NKCC activity restores muscle water during hyperosmotic challenge independent of insulin, ERK, and p38 MAPK

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Gosmanov, Aidar R., Edward G. Schneider, and Donald B. Thomason. NKCC activity restores muscle water during hyperosmotic challenge independent of insulin, ERK, and p38 MAPK. *Am J Physiol Regul Integr Comp Physiol* 284: R655–R665, 2003. First published November 14, 2002; 10.1152/ajpregu.00576.2002.—In isosmotic conditions, insulin stimulation of PI 3-K/Akt and p38 MAPK pathways in skeletal muscle inhibits Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) activity induced by the ERK1,2 MAPK pathway. Whether these signaling cascades contribute to NKCC regulation during osmotic challenge is unknown. Increasing osmolarity by 20 mosM with either glucose or mannitol induced NKCC-mediated ⁸⁶Rb uptake and water transport into rat soleus and plantaris skeletal muscle in vitro. This NKCC activity restored intracellular water. In contrast to mannitol, hyperosmolar glucose increased ERK1,2 and p38 MAPK phosphorylation. Glucose, but not mannitol, impaired insulin-stimulated phosphorylation of Akt and p38 MAPK in the plantaris and soleus muscles, respectively. Hyperosmolarity-induced NKCC activation was insensitive to insulin action and pharmacological inhibition of ERK1,2 and p38 MAPK pathways. Paradoxically, cAMP-producing agents, which stimulate NKCC activity in isosmotic conditions, suppressed hyperosmolar glucose- and mannitol-induced NKCC activity and prevented restoration of muscle cell volume in hyperosmotic media. These results indicate that NKCC activity helps restore muscle cell volume during hyperglycemia. Moreover, hyperosmolarity activates NKCC regulatory pathways that are insensitive to insulin inhibition.

hyperglycemia; adenosine 3',5'-cyclic monophosphate; Akt; Na⁺/K⁺/2Cl⁻ cotransporter; phosphatidylinositol 3-kinase

Cell volume plays a critical role in mediating insulin effects in different mammalian cell types (38). The mechanisms that regulate cell water balance should, therefore, be important to skeletal muscle, a major site of insulin action. Several physiological and pathological conditions are accompanied by increased plasma osmolarity. Ingestion of food, moderate exercise, diabetes-related hyperglycemia, or dehydration can increase plasma osmolarity to 325 mosM or higher (6, 8, 10, 15, 20, 28). In types 1 and 2 diabetes, hyperglycemia may ultimately lead to intracellular water (ICW) depletion (39). The resultant cell shrinkage is thought to contribute to insulin resistance and diabetes complications (24, 38). In response to shrinkage, a cell activates compensatory mechanisms to restore cell volume and function [regulatory volume increase (RVI)] (24, 31). During chronic hyperglycemia, cells may accommodate by accumulating organic osmotics such as sorbitol (24, 31). However, the factors contributing to the initial RVI in insulin-sensitive tissues are not well understood.

In skeletal muscle, as in many other cell types including rabbit cardiac myocytes and rat diaphragm, RVI may be mediated by Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) activity (9, 37, 40). Although direct evidence for RVI and its mechanisms in skeletal muscle tissue is lacking, we and others recently demonstrated the molecular and functional presence of NKCC in rat skeletal muscle (27, 44). We reported that NKCC activity, which is normally minuscule in quiescent skeletal muscle, may be stimulated by catecholamines and contractile activity (12, 45). Conversely, inhibition of NKCC activity can be induced by insulin or disruption of signaling through pertussis toxin-sensitive G proteins (13, 14). The ERK1,2 MAPK cascade stimulates NKCC activity, whereas activation of Akt and p38 MAPK signaling cascades inhibits NKCC function, apparently through inhibition of ERK1,2 MAPK pathway (13, 14, 45). Thus, the intracellular signaling pathways regulating NKCC activity in skeletal muscle are beginning to be identified.

Exposure of mammalian cells to hyperosmotic conditions results in a robust stimulation of ERK1,2 and p38 MAPK pathways (23). Also, activation of Akt in the presence of a high-glucose concentration or hyperosmotic shock in vitro and in vivo is impaired (7, 21, 22, 30, 32, 36). Although these processes may alter cellular metabolism, the physiological relevance of the altered metabolism induced by hyperosmolarity and the role of insulin are not resolved. In particular, with respect to the regulation of cell volume, the interplay among these signaling cascades to provide a meaningful restoration of cell volume is also not understood.

The current study was performed to assess the role of NKCC activity in regulating skeletal muscle cell water volume. In addition, we asked whether the sig-
naling pathways that are necessary for regulating of NKCC function in isosmotic conditions also control cotransporter activity during hyperosmotic challenge. Data reported here demonstrate that NKCC activity is necessary to maintain muscle ICW during hyperosmotic challenge, but it is regulated by mechanisms unique from those controlling NKCC activity under isosmotic conditions.

