ATP-sensitive potassium channels mediate hyperosmotic stimulation of NKCC in slow-twitch muscle

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Gosmanov, Aidar R., Zheng Fan, Xianqiang Mi, Edward G. Schneider, and Donald B. Thomason. ATP-sensitive potassium channels mediate hyperosmotic stimulation of NKCC in slow-twitch muscle. Am J Physiol Cell Physiol 286: C586–C595, 2004. First published October 30, 2003; 10.1152/ajpcell.00247.2003.—In mildly hyperosmotic medium, activation of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC) counteracts skeletal muscle cell water loss, and compounds that stimulate protein kinase A (PKA) activity inhibit the activation of the NKCC. The aim of this study was to determine the mechanism for PKA inhibition of NKCC activity in resting skeletal muscle. Incubation of rat slow-twitch soleus and fast-twitch plantaris muscles in isosmotic medium with the PKA inhibitors H-89 and KT-5720 caused activation of the NKCC only in the soleus muscle. NKCC activation caused by PKA inhibition was insensitive to MEK MAPK inhibitors and to insulin but was abolished by the PKA stimulators isoproterenol and forskolin. Furthermore, pinacidil [an ATP-sensitive potassium (K\(_{ATP}\)) channel opener] or inhibition of glycolysis increased NKCC activity in the soleus muscle but not in the plantaris muscle. Preincubation of the soleus muscle with glibenclamide (a K\(_{ATP}\) channel inhibitor) prevented the NKCC activation by hyperosmolarity, PKA inhibition, pinacidil, and glycolysis inhibitors. In contrast, glibenclamide stimulated NKCC activity in the plantaris muscle. In cells stably transfected with the Kir6.2 subunit of the K\(_{ATP}\) channel opener, inhibition of glycolysis increased NKCC activity in the soleus muscle but not in the plantaris muscle. Preincubation of the soleus muscle with glibenclamide (a K\(_{ATP}\) channel inhibitor) prevented the NKCC activation by hyperosmolarity, PKA inhibition, pinacidil, and glycolysis inhibitors. In contrast, glibenclamide stimulated NKCC activity in the plantaris muscle. In cells stably transfected with the Kir6.2 subunit of the K\(_{ATP}\) channel opener, inhibition of glycolysis activated potassium current and NKCC activity. We conclude that activation of K\(_{ATP}\) channels in slow-twitch muscle is necessary for activation of the NKCC and cell volume restoration in hyperosmotic conditions.

protein kinase A; glibenclamide; glycolysis; Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter; Kir6.2

SKELETAL MUSCLE Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC) activity participates in the transport of osmotically active electrolytes into the tissue (11, 12, 22, 23, 42). During muscle stimulation with catecholamines or contractile activity, NKCC-mediated potassium uptake accounts for ~35% of total stimulated potassium transport (11, 42) but does not affect muscle cell volume (12). Exposure of muscle to hyperosmotic medium also triggers muscle NKCC activity; this activity is necessary for increased water flux and preservation of cell volume (12, 22). Because skeletal muscle contains up to 75% of body potassium and 40% of body water, both potassium- and water-sparing functions of NKCC can play an important role in regulation of whole body water and electrolyte homeostasis. An intriguing feature of the muscle NKCC is that its activity is minuscule under basal, unstimulated conditions (13, 23). Hence, either the intracellular mechanisms for NKCC activation are not active in quiescent muscle or NKCC activity is suppressed. We have previously demonstrated that intracellular signaling through the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway is indispensable for stimulation of NKCC-mediated potassium uptake under isosmotic conditions (11, 13, 14, 42). However, we have also shown that cAMP-dependent protein kinase (PKA) activity can actively suppress the NKCC activity that is stimulated by hyperosmotic challenge (12). Muscle has potent PKA activity that is active in quiescent muscle and is tightly controlled (17). Thus it is possible that the basal level of PKA activity suppresses NKCC activity under resting conditions. PKA is a well-known stimulator of glycogenolysis (9, 18, 43) and subsequent glycolytic ATP production. In cardiac muscle, diminished production of glycolytically derived ATP activates potassium efflux through the ATP-sensitive potassium (K\(_{ATP}\)) channels (40, 41). An efflux of potassium could, in turn, stimulate NKCC to maintain cell volume similar to what occurs with hyperosmotic stimulation (22, 23).

