Musculoskeletal Pathology

Enhanced Na^+/H^+ Exchange Activity Contributes to the Pathogenesis of Muscular Dystrophy via Involvement of P2 Receptors

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A subset of muscular dystrophy is caused by genetic defects in dystrophin-associated glycoprotein complex. Using two animal models (BIO14.6 hamsters and *mdx* mice), we found that Na^+/H^+ exchanger (NHE) inhibitors prevented muscle degeneration. NHE activity was constitutively enhanced in BIO myotubes, as evidenced by the elevated intracellular pH and enhanced $^{22}\text{Na}^+$ influx, with activation of putative upstream kinases ERK42/44. NHE inhibitor significantly reduced the increases in baseline intracellular Ca^{2+} as well as Na^+ concentration and stretch-induced damage, suggesting that Na^+ -dependent Ca^{2+} overload via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may cause muscle damage. Furthermore, ATP was found to be released continuously from BIO myotubes in a manner further stimulated by stretching and that the P2 receptor antagonists reduce the enhanced NHE activity and dystrophic muscle damage. These observations suggest that autocrine ATP release may be primarily involved in genesis of abnormal ionic homeostasis in dystrophic muscles and that Na^+ -dependent ion exchangers play a critical pathological role in muscular dystrophy. (*Am J Pathol* 2007, 171:1576–1587; DOI: 10.2353/ajpath.2007.070452)

Muscular dystrophy is a heterogeneous genetic disease that causes severe skeletal muscle degeneration, characterized by fiber weakness and muscle fibrosis. The genetic defects associated with muscular dystrophy often include mutations in one of the components of the dystrophin-glycoprotein complex, such as dystrophin or sarcoglycans (α -, β -, γ -, and δ -SG).^{1–3} The dystrophin-glycoprotein complex is a multisubunit complex^{2,4,5} that spans the sarcolemma to form a structural link between the extracellular matrix and the actin cytoskeleton.⁶ Disruption of dystrophin-glycoprotein complex significantly impairs membrane integrity or

stability during muscle contraction/relaxation and prevents myocyte survival. This enhanced susceptibility to exercise-induced damage of muscle fibers is observed in dystrophic animals, such as δ -SG-deficient BIO14.6 hamsters and dystrophin-deficient *mdx* mice, genetic homologues of human limb-girdle and Duchenne muscular dystrophy, respectively.

Despite identification of many genes responsible for muscular dystrophy, the pathways through which genetic defects lead to muscle dysgenesis are still poorly understood. Myocyte degeneration has long been attributed to membrane defects, such as increased fragility to mechanical stress. Enhanced membrane stretching results in increased permeability to Ca^{2+} , and the resultant abnormal Ca^{2+} handling has been suggested to be a prerequisite for muscle dysgenesis. A number of studies have indicated chronic elevation in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), beneath the sarcolemma, or within other cell compartments in skeletal muscle fibers or in cultured myotubes from dystrophin-deficient (Duchenne muscular dystrophy) patients and *mdx* mice.^{7–9} Recently, we identified one of the stretch-activated channels, the growth factor responsive channel (GRC, TRPV2), which may be involved in the pathogenesis of myocyte degeneration caused by dystrophin-glycoprotein complex disruption.¹⁰ More recently, we found that Ca^{2+} -handling drugs, such as tranilast and diltiazem, exert protective effects against muscle degeneration in

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both *mdx* mice and BIO14.6 hamsters,¹¹ suggesting that Ca^{2+} -permeable channels primarily contribute to abnormal Ca^{2+} -homeostasis in dystrophic animals. In addition to the Ca^{2+} -entry pathway across the plasma membrane, it is also plausible that modifications of other ion-transport proteins contribute to genesis of the abnormal Ca^{2+} homeostasis in muscular dystrophy. We discovered that plasma membrane Na^+/H^+ exchanger (NHE) inhibitors are highly protective against muscle damage in dystrophic animals. NHE is an important transporter regulating the intracellular pH (pH_i), Na^+ concentration ($[\text{Na}^+]_i$), and cell volume, and catalyzing the electroneutral countertransport of Na^+ and H^+ through the plasma membrane or organelle membranes.^{12–14} The housekeeping isoform, NHE1, is activated rapidly in response to various extracellular stimuli, such as hormones, growth factors, and mechanical stressors.¹² Enhanced NHE activity would cause elevation of $[\text{Na}^+]_i$ and may produce intracellular Ca^{2+} overload via reduced Ca^{2+} extrusion by the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Although Ca^{2+} overload caused by Na^+ -dependent ion exchangers has been studied extensively in ischemic hearts,^{15–17} such phenomena have not been reported in dystrophic skeletal muscles. The protective effects of NHE inhibitors suggest that in addition to the Ca^{2+} -permeable channel(s), Na^+ -dependent ion exchangers may be involved in the pathogenesis of muscular dystrophy, presumably through the sustained increase in $[\text{Ca}^{2+}]_i$.