METHODS

Materials. Insulin, isoproterenol (ISO), and bumetanide were purchased from Sigma (St. Louis, MO). Forskolin (FSK), PD-098059, U-0126, SB-203580, and SB-202190 were obtained from CalBiochem (La Jolla, CA). 86RbCl and [3H]mannitol were from New England Nuclear (Boston, MA). Enhanced chemiluminescence (ECL) kit and [14C]urea were from Amersham Life Sciences (Piscataway, NJ). Phospho-specific antibodies to ERK and anti-ERK-2 were obtained from Amersham Life Sciences (Piscataway, NJ). Phospho-specific Akt and p38 MAPK and anti-Akt and anti-p38 MAPK antibodies to ERK and anti-ERK-2 were obtained from Amersham Life Sciences (Piscataway, NJ). Phospho-Tyr182, and to Akt phosphorylated on Ser 473 were used to assess the effect of cAMP, ISO (30 μM) and FSK (20 μM) on ICW. The bumetanide-sensitive 86Rb rate constant was used as an index of NKCC activity, as described previously (45). The bumetanide-sensitive portion of 86Rb uptake was calculated by subtracting the bumetanide treat-

ment value for the muscle of one hindlimb from the vehicle treatment value of the contralateral muscle.

Cell volume measurement. A modification of the technique described by Hayama et al. (16) was used to measure muscle cell volume. Briefly, the distribution volume of [14C]urea (a membrane-permeable tracer that measures both cell and extracellular volumes) minus the distribution volume of [3H]mannitol (a measure of extracellular volume) was used to measure intracellular muscle cell volume. The muscles were preincubated and incubated as described above. The preincubation and incubation media contained 2 μCi [14C]urea and 8 μCi [3H]mannitol. To terminate radiotracer flux, muscles were quickly rinsed in cold Krebs-Ringer solution and then blotted, weighed, and dissolved by adding 1 ml of Soluene-350 (Packard). Five milliliters of scintillation fluid were added to each scintillation vial, and disintegrations per minute (DPM) for both 14C and 3H were determined. The volume of the muscle cells was calculated by subtracting the [3H]mannitol volume (DPM of 3H in tissue/DPM of 3H in medium) from the [14C]urea volume (DPM of 14C in tissue/ DPM of 14C in medium). The following equation was used to calculate ICW content (in μg/mg tissue)

\[
\text{ICW} = \frac{\text{muscle [14C]urea DPM} - \text{muscle [3H]mannitol DPM}}{\text{bulk [14C]urea DPM/μl} - \text{bulk [3H]mannitol DPM/μl}}
\]

Analysis of ERK1,2 MAPK, p38 MAPK, and Akt phosphorylation. Whole muscle was preincubated as described. Incubation medium did not include 86Rb, 14C, or 3H. After incubation, the muscles were placed in ice-cold lysozyme buffer as before (12), homogenized, and centrifuged at 4°C for 15 min at 5,000 g. Protein concentration of the supernatant was measured by the bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein were mixed with SDS denaturing buffer, warmed to 95°C for 5 min, electrophoresed on a 10% SDS-PAGE gel, and electroblotted onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C in blocking buffer (1.5 mM NaH2PO4, 8 mM Na2HPO4, 0.15 M NaCl, 0.3% Triton X-100, pH 7.4) supplemented with 3% BSA. Then, the membranes were incubated at room temperature for 1.5 h in blocking buffer containing 1% BSA and the specific antibody (1:1,000). Phospho-specific antibodies to ERK1,2, and Akt phosphorylation on Thr202 and Tyr217, were used to detect the catalytically activated forms of the kinases. After incubation with 1% BSA blocking buffer containing horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, the proteins of interest were visualized by chemiluminescent exposure of X-ray film (ECL Plus). Bands were quantitated by video densitometry. Protein phosphorylation was calculated as the ratio of phospho-to-total protein expression, normalized to the basal level value (taken as 1.0).

Statistics. Comparisons within and among treatments for the rate constant data were made by analysis of variance. 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.