We hypothesized that PKA control of energy status in skeletal muscle may affect K\(_{ATP}\) channel activity and, in turn, NKCC activity. Our experiments demonstrate that tonic PKA activity in slow-twitch skeletal muscle, but not fast-twitch muscle, suppresses K\(_{ATP}\) channel activity. Furthermore, activation of K\(_{ATP}\) channels in muscle or in cells transfected with the K\(_{ATP}\) channel stimulates NKCC activity, demonstrating a functional interaction between NKCC and K\(_{ATP}\) channel activity.

METHODS

Materials

\(86\)RbCl and \([^{3}H]\)mannitol were from New England Nuclear (Boston, MA). Enhanced chemiluminescence (ECL) kit and \([^{14}C]\)urea were from Amersham Life Sciences (Piscataway, NJ). Insulin, isoproterenol, glibenclamide, pinacidil, and bumetanide were purchased from Sigma (St. Louis, MO). Forskolin, PD-098059, and U0126 were obtained from CalBiochem (La Jolla, CA). Phospho-specific antibodies to ERK1/2, Akt on Ser\(^{473}\), and p38 MAPK; anti-ERK-1/2, anti-Akt, and anti-p38 MAPK antibodies; and phospho-(Ser/Thr) PKA substrate antibody were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals were from Sigma.

Animal Care and Muscle Preparation

Female Sprague-Dawley rats (90–120 g) were used for all experiments. The rats were housed in light- and temperature-controlled quarters where they received food and water ad libitum. Animals were randomly assigned to experimental groups, and all animals were handled identically. The rats were anesthetized with pentobarbital sodium (45 mg/kg ip) for tissue removal. Hindlimb soleus (predominantly slow-twitch fibers) and plantaris and smaller extensor digito-
rum longus (EDL) (predominantly fast-twitch fibers) muscles were isolated as described previously (13). The Animal Care and Use Committee of the University of Tennessee Health Science Center approved all procedures. All experiments on animals were conducted in accordance with the most recent guiding principles for research of the American Physiological Society (2).

**NKCC-Mediated 86Rb Uptake**

Isolated muscles were preincubated for 15 min at 30°C in preincubation media [oxygenated Krebs-Ringer containing bumetanide (10−5 M) or vehicle (DMSO) for the contralateral muscle; osmolality 298 mosM]. After preincubation, muscles were taken directly to incubation medium (oxygenated Krebs-Ringer containing 1 μCi/ml 86Rb and either bumetanide or vehicle; osmolality 298 mosM) at 30°C that contained 30 μM isoproterenol, 20 μM forskolin, or 100 μM insulin. Incubation was for 10 min. In some experiments, elevation of extracellular osmolality was achieved by addition of 20 mM mannitol to the preincubation and incubation media (medium osmolality 318 mosM). Where indicated, the appropriate pharmacological inhibitors of protein kinases were added to the preincubation and incubation media. For PKA activity inhibition, we applied either 2 μM H-89 or 2 μM KT-5720. To inhibit ERK MAPK activity (42), we used two structurally different inhibitors of MEK1/2 (20 μM PD-098059 and 1 μM U0126). To determine the role of the KATP channel, we added 100 μM pinacidil (to activate channel) and 10 or 25 μM glibenclamide (to block channel) to the media, as necessary (5, 15). In the experiments designed to evaluate the effect of glycolysis inhibition, iodoacetic acid (IAA) was applied at a concentration of 1 mM (15) or glucose was replaced with pyruvate (10 mM). After all incubations, muscles were immediately washed with ice-cold 0.9% saline solution. The muscles were then blotted, weighed, and homogenized in 2 ml of 0.3 M trichloroacetic acid. 86Rb uptake by the muscle was measured by Cerenkov counting. 86Rb transport was expressed as a rate constant, as described previously (42). The bumetanide-sensitive 86Rb uptake was calculated by subtracting the bumetanide treatment value for the muscle of one hindlimb from the vehicle treatment value for the muscle of the contralateral muscle.