Here, we first show that the NHE inhibitors, cariporide and 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), have protective effects against muscle degeneration in dystrophic BIO14.6 hamsters and *mdx* mice. We also show that the NHE activity is constitutively enhanced in dystrophic myotubes and that cariporide significantly reduces both the elevated $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$. Furthermore, we show that P2 receptor stimulation with ATP released by stretching may be the mechanism underlying the constitutive activation of NHE. To our knowledge, this is the first report indicating the pathological importance of Na^+ -dependent ion exchangers in muscular dystrophy.

Materials and Methods

Materials

Cariporide was a gift from Aventis Pharma Chem. Ltd. (Frankfurt, Germany), and EIPA and KB-R7943(KBR) were from the New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). Rabbit polyclonal antibodies against NHE1 and NCX1 were described previously.^{18–20} Rabbit polyclonal antibody against p44/42 MAP kinase and mouse monoclonal antibody against phospho-p44/42 MAP kinase (T202/Y204) were purchased from Cell Signaling (Beverly, MA). Gadolinium chloride (GdCl_3) hexahydrate, ouabain, apyrase, 6-azaophenyl-2',4'-disulfonic acid (PPADS), suramin, and monensin were purchased from Sigma Chemical (St. Louis, MO). Thapsigargin was from Calbiochem (La Jolla, CA). $^{22}\text{NaCl}$ was purchased from NEN Life Science Products (Boston, MA). Fura-2/acetoxymethylester (AM) and fluo4-AM were

from Dojindo Laboratories (Tokyo, Japan) and Molecular Probes (Eugene, OR), respectively.

Animal Experiments

Our study followed institutional guidelines of National Cardiovascular Center for animal experimentation and was performed under the approved protocol. For examination of drug effects, EIPA and cariporide were administered orally in either the drinking water at a drug/body weight ratio of 3 mg/kg per day to 60-day-old BIO14.6 hamsters or 50-day-old *mdx* mice or age-matched normal controls as described.²¹ Suramin was administered by intraperitoneal injection at 25 mg/kg per day.²² After continuous administration for periods indicated in legends to each figure, animals were subjected to measurement of creatine phosphokinase (CK) level in serum, histochemical analysis of muscles, and grip test. For the grip test for *mdx* mice, forelimb grip strength of *mdx* mice was assessed by timing how long they could support their body weight by holding onto a fine wire net. Each group consisted of more than five mice, all of which were analyzed twice on 2 different days.

Histochemical Analysis of Muscles

Skeletal muscles were fixed in phosphate-buffered saline (PBS) containing 10% formalin and embedded in paraffin. Serial 5- μm sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome. The extent of experienced damage occurring in muscles was determined by comparing the number of centrally located nuclei between samples using a light microscopy. Variability of fiber size was obtained by averaging the standard deviations from three to four cross-sectional views of myofibers from three to four animals per group. The extent of fibrosis (blue-staining area) was measured on photographs of Masson's trichrome-stained sections.