RESULTS

High-glucose concentration induces NKCC activation that is insensitive to inhibition by insulin. Under isosmotic conditions, NKCC activity in unstimulated and insulin-stimulated soleus (predominantly slow-twitch)
and plantaris (predominantly fast-twitch) muscle was barely detectable (Fig. 1); NKCC activity is measured as the bumetanide-sensitive $^{86}$Rb uptake rate constant (45). The presence in the incubation media of additional 20 mM glucose (osmolarity of 318 mosM) provoked a significant increase in NKCC activity in both the soleus and plantaris muscle (Fig. 1). To assess whether nonspecific effects of either high osmolarity or enhanced glucose uptake (3) mediate the NKCC stimulatory effect of high-glucose concentration, we performed analogous experiments using nonmetabolizable sugars that are not transported or transported into the cell similar to glucose while preserving the osmolarity of 318 mosM. Mannitol (which is not transported across the cell membrane) induced activation of NKCC similar to that of high-glucose concentration (Fig. 1). Likewise, 3-O-methyl-D-glucose and glucosamine (transported but not metabolized) led to a robust stimulation of bumetanide-sensitive $^{86}$Rb uptake in the skeletal muscle (Fig. 1). Also, we examined the effect of urea (a permeable solute) on NKCC activity. Addition of 20 mM urea did not produce significant changes in NKCC activity (Fig. 1). Recently, we reported that insulin inhibits activation of NKCC in the rat skeletal muscle under isosmotic conditions (13). In sharp contrast to the effects in isosmotic conditions, insulin in a concentration of 100 $\mu$U/ml (~0.6 nmol) did not affect NKCC activity induced by either hyperosmolar glucose, mannitol, 3-O-methyl-D-glucose, or glucosamine (Fig. 1). Importantly, 1,000 $\mu$U/ml of insulin also did not alter bumetanide-sensitive $^{86}$Rb uptake evoked by different sugars (data not shown). Therefore, it appeared that the effects of high-glucose concentration on NKCC activity were independent of specific glucose transport and metabolizing pathways. These results indicate that elevation of osmolarity (by 20 mosM) activates NKCC-mediated $^{86}$Rb uptake, which is independent of regulation by insulin.

NKCC activity regulates ICW of skeletal muscle exposed to hyperosmotic media. Changes in muscle ICW resulting from bumetanide treatment were used to estimate the role of NKCC activity in volume regulation during hyperosmotic challenge. ICW was estimated from the difference between total and extracellular water; these values were determined from muscle equilibrated with $^{14}$Curea and $^{3}$Hmannitol before hyperosmotic challenge. Vehicle- and isosmotically treated muscle provided controls for possible incomplete redistribution of the radiotracers during the 25-min hyperosmotic challenge (half-times are estimated to be 5~10 min). We observed no change in the cell water with bumetanide treatment under isosmotic conditions (Fig. 2), consistent with the miniscule NKCC activity (Fig. 1 and Refs. 13, 14, 45). Exposure of the basal and insulin-stimulated muscles to either the hyperosmolar glucose or mannitol did not provoke significant changes in total cell water (Fig. 2). In basal and insulin-stimulated muscle, hyperosmolar glucose or mannitol significantly decreased cell water when NKCC activity was inhibited with bumetanide (Fig. 2). These data demonstrate that muscle cells have a compensatory mechanism to maintain cell volume in conditions of hyperosmolarity that involve NKCC stimulation.

High-glucose concentration and mannitol differentially regulate basal and insulin-stimulated phosphorylation of ERK1,2 MAPK, p38 MAPK, and Akt. In isosmotic conditions, NKCC activity in skeletal muscle is regulated by ERK1,2 MAPK, p38 MAPK, and Akt signaling pathways (12~14). A schematic of these pathways is shown in Fig. 3. Hence, we analyzed the significance of these cascades for NKCC activation by hyperosmolar glucose and mannitol. Incubation of both the soleus and plantaris muscles in medium containing
an additional 20 mM glucose resulted in a significant 1.5- to 2.0-fold activation of ERK1,2 phosphorylation (P < 0.05) (Fig. 4). The effect of insulin, which stimulates ERK1,2 phosphorylation in isosmotic medium (13, 43), was not additive with the increase induced by high-glucose concentration (Fig. 4). Importantly, mannitol (used as an osmotic control) was unable to increase ERK1,2 activation in either the soleus or plantaris muscle. Furthermore, mannitol did not alter insulin-stimulated ERK1,2 phosphorylation (Fig. 4). Short-term exposure of the muscles to a hyperosmotic medium or insulin did not alter expression of the ERK-2 protein (Fig. 4).

Activation of p38 MAPK likely participates in the changes of intracellular metabolism induced by osmotic shock or insulin (23). Indeed, incubation of the slow-twitch soleus muscle with hyperosmolar glucose, mannitol, or insulin alone increased p38 MAPK phosphorylation (Fig. 5). In contrast, none of these stimuli activated phosphorylation of p38 MAPK in the fast-twitch plantaris muscle (Fig. 5). Of particular interest, preincubation with hyperosmolar glucose, but not mannitol, impaired insulin-stimulated phosphorylation of p38 MAPK in the soleus muscle (Fig. 5). Expression of p38 MAPK was not changed in the muscles treated with insulin or hyperosmolarity (Fig. 5).

Consistent with our previous report (13), insulin caused phosphorylation of Akt on Ser473 by fourfold in both the soleus and plantaris muscles (P < 0.05) (Fig. 6). Elevation of the osmolarity with mannitol did not alter phosphorylation of Akt in either muscle, whereas a high-glucose concentration in the medium led to a twofold decrease of Akt phosphorylation in the plantaris muscle (P < 0.05) (Fig. 6). Insulin still stimulated Akt phosphorylation in the hyperosmolar medium containing mannitol (Fig. 6). However, incubation of the plantaris muscle in the high-glucose medium prevented the stimulating effects of insulin on Akt phosphorylation (Fig. 6). Akt protein expression was not altered by short-term incubation with insulin or hyperosmolarity (Fig. 6).