**NKCC-Mediated Cell Volume Changes**

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, as before (12). Briefly, the muscles were preincubated and incubated as described above with no 86Rb included. The preincubation and incubation media contained 2 μCi [14C]urea and 8 μCi [3H]mannitol. Upon termination of incubations, muscles were blotted, weighed, and dissolved by addition of 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 calculation of intracellular water content (μl/mg tissue) was performed as described previously (12).

**Western Blotting**

Whole muscle was preincubated as described. Incubation medium did not include 86Rb, 14C, or 3H. After incubation, the muscles were placed in ice-cold lysis buffer as before (13), homogenized, and centrifuged at 4°C for 15 min at 5,000 g. Protein concentration of the supernatant was measured by the bicinchoninic acid (BCA) 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 difluoride membranes. The membranes were incubated overnight at 4°C in blocking buffer (1.5 mM Na2HPO4, 8 mM NaHPO4, 0.15 M NaCl, 0.3% Triton X-100, pH 7.4) supplemented with 3% BSA. The membranes were then 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 dually phosphorylated on Thr202 and Tyr204, to p38 MAPK dually phosphorylated on Thr180 and Tyr182, and to Akt phosphorylated on Ser473 were used to detect the catalytically activated forms of the kinases. A phospho-PKA substrate antibody was used to detect proteins that can be phosphorylated by PKA on serine and threonine residues. After incubation with 1% BSA blocking buffer containing horseradish peroxidase-conjugated antio 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 phosphorylated to total protein expression, normalized to the basal level value (taken as 1.0).

**Patch-Clamp Recordings and NKCC-Mediated 86Rb Uptake in HEK-293 Cells Expressing Kir6.2 Channels**

The Kir6.2 channels were expressed in the absence of sulfonylurea receptor (SUR) regulatory subunits. Kir6.2 was encouraged to traffic to the surface membrane with the addition of an enhanced green fluorescence protein (EGFP) at the COOH terminus of the Kir6.2 subunit. The channel formed by the Kir6.2-EGFP fusion subunits has gating kinetics and regulation characteristics similar to those of the unmodified Kir6.2 channel in the absence of SUR; however, its surface expression is greatly increased (19). Therefore, we used the Kir6.2-EGFP channel as a control background (designated as Kir6.2-GFP) in this study. Kir6.2-GFP was a gift from Dr. Weiss (UCLA, Los Angeles, CA). A HEK-293 cell line was maintained in continuous culture. Kir6.2-GFP in mammalian expression vectors was stably transfected into the cells with an Effectene transfection reagent (Aiae gen, Hilden, Germany). The transfected cells were cultured in a medium containing 800 μg/ml G418 for 3 wk before they were sorted with a Coulter ELITE-ESP fluorescence-activated flow cytometer. The sorted fluorescent cells were kept in culture under the presence of 400 μg/ml G418.

**Patch-clamp recordings.** The intracellular solution and bath solution contained 140 mM KCl, 5.5 mM HEPES, and 2 mM EGTA, pH 7.3. The extracellular solution contained 130 mM NaCl, 10 mM KCl, 1.8 mM CaCl2, 0.48 mM MgCl2, and 5.5 mM HEPES, pH 7.4. Currents were recorded at a membrane potential of 0 mV. IAA (1 mM) was administered in the bath solution, which was applied via a gravity-driven perfusion system to the extracellular surface of the cell in the cell-attached configuration and to the intracellular side of the membrane in the inside-out configuration. It took <20 s (an average of ~5 s) to exchange the bath solution.

**NKCC-mediated 86Rb uptake.** HEK-293 cells were preincubated in 1.0 ml of HEPES-buffered saline for 15 min at room temperature. After preincubation, 1.0 ml of the medium containing 1 μCi/ml of 86RbCl was added to wells for 10 min. Preincubation and incubation media contained NKCC inhibitor bumetanide (10−5 M) or vehicle (DMSO). In some experiments, cells were preincubated and incubated in the presence of 1 mM IAA to inhibit ATP production. 86Rb uptake was terminated by removing the experimental medium and rinsing the wells three times with ice-cold 0.1 M MgCl2. Thereafter, 1.0 ml of 0.1 N HNO3 was added to each well; 30 min later, 0.1 ml of solution was taken for protein measurement and 0.9 ml was collected and its radioactivity determined by Cerenkov counting. The protein concentration in each well was measured by BCA assay. 86Rb uptake rate constants were calculated as described before (42). The bumetanide-sensitive 86Rb uptake constant was used as an index of NKCC activity (42) and was calculated by subtracting the average of bumetanide treatment values from the average of vehicle treatment values. Each experiment,
Inhibitors of PKA Activity Stimulate NKCC-Mediated $^{86}$Rb Uptake in Slow-Twitch Skeletal Muscle

