAPK. Both the insulin and β-adrenergic receptor can stimulate the ERK MAPK cascade, and this stimulation is modulated through simultaneous stimulation of PI 3-kinase and p38 MAPK (25,26,35,36). We have previously shown that activation of ERK is necessary for NKCC activity in skeletal muscle (13), so PI 3-kinase and p38 MAPK were candidate control points for interaction between signals originating at the insulin and β-adrenergic receptors. The data presented demonstrate several novel mechanistic details regarding β-adrenergic regulation of potassium transport. First, ISO-stimulated PI 3-kinase activity increased NKCC activity only in the predominantly slow-twitch soleus muscle by activating the ERK pathway (Figs. 2 and 5). To our knowledge, these data are the first to demonstrate the dependence of β-adrenergic receptor-mediated ERK phosphorylation on PI 3-kinase activity in skeletal muscle. However, although an inhibitor of p38 MAPK altered total ISO-stimulated K-transport in both muscles, NKCC activity was not affected (Fig. 2).

In contrast to the stimulation of NKCC activity by β-adrenergic receptor–stimulated PI 3-kinase activity in slow-twitch muscle, insulin receptor stimulation appears to have inhibited NKCC activity through activation of PI 3-kinase (Fig. 3). The PI 3-kinase activation apparently inhibited the phosphorylation of the ERK necessary for NKCC activity (Fig. 7). The inhibitory action of insulin-stimulated PI 3-kinase on NKCC activity may be stronger than the excitatory action of β-adrenergic receptor–stimulated PI 3-kinase activity because insulin inhibited the ISO-stimulated NKCC activity (Fig. 1). Recently, insulin-stimulated PI 3-kinase activity has been proposed as a site of counterregulatory action on ISO effects (37). However, our findings indicated that inhibition of PI 3-kinase activity during treatment with both hormones was not sufficient to restore ISO-stimulated NKCC activity (Fig. 4). Fiber-type specificity of the signaling pathways became apparent with the inhibition of insulin-stimulated p38 MAPK activity. It is known that activation of p38 MAPK inhibits some cellular responses (16). For example, an interleukin (IL)-1–mediated increase in p38 MAPK phosphorylation leads to β-adrenergic hyporesponsiveness in cultured airway smooth muscle cells, and SB203580 significantly reduces the IL-1 effect (38). Only in the slow-twitch soleus muscle did SB203580 allow a large stimulation of NKCC activity and ERK phosphorylation by insulin alone (Figs. 3 and 7). In contrast, only in the fast-twitch plantaris muscle was the inhibition of p38 MAPK sufficient to overcome the inhibition of NKCC activity by insulin and ISO combined (Fig. 4). Despite the apparent implication of p38 MAPK activity for inhibition of NKCC activity in the soleus muscle, insulin, like ISO, increased p38 MAPK phosphorylation only in the plantaris muscle (Fig. 6). There are several explanations for this. It may be that the effect of SB203580 is not related to inhibition of p38 MAPK activity. Several lines of evidence have suggested that SB203580 can activate the Raf/MAPK kinase/ERK pathway independent of its action on p38 MAPK activity (28,39). Such an effect of SB203580 would be consistent with the combination of insulin and SB203580 stimulating NKCC (Fig. 3) and ERK phosphorylation in soleus muscle (Fig. 7). However, a stimulatory effect of SB203580 on the ERK pathway that is independent of p38 MAPK activation is not consistent with the lack of stimulation of NKCC activity and ERK observed when SB203580 was used alone. Another explanation of the effect of insulin in combination with SB203580 in the soleus muscle is that there is a tonic level of activation of p38 MAPK that does not inhibit NKCC activity (consistent with the lack of effect of SB203580 alone). A tonic level of activation could make it difficult to observe further activation by insulin, and this small, necessarily compartmental p38 MAPK activation inhibits NKCC activation such that the inhibition is relieved by SB203580. Thus, although it is not clear from the SB203580 action whether p38 MAPK exerts an inhibitory effect on NKCC activity, it is clear that a phenotypic difference exists for the affected pathways, in addition to those discussed previously for the PI 3-kinase pathway.

Our current findings are in contrast with a previous report indicating that PI 3-kinase inhibition abolishes the activation of ERK by insulin (40). In that study, the investigators used a supraphysiological concentration of insulin (20,000 μU/ml), whereas we obtained significant stimulation of 86Rb uptake and MAPK and Akt activation with a physiological concentration of 100 μU/ml (~1 nmol) of hormone. One possibility is that high dosages of insulin may also activate the IGF-I receptors (41,42). Activation of IGF-I receptors may compete with insulin-mediated cellular events and could cross-talk with insulin-signaling pathways. This possibility is consistent with our data and the report of Shepherd et al. (43), in which inhibition of PI 3-kinase activity did not affect the phosphorylation of ERK in human skeletal muscle in response to 1,000 μU/ml insulin. Moreover, in our study we measured insulin-stimulated protein kinase B/Akt Ser (473) phosphorylation as a suitable reporter of hormone-mediated PI 3-kinase activity. In both muscles, insulin action resulted in a two- to fivefold stimulation of Akt phosphorylation (Fig. 8). Furthermore, inhibition of PI 3-kinase activity abolished insulin-mediated Akt phosphorylation, in agreement with previously reported data (43,44). There are several reports that have shown PI 3-kinase/Akt pathway inhibition of Raf-1/MEK/ERK in myocytes and human embryonic kidney 293 cells (29,45). In this regard, we propose that Akt activity may be a crucial element in insulin-dependent inhibition of NKCC activity in skeletal muscle.

In conclusion, we have presented data showing the inhibitory influence of insulin-stimulated PI 3-kinase activity on ERK MAPK-dependent NKCC activity in skeletal muscle. Our data also indicated that insulin inhibition of NKCC activity can be relieved in a muscle phenotype–
specific manner by the p38 MAPK inhibitor SB203580. However, it is not clear that p38 MAPK is an active site of this inhibition. Implicit in these data are a broader physiological consequence of the phenotypic differences because of the greater overall potassium transport and NKCC activity of the slow-twitch soleus muscle in response to hormonal stimulation. A growing body of evidence indicates that changes in skeletal muscle fiber phenotype toward a decrease in the highly insulin-sensitive slow-twitch fibers may facilitate insulin resistance and diabetes (46–48). Therefore, the differences in skeletal muscle phenotype—specific characteristics of potassium uptake may contribute to the hyperkalemia that appears in patients with diabetes and insulin resistance. Further, we suggest that skeletal muscle, as a major site of insulin action, might be a prominent locus of the hormone's regulatory effects on catecholamine action with respect to potassium uptake.

ACKNOWLEDGMENTS

The research was supported by an American Diabetes Association Research award to D.B.T.

The authors are grateful to L.A. Malinick for assistance with publication graphics.

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