Together, these findings indicate that ERK1,2 MAPK activation might be involved in NKCC activation by high-glucose concentration or mannitol in the presence or absence of insulin. Because ERK1,2 MAPK and p38 MAPK can regulate NKCC activity in the skeletal muscle (12–14, 45), we took advantage of specific inhibitors of these signaling pathways to test their role in the hyperosmotic activation of NKCC.

**ERK1,2 MAPK and p38 MAPK pathway inhibitors do not alter NKCC activation by high-glucose concentration or mannitol.** Pharmacological inhibitors of MEK1,2 (PD-098059) and p38 (SB-203580) MAPKs have been demonstrated to be effective blockers of hyperosmolality-induced ERK and p38 MAPK activation, respectively, in various cell types (3, 4, 11). We examined the effects of MEK1,2 inhibition on NKCC activity stimulated by hyperosmolality. PD-098059 did not alter activation of NKCC by either high-glucose concentration or mannitol, regardless of the presence or absence of insulin stimulation (Table 1). Consistent with these data, U-0126, a structurally different inhibitor of the ERK1,2 MAPK pathway, had no effect on hyperosmolality-induced activation of bumetanide-sensitive 86Rb uptake in the skeletal muscle (data not shown).

**Fig. 2.** Effect of hyperosmolar glucose and mannitol on intracellular water in skeletal muscle. Isolated soleus (A) and plantaris (B) muscle pairs were preincubated and incubated as in Fig. 1 (total incubation time was 25 min) with [14C]urea and [3H]mannitol added to all solutions to measure total and extracellular water, respectively. 86Rb was omitted from the solutions. Intracellular water was calculated as the difference between the total and extracellular volumes and expressed as the percentage of the isotonic control (basal) value. Results are means ± SE of 8–10 experiments. *P < 0.05 compared with vehicle-treated muscle. INS, insulin.

**Fig. 3.** Schematic of the known pathways that can regulate skeletal muscle Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) activity under isosmotic conditions. The schematic is a composite of previous reports investigating the pathways in slow-twitch and fast-twitch skeletal muscle (12–14). The specific pathway components tested in this study are emphasized in bold. InsR, insulin receptor; AR, adrenergic receptors.
shown, $n = 5$). Inhibition of p38 MAPK by SB-203580 also did not affect NKCC activity in the skeletal muscle exposed to hyperosmolar glucose or mannitol (Table 1). Similarly, another inhibitor of p38 MAPK activation, SB-202190, did not affect NKCC activation by hyperosmotic media (data not shown, $n = 5$).

To verify that pharmacological inhibitors were indeed active in our experimental conditions, we assessed effect of these inhibitors on ERK MAPK and p38 MAPK phosphorylation. As shown in Fig. 7, PD-098059 abrogated the hyperosmolar glucose-induced phosphorylation of ERK MAPK in both muscles. Incubation of muscles with SB-203580 resulted in a significant decrement in hyperosmolar glucose- and mannitol-induced p38 MAPK phosphorylation in slow-twitch soleus muscle (Fig. 8). In isosmotic conditions, these

![Fig. 4. Effect of hyperosmolar glucose and mannitol on the phosphorylation of ERK1,2 MAPK.](http://ajpregu.physiology.org/)

![Fig. 5. Effect of hyperosmolar glucose and mannitol on the phosphorylation of p38 MAPK.](http://ajpregu.physiology.org/)
Fig. 6. Effects of hyperosmolar glucose and mannitol on the phosphorylation of Akt on Ser473. Isolated soleus (A) and plantaris (B) muscles were preincubated and incubated as in Fig. 1 except that [86Rb] was omitted from the incubation solution. Equal amounts of protein (100 µg) from whole cell lysates were immunoblotted with an antibody to phospho-Akt Ser473. The immunoblots were then stripped and reprobed with anti-Akt antibody. Representative blots are shown. The intensity of each band was quantified by densitometry, and the phospho/total ratio was calculated. Values are expressed relative to the level of phosphorylation in the basal state (taken as 1.0). Data were obtained from 6 to 12 different muscles (means ± SE). *, †Significant (P < 0.05) differences from basal and insulin-stimulated state, respectively.

Table 1. Effects of ERK 1,2 MAPK and p38 MAPK pathway inhibitors on hyperosmolarity-induced activation of bumetanide-sensitive [86Rb] uptake