Consistent with our previous findings (12), a 20 mosM elevation of extracellular osmolarity by addition of mannitol resulted in activation of the NKCC in both the soleus and plantaris muscles (Fig. 1). In isolated skeletal muscle, hyperosmotic stimulation of NKCC activity is abolished by agents that stimulate PKA activity (12). To test whether inhibition of PKA activity affects NKCC activity under isosmotic conditions, we applied two structurally different inhibitors of PKA, H-89 and KT-5720. Preincubation of the slow-twitch soleus muscle with either compound significantly increased NKCC-mediated $^{86}$Rb uptake (Fig. 1). In contrast, NKCC activity in fast-twitch plantaris muscle did not respond to pretreatment with PKA inhibitors (Fig. 1). To determine whether PKA inhibitor had the same effect on smaller fast-twitch muscle, we also measured the effect of H-89 on NKCC activity in the EDL muscle. In the basal state, the smaller EDL muscle also did not exhibit significant NKCC activity with H-89 treatment (data not shown).

To determine whether PKA inhibitors indeed affected the basal level of PKA activity in the soleus muscle, we employed a complementary approach using an anti-phospho-PKA substrate antibody. This antibody specifically recognizes proteins containing a phospo-Ser/Thr residue with Arg at the −3 position. Protein kinases A, G, and C phosphorylate their substrates on Ser/Thr residues. We also combined forskolin [a PKA stimulator (14)] and PKA inhibitors to test for modulation of PKA activity. A Western blot of total muscle protein incubated with the anti-phospho-PKA substrate antibody detected several immunoreactive bands in untreated soleus and plantaris muscles, and these bands increased in intensity with forskolin treatment (Fig. 2A). Application of H-89 decreased the basal level of phosphorylation only in the soleus muscle (Fig. 2B). The forskolin-induced phosphorylation was inhibited by H-89 in both muscles (Fig. 2B). Together, these results (Figs. 1 and 2) demonstrated that inhibition of PKA activity in the isosmotically incubated soleus muscle stimulates NKCC activity on a magnitude similar to NKCC activation by hyperosmolarity.

Modulation of H-89-Induced NKCC Activation With MEK MAPK Inhibitors, Insulin, and PKA-Activating Agents

In rat skeletal muscle, NKCC activity is stimulated in isosmotic medium by contractile activity and adrenergic receptor stimulation (42); NKCC activity is also stimulated by exposure to hyperosmotic medium (12). Signaling through the
ERK1/2 MAPK pathway is necessary for activation of the NKCC in isosmotic conditions, but it is not required for hyperosmotic activation of the NKCC (12, 42). Therefore, we assessed whether ERK1/2 MAPK participates in H-89-induced NKCC activation in the soleus muscle under isosmotic conditions. H-89 increased phosphorylation of ERK1/2 and its upstream activator MEK1/2 by 25 and 79% (P < 0.05), respectively (Fig. 3). In the plantaris muscle, H-89 did not produce significant changes in MEK1/2 MAPK phosphorylation and decreased ERK1/2 MAPK phosphorylation by 30% (P < 0.05) (Fig. 3). Although the MEK1/2 inhibitor PD-98059 by itself did not alter MAPK phosphorylation in either the soleus or plantaris muscles, it abrogated H-89-induced MEK and ERK phosphorylation in the soleus muscle (Fig. 3). Similarly, in the presence of H-89, PD-98059 decreased MAPK phosphorylation in the plantaris muscle two- to threefold compared with basal levels of phosphorylation (Fig. 3). None of the treatments altered total MAPK expression (not shown). The ability of 20 μM PD-98059 to lower MEK1/2 and ERK1/2 MAPK phosphorylation below a basal level in the presence of H-89 was unexpected. It has been previously reported that a higher (50 μM) concentration of PD compound significantly decreases basal level of ERK1/2 MAPK phosphorylation in rat skeletal muscle (36). Hence, our data would argue that H-89 action sensitizes muscle MAPK signaling to PD-98059 action, consistent with our previous report of a synergism between PKA and the ERK MAPK pathway (14). We have previously shown that activation of protein kinase B/Akt and p38 MAPK alters the ERK MAPK activity that is necessary for NKCC activation. Pharmacological treatment with H-89 and/or PD-98059 did not change Akt phosphorylation on Ser473 and p38 MAPK phosphorylation in either the soleus or plantaris muscle (data not shown).