Culture of Myotubes

Myotubes in culture were prepared as described previously.^{10,11} In brief, myoblast cells were isolated from the gastrocnemius muscles of normal or BIO14.6 hamsters by enzymatic dissociation. Minced muscles (0.3 g) were incubated for 45 minutes at 37°C in 1 ml of Ham's F-12 medium containing 2 U/ml dispase and 1% collagenase. After filtration through a fine mesh nylon filter and preplating to remove fibroblasts, cells were plated with ~80% confluence onto collagen I-coated culture dishes in growth medium consisting of Ham's F-12 medium supplemented with 20% fetal calf serum and 2.5 ng/ml basic fibroblast growth factor (Promega BRL, Madison, WI) and 1% chick embryo extract (Life Technologies, Inc., Grand Island, NY). One or 2 days after plating, medium was changed to Dulbecco's modified Eagle's medium containing 2% horse serum (Hyclone Laboratories, Logan, UT) to initiate differentiation. Myoblasts begin to fuse and form myotubes in culture within 24 hours. We used the myotubes 2 to 5 days after the switch to differentiation medium.

Measurement of $^{22}\text{Na}^+$ Uptake

Normal and BIO14.6 myotubes cultured on collagen I-coated silicon membranes or in 24-well dishes were incubated at 37°C for 30 minutes in uptake solution containing 50 mmol/L NaCl, 96 mmol/L choline chloride, 1 mmol/L MgCl_2 , 0.1 mmol/L CaCl_2 , 10 mmol/L glucose, 0.1% bovine serum albumin, 10 mmol/L HEPES/Tris, pH 7.4, 37 kBq/ml $^{22}\text{NaCl}$, and 1 mmol/L ouabain. In some wells, the uptake solution contained 0.1 mmol/L EIPA or/and 0.25 mmol/L GaCl_3 . After 30 minutes, cells were rapidly washed four times with ice-cold PBS to terminate $^{22}\text{Na}^+$ uptake. Cells were lysed in 0.1 N NaOH, and aliquots were taken for determination of protein and radioactivity.

Measurement of pH_i and $[\text{Na}^+]_i$

Myoblasts from skeletal muscles were seeded onto 25-mm glass coverslips coated with collagen I (Becton, Dickinson and Company, Franklin Lakes, NJ) and differentiated into myotubes. Myotubes were loaded with 3 $\mu\text{mol/L}$ 2',7'-bis-(bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) in balanced salt solution (BSS) (146 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 10 mmol/L glucose, 0.1% bovine serum albumin, and 10 mmol/L HEPES/Tris, pH 7.4) for 10 minutes at room temperature. The coverslip was mounted on a flow chamber and continuously perfused with solutions at 0.6 ml/minute with a Perista pump. Changes in intracellular pH (pH_i) were estimated by ratiometric scanning of changes in BCECF fluorescence. Fluorescence was monitored by alternatively exciting at 440 and 490 nm through a 505-nm dichroic reflector and 510- to 530-nm band-path emission filter. Fluorescence images were collected every 10 seconds using a cooled charge-coupled device camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan) mounted onto an inverted microscope (IX 71; Olympus, Tokyo, Japan) with a $\times 20$ objective (UApo/340; Olympus) and were then processed with AQUACOSMOS software (Hamamatsu Photonics). The pH_i value was calibrated with high K^+ solution containing 5 $\mu\text{mol/L}$ nigericin adjusted to various pH values. For measurement of $[\text{Na}^+]_i$, myotubes were incubated with 10 $\mu\text{mol/L}$ sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM) and Pluronic F-127 (0.05% w/v) in BSS for 120 minutes at room temperature. After washout, SBFI-AM was de-esterified for 20 minutes. SBFI fluorescence was monitored by alternatively exciting at 340 and 380 nm at 1 Hz through a 505-nm dichroic reflector and 510- to 530-nm band-path emission filter. In some experiments we used BSS buffered with 10 mmol/L NaHCO_3 (pH 7.4), saturated with 5% CO_2 and 95% O_2 gas. Fluorescence images were collected as described above for pH_i measurement. The fluorescence ratio at 340:380 was calculated with AQUACOSMOS software and $[\text{Na}^+]_i$ was calibrated at the end of each experiment in solutions containing 0, 10, or 20 mmol/L extracellular NaCl in the presence of 10 $\mu\text{mol/L}$ gramicidin, 1 mmol/L ouabain, and 2 $\mu\text{mol/L}$ monensin.