<table>
<thead>
<tr>
<th>Bumetanide-Sensitive, NKCC-Mediated [86Rb] Uptake, (g/ml)⁻¹·min⁻¹×10⁻³</th>
<th>Glucose</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soleus</td>
<td>Plantaris</td>
</tr>
<tr>
<td>Basal</td>
<td>9.5 ± 2.4</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>Basal + PD-98059</td>
<td>8.9 ± 3.0</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>Insulin + PD-98059</td>
<td>8.8 ± 2.1</td>
<td>5.7 ± 2.2</td>
</tr>
<tr>
<td>Basal + SB-203580</td>
<td>9.8 ± 3.1</td>
<td>4.9 ± 2.2</td>
</tr>
<tr>
<td>Insulin + SB-203580</td>
<td>12.0 ± 3.0</td>
<td>5.4 ± 1.9</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6–8. Muscles were treated as described in Methods and Fig. 1, with or without 100 µU/ml insulin added during the incubation period. Kinase inhibitors, 20 µM PD-98059 or 10 µM SB-203580, were present in both preincubation and incubation media. Bumetanide-sensitive [86Rb] uptake was calculated as the difference between vehicle- (total uptake) and bumetanide-treated muscles. NKCC, Na⁺-K⁺-2Cl⁻ cotransporter.


dicates that NKCC activity is inhibited by an increased intracellular level of cAMP in vascular smooth muscle cells and PC12 cells (25, 33). On the other hand, we previously reported that cAMP-elevating agents could stimulate NKCC activity in skeletal muscle under isosmotic conditions (12, 13). Thus, we assessed the effects of ISO and FSK [which elevate cAMP concentration in the skeletal muscle (19, 35)] on the hyperosmolarity-induced activation of NKCC. Both ISO and FSK abolished hyperosmolar glucose- and mannitol-induced stimulation of bumetanide-sensitive [86Rb] uptake in soleus and plantaris muscles (Table 2).

Inhibition of NKCC activity by cAMP-elevating agents causes water loss in skeletal muscle exposed to hyperosmotic media. We hypothesized that if NKCC activity does indeed participate in cell water maintenance during hyperosmotic challenge, the ability of ISO and FSK to abolish hyperosmolarity-induced, NKCC-mediated [86Rb] uptake should manifest as a loss of ICW. As shown in Fig. 9, both ISO and FSK caused a significant loss of cell water and abrogated the bumetanide-sensitive component of cell water.

Correlation of NKCC activity with maintenance of ICW during hyperosmotic challenge. We analyzed the data of Figs. 1, 2, 9, and Table 2 to demonstrate the predictive value of the two measures of muscle NKCC activity, bumetanide-sensitive [86Rb] uptake, and bumetanide-sensitive ICW on the fate of ICW during hyperosmotic challenge. As shown in Fig. 10, for the slow-twitch soleus muscle, both measures of NKCC activity similarly indicated the ability of the muscle to maintain ICW in the face of a hyperosmotic challenge.

DISCUSSION

Overt hyperglycemia in subjects with types 1 and 2 diabetes mellitus is a hallmark of hyperglycemic crisis or diabetic ketoacidosis. This hyperglycemia is associated with an increased rate of mortality (10, 18). It has been proposed that high plasma glucose concentration per se might impair metabolism at the cellular level (a phenomenon called glucotoxicity), which would decrease sensitivity of the tissues to the insulin and, in turn, further exacerbate the pathophysiological consequences of hyperglycemia (18). However, the cellular mechanisms by which hyperglycemia could disturb cellular metabolism are poorly understood. In particular, the factors mediating hazardous effects of hyperglycemia on insulin-sensitive tissues such as skeletal mus-
cle are not well defined. Recently, cell hydration status was proposed as a critical factor for mediating insulin-stimulated events in the cell (38). Indeed, cell shrinkage, or cell water loss, which may occur as a result of hyperglycemic hyperosmolarity, may diminish insulin effects in adipocytes and liver (7, 39). The main result of our study is that acute hyperglycemia is unlikely to produce more than a transient decrease in skeletal muscle cell water. The protection against water loss is achieved, in part, by activation of NKCC to allow the cell to compensate for a volume decrease in hyperosmolar conditions. In addition, we demonstrated that hyperglycemia apparently desensitizes skeletal muscle to the insulin action independently of osmotic effects exerted by the high-glucose concentration.

Regulation of cell volume is a precise and self-regulated process that requires rapid movement of solutes and osmotically active water in the appropriate direction (24, 29, 31). In the case of hyperosmotic challenge to nonepithelial tissues, NKCC and Na\(^+\)/H\(^-\) exchanger isoform 1 (NHE-1) have been shown to be critical for restoring cell water after cell shrinkage (24, 29, 31, 37). Results of several previous studies on mammalian striated muscle (9, 40), as well as the recent demonstration of the morphological and functional presence of NKCC in rat skeletal muscle (27, 44), led us to hypothesize

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**Fig. 7.** Effects of MEK and p38 MAPK pathway inhibitors on ERK phosphorylation in hyperosmotic media. Isolated soleus (A) and plantaris (B) muscles were preincubated and incubated as in Fig. 1 except that \(^{38}Rb\) was omitted from the incubation solution. Kinase inhibitors, 20 \(\mu M\) PD-098059 or 10 \(\mu M\) SB-203580, were present in both preincubation and incubation media. Equal amounts of protein (25 \(\mu g\)) from whole cell lysates were immunoblotted with an antibody to phospho-ERK1,2. The immunoblots were stripped and reprobed with anti-ERK-2 antibody. Representative blots are shown. The intensity of each band was quantified by densitometry, and the phospho/total ratio was calculated. Values are expressed relative to the level of phosphorylation in the basal state (taken as 1.0). Data were obtained from 6 different muscles (means \(\pm\) SE). *Significant difference \((P < 0.05)\) from basal state. †Significant difference \((P < 0.05)\) from the hyperosmotic state without addition of kinase inhibitor.