Because the H-89-induced NKCC activity in the soleus muscle was accompanied by a significant increase in MAPK phosphorylation and PD-98059 blocked this increased phosphorylation, we assessed the effect of MAPK inhibition on NKCC-mediated 86Rb uptake. Neither PD-98059 nor another MEK inhibitor, U0126 (not shown), were able to abolish H-89-induced NKCC activation in the soleus muscle (Fig. 4). Insulin can also inhibit MAPK-dependent NKCC activation (13). Consistent with the lack of a MEK inhibitor effect, insulin did not abolish the NKCC stimulation by H-89 in the soleus muscle.
muscle (Fig. 4). The β-adrenergic agonists isoproterenol and forskolin, which stimulate adenyl cyclase activity, each abolished the H-89-induced NKCC activation in soleus muscle (Fig. 4). These data indicate that H-89 inhibition of PKA activity does not employ the ERK1/2 MAPK pathway for NKCC activation. Rather, the same agents that inhibit hyperosmolarity-induced NKCC activation (isoproterenol and forskolin) (12) inhibit H-89-induced NKCC activity.

**Regulation of NKCC-Mediated 86Rb Uptake and Cell Water by ATP-Sensitive Potassium Channels**

PKA activation in muscle cells can induce ATP production due to stimulation of glycosogenolysis and glucose entry into glycolysis (9, 18, 33, 43). Small changes in intracellular ATP concentration can affect K+ transport across the sarcolemma by K<sub>ATP</sub> channels (34). Mammalian skeletal muscle K<sub>ATP</sub> channels can be opened by pinacidil and blocked by glibenclamide (5, 15, 25). The potassium channel opener pinacidil increased NKCC-mediated 86Rb uptake in the slow-twitch soleus muscle (Fig. 5). Inhibition of ATP production in muscle by either 1 mM IAA or replacement of glucose by pyruvate also stimulated NKCC activity (Fig. 5). The magnitude of the pinacidil-induced and metabolic inhibition-induced NKCC activation in the soleus muscle was comparable to the NKCC stimulation caused by H-89 or hyperosmolarity (Fig. 5). Using the same experimental design, we analyzed NKCC activity in the presence of the K<sub>ATP</sub> channel blocker glibenclamide (Fig. 5). Glibenclamide (25 μM) treatment abolished the NKCC activity induced by H-89, the metabolic inhibitors, or hyperosmolarity (Fig. 5); glibenclamide significantly decreased the pinacidil-stimulated NKCC activity (Fig. 5). A glibenclamide concentration of 25 μM was chosen to ensure binding to the low-affinity binding sites on the K<sub>ATP</sub> channel (16). It is worth noting that even a lower concentration of glibenclamide (10 μM) prevented hyperosmolarity-induced NKCC activation in the soleus muscle (Fig. 6). In the fast-twitch plantaris muscle, all treatments directed to produce K<sub>ATP</sub> channel activation failed to stimulate NKCC (Fig. 5). In contrast, treating the plantaris with 25 μM glibenclamide resulted in a significant activation of NKCC-mediated 86Rb uptake (Fig. 5B). Consistent with the plantaris muscle results, glibenclamide in a concentration of 25 μM, but not 10 μM, markedly increased NKCC-mediated 86Rb uptake in the smaller fast-twitch EDL muscle (Fig. 6).