Measurement of $[\text{Ca}^{2+}]_i$

For Ca^{2+} imaging, cells were plated on glass and cultured and loaded with fluo-4 by incubation for 30 minutes at 37°C in 4 $\mu\text{mol/L}$ acetoxymethyl ester (Molecular Probes) in BSS as described previously.¹⁰ In brief, fluorescence signals in cells were detected by confocal laser-scanning microscopy using a MRC-1024ES system (Bio-Rad, Richmond, CA) mounted on an Olympus BX50WI microscope with a $\times 60$ water immersion lens. The frequency of image acquisition was selected as one image per <1 second. Analysis of single-frame or single-cell integrated signal density was performed with LaserSharp software (Bio-Rad, Hertfordshire, UK). The Ca^{2+} level was represented as $\Delta\text{F}/\text{F}_0$, where F_0 is the resting fluo-4 fluorescence and ΔF is the difference between peak steady-state fluorescence within 1 to 2 minutes after stimulation and resting fluorescence. In some experiments, we also loaded cells with 4 $\mu\text{mol/L}$ fura-2 acetoxymethyl ester as described above and measured $[\text{Ca}^{2+}]_i$ by a ratiometric fluorescence method using a fluorescence image processor (Aquacosmos; Hamamatsu Photonics). The excitation wavelength was alternated at 340 and 380 (1 Hz), and we measured the fluorescence light emitted at 510 nm. The fluorescence ratio at 340:380 was calculated.

Application of Cell-Stretch to Myotubes in a Silicone Chamber

Mechanical stretching was applied to myotubes using a silicon chamber as described previously.¹⁰ After cells were allowed to attach to the chamber bottom, uniaxial sinusoidal stretching was applied to the chamber at a constant strength from 5 to 20% elongation at 1 Hz for indicated periods. The relative elongation of the silicone membrane was uniform across the whole membrane area.

CK Activity and ATP Assay

After stretching of myotubes, CK activity in the medium was determined using an *in vitro* colorimetric assay kit (CK test kit; Wako Pure Chem. Co., Osaka, Japan) according to the protocol provided by the manufacturer. For ATP measurement, myotubes were washed twice with 0.5 ml of BSS 1 hour before stretching. BSS (0.5 ml) was added to the chamber, and uniaxial sinusoidal stretching was applied as above. Aliquots (100 μl) of the BSS solution were taken at selected times to measure the ATP level. The concentration of ATP released from the myotubes was measured using the luciferin-luciferase reaction (ENLITEN; Promega).

Other Procedures

Quantitative immunoblotting analysis and immunocytochemistry were performed as described previously.^{10,11,23,24} Protein concentration was measured using a bicinchoninic acid assay system (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. Unless

otherwise stated, experiments were performed at $25 \pm 1^\circ\text{C}$ and data are represented as means $\pm$ SD of at least three determinations. We used unpaired *t*-test, one-way analysis of variance followed by Dunnett's test, or two-way analysis of variance for statistical analyses. Values of $P < 0.05$ were considered statistically significant.

Results

NHE Inhibitors Prevent Skeletal Muscle Dysfunction and Cell Damage in Dystrophic Hamsters and Mice

Oral EIPA protected against muscle degeneration, as shown in sections stained with H&E (Figure 1A). We measured the number of fibers with central nuclei, which was often used as an index for regeneration to compensate for the fiber breakdown. The number of centrally located nuclei was markedly reduced by treatment with EIPA (Figure 1Ba) or cariporide (see Figure 8, B and C). Among several other abnormal morphological features, dystrophic muscle fibers are known to display greater variations in their cross-sectional area because muscles contain fibers with different sizes, such as necrotic, splitting, and regenerating fibers. EIPA markedly reduced this fiber size variability as determined by the SD of the cross-sectional areas of myofibers (Figure 1Bb). In addition, NHE inhibitor considerably reduced the area of fibrosis stained with Masson's trichrome (see Figure 8Cb). These results suggest that NHE inhibitor prevented muscle degeneration and blocked the resultant regeneration as evidenced by the reduced centrally located nuclei. Furthermore, EIPA markedly reduced CK level in the serum of BIO14.6 hamsters, which is also a marker for muscle degeneration (Figure 1C). In *mdx* mice, the extent of muscle degeneration reaches the first peak in ~ 21 to 28 days and then declines because of regeneration and reaches the second peak in ~ 72 days, when muscle degeneration was checked by serum CK level. Therefore, we started the treatment with cariporide in 50-day-old mice to see whether muscle damage during the second period is reduced. As shown in Figure 1D, treatment with cariporide for 22 days (Figure 1Db) markedly prevented muscle damage. Cariporide reduced inflammatory infiltrate (Figure 1D), fibrosis (data not shown, but see Figure 8), and the number of myofibers with central nuclei particularly in mice treated for 60 days (Figure 1E). In control *mdx* mice, serum CK levels reached a peak in 72 days of age (22 days after start of experiment) and remained at relatively high level until 145 days of age (95 days after start of experiment, Figure 1F). Treatment with cariporide considerably reduced serum CK level in all investigated ages in *mdx* mice (Figure 1F). Together, these results suggest that the degenerative and accompanying regenerative episodes become rare on treatment with NHE inhibitor. Furthermore, we also evaluated the muscle performance of mice by timing how long they could support their body weight holding onto a fine wire net. Cariporide significantly improved the results of this grip test in *mdx* mice (Figure 1G). These observations collectively sug-