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**Fig. 8.** Effects of MEK and p38 MAPK pathway inhibitors on p38 MAPK phosphorylation in hyperosmotic media. Isolated soleus (A) and plantaris (B) muscles were preincubated and incubated as in Fig. 1 except that \(^{38}Rb\) was omitted from the incubation solution. Kinase inhibitors, 20 \(\mu M\) PD-098059 or 10 \(\mu M\) SB-203580, were present in both preincubation and incubation media. Equal amounts of protein (50 \(\mu g\)) from whole cell lysates were immunoblotted with an antibody to phospho-p38 MAPK. The immunoblots were stripped and reprobed with anti-p38 MAPK antibody. Representative blots are shown. The intensity of each band was quantified by densitometry, and the phospho/total ratio was calculated. Values are expressed relative to the level of phosphorylation in the basal state (taken as 1.0). Data were obtained from 6 different muscles (means \(\pm\) SE). *Significant difference \((P < 0.05)\) from basal state. †Significant difference \((P < 0.05)\) from the hyperosmotic state without addition of kinase inhibitor.
that NKCC is a volume regulator in this tissue. Increasing the medium osmolarity (from 298 to 318 mosM) by adding 20 mM of glucose resulted in a significant activation of NKCC-mediated $^{86}$Rb uptake (Fig. 1). It is of note that this elevation of glucose level mimics the glucose concentration in the plasma of the patients with diabetes mellitus experiencing a hyperglycemic crisis (18). Interestingly, the nature of the sugar that increased osmolarity (metabolizing vs. non-metabolizing, transported vs. nontransported) did not vary the magnitude of the response (Fig. 1). Thus, we can assume that the osmotic, rather than metabolic, effect of the high-glucose concentration mediates NKCC activation in rat skeletal muscle. In isosmotic solutions, neither bumetanide-sensitive $^{86}$Rb uptake nor NKCC-mediated ICW changes were significant (Figs. 1 and 2). In agreement with our current findings demonstrating activation of NKCC-mediated $^{86}$Rb uptake by hyperosmolarity (Fig. 1), we observed that

Table 2. Effect of isoproterenol and forskolin on hyperosmolarity-induced NKCC activation in the skeletal muscle

<table>
<thead>
<tr>
<th>Bumetanide-Sensitive, NKCC-Mediated $^{86}$Rb Uptake, (g/ml)$^{-1}$min$^{-1}$×10$^{-3}$</th>
<th>298 mosM</th>
<th>318 mosM (Glucose)</th>
<th>318 mosM (Mannitol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td><strong>Plantaris</strong></td>
<td><strong>Soleus</strong></td>
<td><strong>Plantaris</strong></td>
</tr>
<tr>
<td>Basal</td>
<td>0.5 ± 0.7</td>
<td>0.1 ± 0.5</td>
<td>9.5 ± 2.4</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>9.2 ± 2.3</td>
<td>4.2 ± 0.7</td>
<td>2.8 ± 1.5*</td>
</tr>
<tr>
<td>Forskolin</td>
<td>4.5 ± 0.6</td>
<td>0.2 ± 0.5</td>
<td>3.0 ± 1.2*</td>
</tr>
</tbody>
</table>

Data are means ± SE; $n = 6–8$. Muscles were treated as described in METHODS and Fig. 1, with 30 μM isoproterenol or 20 μM forskolin added during the incubation period. *$P < 0.05$ compared with the basal value.

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Fig. 9. Effects of hyperosmolar glucose and mannitol on bumetanide-sensitive cell volume changes in the presence of isoproterenol (ISO) or forskolin (FSK). Isolated soleus (A) and plantaris (B) muscle pairs were preincubated and incubated as in Fig. 2. ISO (30 μM) or FSK (20 μM) was added to the incubation media. Intracellular water was calculated as the difference between the total and extracellular volumes and expressed as the percentage of the isotonic control (basal) value; control data are the same as those reported in Fig. 2. Data were obtained from 6 to 12 different muscles (means ± SE). *$P < 0.05$ compared with vehicle-treated muscle.