Our recent studies revealed that NKCC activity will necessarily affect muscle cell volume under hyperosmotic conditions (12). In the basal, isosmotic conditions, NKCC inhibition with bumetanide did not affect cell water content in slow or fast muscle (Fig. 7). Exposure to hyperosmotic solution (Krebs-Ringer solution + 20 mM mannitol) activates NKCC-mediated water transport, and NKCC inhibition with bumetanide significantly decreases cell water by 10–15% (P < 0.05) (Fig. 7 and Ref. 12). Pretreatment of the soleus muscle with pinacidil or IAA both activated NKCC-mediated water influx to a similar extent (Fig. 7). Consistent with glibenclamide induction of NKCC-mediated 86Rb uptake in the plantaris muscle, glibenclamide stimulated bumetanide-sensitive water transport in the plantaris muscle (Fig. 7).

NKCC-Mediated 86Rb Uptake in HEK-293 Cells Stably Transfected With Kir6.2 Subunit of Potassium Channel

Naive HEK-293 cells do not possess potassium channels. To further investigate the role of potassium channel activation in NKCC regulation, we transfected HEK-293 cells with the Kir6.2 subunit of K<sub>ATP</sub> channels fused with green fluorescent protein (Kir6.2-GFP) (Fig. 8A). Addition of IAA to Kir6.2-transfected cells elevated potassium conductivity (Fig. 8B). This IAA-induced conductivity had the characteristics of Kir6.2 conductivity, because it was maximally activated under 0 mM ATP conditions and was suppressed by ATP (Fig. 8B). In the normal and transfected cells, NKCC accounts for ~25% of total 86Rb uptake (Fig. 8C). Brief incubation of normal HEK-293 cells with IAA resulted in a twofold decrease in NKCC activity, which is consistent with the ATP requirement for adequate NKCC function in in vitro-incubated cells (Fig. 8A).
A growing body of evidence demonstrates a functional relationship between the NKCC and K\(^+\) channels. Masset et al. (24) recently reported that K\(_{ATP}\) channels in endothelial cells mediate the dilation of skeletal muscle arterioles to hyperosmotic solutions. In aortic endothelial cells, activation of K\(^+\) channels by either Ca\(^{2+}\) mobilization or shear stress activates the NKCC, likely to restore cell volume after initial shrinkage (30, 37). In contrast, inhibition of Ca\(^{2+}\) influx in vascular smooth muscle by PKA decreases NKCC-mediated water transport (31, 32). K\(_{ATP}\) channel activity can also modulate NKCC-dependent electrolyte transport in the loop of Henle. The K\(_{ATP}\) channel opener minoxidil stimulates NKCC activity, resulting in an increased rate of Na\(^+\), K\(^+\), and Cl\(^-\) absorption (39). Conversely, the K\(_{ATP}\) channel blocker glibenclamide has natriuretic properties (8). Thus a general mechanism appears to exist whereby K\(^+\) channel activation leads to loss of K\(^+\), stimulating a compensatory increase in NKCC activity that transports both K\(^+\) and Cl\(^-\) (29).

The intracellular mechanisms that regulate NKCC activity in skeletal muscle are just beginning to be identified. Under isosmotic conditions, signaling through the ERK MAPK path-

Fig. 6. Hyperosmotic stimulation of NKCC activity in the soleus muscle was also blocked by 10 \(\mu\)M GLI. A: the stimulatory effect of glibenclamide on plantaris and extensor digitorum longus (EDL) muscle NKCC activity was absent at the lower GLI concentration. B: nevertheless, both 10 and 25 \(\mu\)M GLI were sufficient to inhibit the hyperosmolarity-induced NKCC activity in the soleus muscle. Isolated soleus, plantaris, and EDL muscle pairs were preincubated 15 min in Krebs-Ringer solution in the presence or absence of 20 mM mannitol (HyperOsm) with or without 10 or 25 \(\mu\)M GLI. Thereafter, the muscles were incubated an additional 10 min in solutions identical to preincubation with the addition of \(^{86}\)Rb. The solutions for 1 muscle of each pair contained 10\(^{-8}\) M bumetanide, whereas the solutions for the contralateral muscle contained vehicle. Bumetanide-sensitive \(^{86}\)Rb uptake was calculated as the difference between rate constants for vehicle and bumetanide-treated muscles. Results are means ± SE of 6 experiments. *\(P < 0.05\) compared with basal state.