Figure 1. NHE inhibitors protect against muscle degeneration in dystrophic muscle. **A:** Histological staining of skeletal muscles. Gastrocnemius muscles were taken from normal (**a** and **c**) or BIO14.6 (**b** and **d**) hamsters administered with EIPA (**c** and **d**) or no EIPA (**a** and **b**) for 14 days and sections were stained with hematoxylin (red) and eosin (blue). EIPA was administered orally to 60-day-old BIO14.6 hamsters up to 74 days. **B:** Quantitation of fibers containing central nuclei in the gastrocnemius muscles of normal and BIO14.6 hamsters administered with EIPA or no EIPA (con). Centrally located nuclei (**a**) and variability in fiber size (**b**) were measured as described in Materials and Methods. Data are means $\pm$ SD ($n = 3$ to 4). $*P < 0.05$ versus BIO14.6/control. **C:** Effect of EIPA on CK level in serum from BIO14.6 hamsters. Data are means $\pm$ SD ($n = 5$). $*P < 0.05$. In normal hamsters, the serum CK level was less than 0.3 U/ml. **D:** H&E staining of muscle sections from *mdx* mice administered with cariporide (**b**) for 22 days and age-matched (72-day-old) control *mdx* mice (**a**). **E:** Quantitation of fibers containing central nuclei in the muscle sections from *mdx* mice administered with cariporide (car) or no drug (con) for 22 or 60 days. **F:** Effect of cariporide on serum CK level in normal and *mdx* mice. Drug administration was started in 50-day-old mice (treatment, 0 day) and continued up to 145 days (treatment, 95 days). Data are means $\pm$ SD ($n = 5$). $*P < 0.05$. **G:** Effect of cariporide on muscle performance measured with grip test. Cariporide-treated (car) for 22 days or control mice (con) gripped a wire mesh with their forefeet and the time until they let go was measured. Data are means $\pm$ SD ($n = 5$). $*P < 0.05$. It is not statistically significant between normal and *mdx*/cariporide. Scale bars = 100 μm .

gest that inhibition of NHE activity confers a significant protective effect against skeletal muscle dysfunction in dystrophic animals.

Enhanced Na^+/H^+ Exchange and Extracellular Signal-Regulated Kinases Activities in Myotubes from Dystrophic Hamsters

Our *in vivo* data prompted us to study the mechanism of the involvement of NHE in skeletal muscle dysgenesis. Immunoblotting analysis revealed that skeletal muscles expressed NHE1, and its expression level was not very different between skeletal muscles from normal and BIO14.6 hamsters (Figure 2A); the normalized relative

Figure 2. NHE1 expression and intracellular pH in normal and BIO14.6 myotubes. **A:** Immunoblotting analysis of NHE1 in normal and BIO14.6 hamster skeletal muscles and their cultured myotubes. Skeletal muscle (40 μ g each) and myotube (10 μ g each) homogenates were subjected to SDS-PAGE followed by immunoblotting analysis using the indicated antibodies. **B** and **C:** Typical traces of change in BCECF fluorescence intensity indicated as the ratio of excitation wavelength 490:440 in a single myotube from normal or BIO14.6 hamsters. Cells were acidified by NH_4^+ prepulse and then pH_i recovery was started by switching to Na^+ -containing solution. To calibrate pH_i , cells were perfused with high K^+ solution containing 5 $\mu\text{mol/L}$ nigericin adjusted to various pH values. **D:** Summarized data for resting level of pH_i . Data are means $\pm$ SD ($n = 10$ to 12). * $P < 0.05$.