Fig. 10. Correspondence of measures of NKCC activity (bumetanide-sensitive cell water, bumetanide-sensitive $^{86}$Rb uptake) and total intracellular water during hyperosmotic challenge. Soleus muscle data from Figs. 1, 2, 9, and Table 2 were analyzed to determine the relationship between measures of NKCC activity and total cell water under hyperosmotic conditions (glucose and mannitol). The bumetanide-sensitive portion of intracellular water was calculated by subtracting the intracellular water value of the bumetanide-treated muscle of 1 hindlimb from the value of the vehicle-treated contralateral muscle; the bumetanide-sensitive intracellular water is expressed as the percentage of vehicle-treated cell volume. The measures of NKCC activity correlate and are predictive of changes in intracellular water with $P < 0.0001.$
treatment of the skeletal muscle with a hyperosmotic medium causes a robust activation of NKCC-mediated water movement into the cell that accounts for up to 14% of total cell volume (Fig. 2). More importantly, no detectable changes in the total cell water content had occurred by the end of incubation period in hyperosmotic media (Fig. 2). In 1965, Blinks (5) noticed that incubation of isolated skeletal muscle in hyperosmotic medium results in instant cell shrinkage followed by rapid volume restoration. Our results agree with Blinks’ findings, emphasizing that NKCC may mediate such a cell volume restoration. Lindinger et al. (26) drew the same conclusion from data on potassium flux and net water flux in isolated, perfused rat hindlimbs in which NKCC activity was blocked during hyperosmotic challenge. Their data indicate that hypertonic perfusate causes a transient, NKCC-mediated potassium influx and volume restoration, whereas the same perfusate administered following blockade of NKCC activity results in a rapid water loss that is nearly complete within 5 min. It is unlikely that the \[^{14}\text{C}]\text{urea} radiotracers used in our experiments equilibrate as rapidly as the cell water, so we probably underestimated the magnitude of ICW changes and, in turn, the magnitude of the contribution by NKCC activity to maintaining ICW. The inability of NKCC to mediate either \[^{86}\text{Rb}]\text{uptake} or water transport in isosmotic conditions in skeletal muscle is also consistent with a previous observation that, in most tissues, processes that mediate regulatory volume increase are inactive under basal conditions (31).

Data reported here also demonstrate that different cellular mechanisms regulate NKCC activation in the isosmotic and hyperosmotic conditions.

We previously provided evidence that the ERK1,2 MAPK pathway is necessary for NKCC stimulation by catecholamines and contractile activity in isosmotically incubated rat skeletal muscle (45). In sharp contrast, however, the ERK MAPK pathway was not required for the NKCC activity induced by hyperosmolar glucose. First, pharmacological blockade of ERK signaling by PD-098059 or U-0126 compounds was ineffective in preventing hyperosmolality-mediated NKCC activation (Table 2), although blockade of the ERK signaling pathway prevented stimulation of ERK MAPK (Fig. 7). Second, in contrast to hyperosmolar glucose, hyperosmolar mannitol did not increase ERK1,2 phosphorylation in either of the muscle phenotypes, yet both glucose and mannitol stimulated NKCC activity (Figs. 1 and 4). Bandopadhyay et al. (3) recently reported that a 20-mM elevation of glucose concentration in the bathing solution for ex vivo preparations of rat fast-twitch and slow-twitch skeletal muscle increases the ERK activity independent of hyperosmolality. It appears that high glucose activates the ERK MAPK pathway through stimulation of Ca\(^{2+}/\)Pyk-2/Grb2/SOS/Raf/MEK. Although Kawano et al. (17) reported that ERK and p38 MAPK phosphorylation is not affected by glucose concentration, differences in experimental design between their study and the present study may explain why they did not detect a change in phosphorylation with two different glucose concentrations. In their design, each of the epitrochlearis muscle pair was exposed to hyperosmotic solution. Thus, if kinase phosphorylation is dependent on osmolality, a change would not be apparent because there was no difference in the osmolality of the bath between the muscles. In our design, we compared hyperosmolar with isosmolar treatment of the soleus and plantaris muscle pairs. Our data that only hyperosmolar glucose stimulates ERK phosphorylation are supported by recent experiments demonstrating that high glucose, but not hyperosmolality, stimulates the ERK MAPK pathway in adipocytes and myotubes (2, 3).

Additional evidence that the ERK pathway is not involved in the hyperosmotic stimulation of NKCC activity was that insulin, which we showed inhibits ERK-dependent NKCC activity (13), did not inhibit ERK1,2 activation or NKCC activity stimulated by hyperosmolality. We recently showed that insulin inhibits ERK pathway-dependent NKCC activity through the Akt and p38 MAPK cascades (Fig. 3 and Refs. 12–14). Final evidence for the hyperosmolality-activated NKCC stimulatory pathway being different from isosmotic regulatory pathway is the muscle fiber type-specific inhibition of the insulin-mediated activation of p38 MAPK and Akt pathways. In the presence of high-glucose level or p38 MAPK activity blockers, inhibition of p38 MAPK or Akt activation did not affect NKCC activity (Figs. 5, 6, 8, and Table 2). Although the mechanisms previously characterized for NKCC activity regulation under isosmotic conditions do not seem to be involved in the hyperosmolality-induced NKCC activity, the present study has revealed fiber type-specific mechanisms of insulin-mediated signaling that have broader implications for muscle physiology. Several studies show that p38 MAPK and PI 3-K/Akt pathways are necessary for insulin-stimulated glucose transport by skeletal muscle (41, 43). On the other hand, high-glucose concentration in vitro and in vivo diminishes muscle insulin-mediated glucose uptake and signaling (7, 21, 22, 30, 32, 36). Our study supports these findings and demonstrates that Akt activation is inhibited in fast-twitch muscle during short-term exposure of the muscle to high glucose. This inhibition likely occurs through a PI 3-K-independent mechanism (22). Inhibition of p38 MAPK activation by high glucose in slow-twitch muscle perhaps represents a new mechanism of regulation of insulin signaling in a hyperglycemic environment. Together, these data indicate signaling mechanisms in skeletal muscle that appear to be unique to a hyperosmotic environment.