Fig. 7. Increased NKCC activity prevented loss of water from slow-twitch muscle in which K\(_{ATP}\) channels were opened. Isolated soleus (A) and plantaris (B) muscle pairs were incubated for 25 min with \[^{14}\text{C}]\text{urea and }[^{3}\text{H} \text{mannitol added to all solutions to measure total and extracellular water, respectively. }[^{86}\text{Rb was omitted from the solutions. Pinacidil (100 \(\mu\)M), IAA (1 \(\mu\)M), GLI (25 \(\mu\)M), or mannitol (HyperOsm; 20 mM) were also added to the incubation medium. The solutions for 1 muscle of each pair contained 10\(^{-5}\) M bumetanide, whereas the solutions for the contralateral muscle contained vehicle. 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 6 experiments. *\(P < 0.05\) compared with vehicle-treated muscle. 8C and Ref. 35). However, incubation of Kir6.2-transfected cells with IAA led to significant activation of NKCC-mediated \(^{86}\)Rb uptake compared with both normal and transfected cells that were not treated with IAA (Fig. 8C).

DISCUSSION

This report demonstrates that, in quiescent slow-twitch skeletal muscle, a basal level of PKA activity suppresses NKCC activity, possibly through tonic inhibition of the K\(_{ATP}\) channel. In fast-twitch muscle, on the other hand, this mechanism appears to be absent. In addition, the data demonstrate that the mechanism of NKCC activation in slow-twitch muscle under hyperosmotic conditions requires K\(_{ATP}\) channel opening. This phenotype-specific interaction between NKCC and K\(_{ATP}\) channel represents a novel mechanism for potassium and water regulation in skeletal muscle cells.
way is necessary for NKCC stimulation by adrenergic receptor activation and contractile activity in both slow- and fast-twitch muscle (Fig. 9 and Refs. 14, 42). However, the data reported here indicate that H-89 stimulation of NKCC activity in slow-twitch muscle (Fig. 1) does not depend on the ERK MAPK pathway (Fig. 4); H-89 did not have an effect on NKCC activity in the predominantly fast-twitch plantaris muscle (Figs. 1 and 4). Importantly, the H-89-induced NKCC activation was accompanied by stimulation of bumetanide-sensitive water influx in slow-twitch muscle (Fig. 7). These data are consistent with our recent demonstration that the ERK MAPK signal cascade does not participate in NKCC-mediated volume restoration under hyperosmotic conditions (Fig. 9 and Ref. 12). Rather, the volume restoration function of the NKCC in slow-twitch skeletal muscle is mediated through downregulation of PKA activity (12). Hence, we hypothesized that PKA activity tonically inhibits NKCC activity in quiescent slow-twitch muscle under isosmotic conditions. This tonic inhibition of PKA activity would provide an additional, novel mechanism for stimulating NKCC activity in slow-twitch skeletal muscle.

Regulation of NKCC Activity in Predominantly Slow-Twitch Soleus Muscle

Some of the targets of PKA in muscle are involved in the control of energy homeostasis. Catecholamine-induced PKA activation stimulates glycogenolysis and glycolysis in rat skeletal muscle at the level of glycogen synthase and phosphofructosekinase (9, 18, 43). Evidence obtained from eukaryotic cells also demonstrates that PKA can directly phosphorylate and activate pyruvate kinase (33), another key enzyme regulating glycolysis. Thus inhibition of PKA activity may lead to a decrease in the rate of glycolytic ATP production. In cardiac muscle, ATP derived from glycolysis is necessary to inhibit potassium efflux through $K_{\text{ATP}}$ channels (40, 41). There is functional and molecular evidence for $K_{\text{ATP}}$ channels in

Fig. 8. Activation of potassium channels in Kir6.2-transfected HEK-293 cells stimulated NKCC activity. HEK-293 cells expressing a Kir6.2-green fluorescent protein (GFP) fusion protein (A) exhibited increased potassium conductance in response to 1 mM IAA (B). The potassium conductance increased in response to 0 mM ATP (inside-out) and decreased in response to 10 mM ATP. C: normal (control) and transfected cells were preincubated with 10$^{-5}$ M vehicle or bumetanide for 15 min with or without 1 mM IAA. The cells were then incubated for 10 min in $^{86}$Rb-containing solution with the continued presence of vehicle, bumetanide, and IAA, as necessary. NKCC-mediated $^{86}$Rb uptake was calculated as the difference between uptake values of vehicle and bumetanide-treated cells and presented as a percentage of the total (vehicle treated) $^{86}$Rb uptake. Data are means ± SE of 4 independent experiments; 3–4 wells were used for each treatment in each experiment. *P < 0.05 compared with vehicle treatment.
Activation in slow-twitch muscle, and this was also sensitive to glibenclamide abrogated the H-89-induced NKCC activation (Fig. 5). However, the mechanism of K\textsubscript{ATP} channel regulation by ATP is not necessarily a simple [ATP] dependence. The K\textsubscript{ATP} channel of the muscle sarcolemma is a pore containing four Kir6.2 subunits and four SUR2A subunits (reviewed in Ref. 4).

The latter stimulates NKCC activity and water transport into the muscle cell. The mechanism for KATP channel-dependent NKCC activity may be functionally active even when SUR is absent (38).

Interestingly, H-89, pinacidil, and metabolic inhibitors did not alter the basal level of NKCC activity in the predominantly fast-twitch plantaris muscle (Fig. 5). More intriguing is the fact that 25 \textmu M glibenclamide was able to activate NKCC-mediated \textsuperscript{86}Rb uptake in plantaris muscle (Figs. 5 and 6). In vitro studies do not support the presence of a basal level of K\textsubscript{ATP} channel activity in skeletal muscle (5), so one possibility is a nonspecific effect of the 25 \textmu M glibenclamide. It is unlikely that, at this concentration, glibenclamide alters voltage-gated or Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels or membrane potential (5, 20). However, there is a possibility that glibenclamide at concentrations higher than 20 \textmu M may increase intracellular [Ca\textsuperscript{2+}] and Ca\textsuperscript{2+} sensitivity of the muscle (10, 26). Supporting the latter is our finding demonstrating no effect of 10 \textmu M glibenclamide on NKCC activity in the fast-twitch plantaris and EDL muscles (Fig. 6). Hence, current findings argue for the possibility of NKCC activation by high concentrations of glibenclamide via Ca\textsuperscript{2+}-dependent mechanisms. It is well-known that elevation of intracellular [Ca\textsuperscript{2+}] is one of the mechanisms that lead to NKCC stimulation in different cell types (27). Activation of Ca\textsuperscript{2+}-dependent of protein kinase C, Ca\textsuperscript{2+}/calmodulin-dependent kinase II, or a yet unidentified action of [Ca\textsuperscript{2+}] on cation and anion channels may stimulate Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransport. The ability of glibenclamide to accelerate potassium influx in muscle is demonstrated in the recent report by Nielsen et al. (28) showing that glibenclamide decreases muscle loss of potassium into the interstitial space in human muscle, which is \textasciitilde50% fast-twitch fibers. Also, studies by...
Lindinger et al. (22) using a rat hindlimb perfusion model show that glibenclamide infusion is accompanied by an increase in net inward water flux and glucose uptake into the muscle; the rat hindlimb is predominantly composed of fast-twitch muscle fibers (3). Future in vitro and in vivo studies will have to address the mechanism of glibenclamide effect on muscle potassium and water fluxes because, as was shown in the current study, the enhancement of potassium influx in the basal conditions might be dose dependent and not involve action on \( K_{ATP} \) channels.

In conclusion, NKCC activity in muscle is the only known mechanism for sparing both potassium and cell volume. From our data we conclude that activation of \( K_{ATP} \) channels in response to decreased PKA activity is an important component for activation of the NKCC in rat slow-twitch fibers. Decreased PKA activity, as occurs in hyperosmolarity, could decrease glycolytic ATP production and open \( K_{ATP} \) channels. In response to the potential loss of potassium through the \( K_{ATP} \) channels, activation of the NKCC appears to provide a counteregulatory mechanism for the return of potassium and osmotic equivalents to the cell (Fig. 9). It remains to be determined whether the mechanism for activation of the NKCC involves a direct interaction of the NKCC and the \( K_{ATP} \) channel or is an indirect consequence of changes in potassium fluxes associated with opening of the \( K_{ATP} \) channel. On the other hand, in fast-twitch muscle different mechanisms are responsible for the NKCC activation by hyperosmolarity.

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