The involvement of pathways for NKCC regulation during hyperosmotic challenge that are different from those activated in isosmotic conditions is supported by analogy with recent studies assessing the modulation of shrinkage-induced NHE-1 activity and glucose transport. In epithelial cells of cornea, it has been shown that ERK1,2 and p38 MAPK activation are indispensable for NHE-1 activation by growth factors in isosmotic conditions (46). However, activation of NHE-1 by a hyperosmotic stimulus is independent of
ERK1,2 and p38 MAPK signaling, although activation of these cascades takes place in the shrunken fibroblasts (4, 11). There is additional support for the presence of different mechanisms for controlling the same molecular transport by different means in isosmotic and hyperosmotic environment. When stimulated by insulin in isosmotic solution, intracellular transport of glucose in skeletal muscle is not sensitive to the inhibition of the ERK1,2 MAPK pathway, but it is predominantly mediated by PI 3-KAkt and p38 MAPK pathways (41, 43). In contrast, a substantial body of evidence indicates that fat or skeletal muscle cells incubated in hyperosmotic medium containing high-glucose levels have a significant activation of glucose uptake mediated by ERK1,2 pathway (3). Furthermore, in contrast to our recent reports that uncovered an NKCC inhibitory role of Akt and p38 MAPK cascades (13, 14), here we demonstrated that these signaling cascades are probably not involved in the regulation of NKCC activity in hyperosmotic conditions (Figs. 5 and 6).

A different pattern of NKCC-activating pathways by hyperosmolarity has also been demonstrated by the ability of cAMP-producing agents to inhibit hyperosmotic induced NKCC activity. Recently, we showed that ISO and FSK (which significantly elevate cAMP levels in the skeletal muscle (19, 35)) stimulate NKCC activity in skeletal muscle under isosmotic conditions (14). In the current study, both ISO and FSK abolished NKCC-mediated 86Rb uptake and cell volume restoration during hyperosmotic challenge, consistent with reports by others (25, 33). This effect of cAMP-producing agents was similar for hyperosmotic solutions containing either high-glucose concentration or mannitol (Table 1 and Fig. 9), once again underscoring the universality of mechanisms for cell water regulation regardless of causal agent. Given that insulin effects are severely impaired in the water-depleted cells (38), our findings may explain why β-blockers make insulin more effective during hyperglycemia (34). The mechanism by which cAMP elevation downregulates solute transport by NKCC may include reorganization of cytoskeleton (29, 31, 42). It has been suggested that the actin cytoskeleton may participate in the regulation of cell volume changes induced by hyperosmolarity. In several cell lines, actin depolymerization, activation of Rho pathway, or inhibition of myosin light chain kinase inhibits regulation of NKCC and NHE activation in response to hypertonic stress (29, 31). Alteration in signaling through several intracellular cascades, including MAPKs and protein kinase A (PKA), is associated with cell volume regulation (29, 38). These data indicate that these kinases may be linked to cytoskeletal organization. The results of our study indicate that MAPK activation is not involved in the hyperosmotic regulation of NKCC in the skeletal muscle. It is possible that inactivation of PKA is responsible for the observed NKCC stimulation by hyperosmolarity; stimulation of adenyl cyclase inhibited hyperosmolar stimulation of NKCC activity. Support for PKA involvement in solute transport comes from a recent study by Szaszi et al. (42) assessing regulation of NHE activity by PKA. Their data show that PKA inhibition of the Rho-Rho kinase complex leading to reorganization of the actin cytoskeleton is linked to NHE inhibition.

In conclusion, we found that high-glucose concentration was a powerful stimulus for NKCC activation in skeletal muscle. This NKCC stimulation was necessary to maintain normal cell volume. Unlike NKCC regulation in isosmotic conditions, hyperosmolarity-mediated transporter activation was not sensitive to modulation by insulin or ERK and p38 MAPK pathway inhibitors and was inhibited by cAMP-elevating agents. Therefore, we speculate that skeletal muscle possesses either two different pools of NKCC or two distinct NKCC-regulating signaling pathways that can be differentially activated depending on changes in the muscle’s environment. Furthermore, our data indicate that the osmotic action of hyperosmolar glucose on cell volume is largely compensated by the NKCC-mediated cell water restoration.

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Osmotic Regulation of Muscle NKCC