amount of NHE1 in BIO14.6 was 0.92 ± 0.07 ($n = 3$) versus normal muscles. NHE1 is distributed mainly in the sarcolemma of the skeletal muscles from normal and dystrophic hamsters (Supplemental Figure 1; see <http://ajp.amjpathol.org>). Moreover, we did not detect a large difference in the expression level (Figure 2A; 0.95 ± 0.08 versus normal myotubes, $n = 3$) of NHE1 between cultured myotubes from normal and BIO14.6 hamsters. We next measured the NHE activity after NH_4^+ prepulse by ratiometric fluorescence measurement with BCECF. In normal myotubes after NH_4^+ prepulse, the addition of external Na^+ induced rapid pH_i recovery, reaching only $\text{pH}_i \sim 7.0$ (Figure 2B). This pH_i recovery was attributable to the NHE activity because it was blocked completely by cariporide (Figure 3A). Because half-maximal inhibition occurred at relatively low cariporide concentration ($<1 \mu\text{mol/L}$), the NHE1 isoform was thought to be mainly involved in pH_i recovery. In contrast, in BIO14.6 myotubes pH_i recovered toward the higher pH_i range (>7.2) (Figure 2C). Myotubes from BIO14.6 hamsters exhibited significantly higher resting pH_i compared with normal animals (Figure 2D). Interestingly, although the PKC activator PMA markedly accelerated the pH_i recovery in normal myotubes, PMA accelerated only the initial pH_i recovery phase in BIO14.6 myotubes (Figure 3A). Figure 3B shows the pH_i dependence of pH_i recovery rate measured in myotubes. The pH_i dependence was shifted to the alkaline side in BIO14.6 as compared with normal myotubes. In normal myotubes, PMA greatly shifted the pH_i dependence to the alkaline side. In contrast in

Figure 3. High Na^+/H^+ exchange activity in BIO14.6 myotubes. **A:** Time courses of Na^+ -induced pH_i recovery in normal and BIO14.6 myotubes. Myotubes were subjected to NH_4^+ prepulse, and then pH_i recovery was induced by exposing myotubes to Na^+ -containing solution. In some experiments, myotubes were exposed to 1 $\mu\text{mol/L}$ PMA or 10 $\mu\text{mol/L}$ cariporide throughout the NH_4^+ prepulse and pH_i recovery phases. **B:** The pH_i dependence of the pH_i recovery rates. The pH_i recovery rate was calculated from the increment in pH_i every 10 seconds and plotted against pH_i . Data are means $\pm$ SD of five independent experiments.

BIO14.6 myotubes, PMA did not induce a large alkaline shift of pH_i dependence although it elevated the recovery rate at each pH_i . These observations may reflect the high levels of activated NHE in BIO14.6 myotubes, inducing an alkaline shift of pH_i dependence resulting in a marginal effect of PMA.

The above findings suggest that some signaling pathways are constitutively activated and lead to the enhanced NHE activity in dystrophic myotubes. We examined the activity of extracellular signal-regulated kinases (ERKs) (ERK42/44) because ERKs were previously demonstrated to mediate the NHE activation in response to hormones or growth signals.^{25–28} As shown in Figure 4, both PMA and mechanical stretch increased the level of phosphorylation of ERKs in normal myotubes (approximately three to four times). In contrast, phosphorylation of ERKs was high in BIO14.6 myotubes, even in the absence of extrinsic stimuli, and PMA and stretch did not further increase ERKs phosphorylation (Figure 4, A and B). These results suggest that ERKs are already activated in BIO14.6 myotubes.

Involvement of NHE in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ Abnormalities in Dystrophic Myotubes

We next measured EIPA-inhibitable $^{22}\text{Na}^+$ uptake into myotubes, another index for the NHE activity. In normal myotubes, EIPA inhibited $^{22}\text{Na}^+$ uptake by more than 60%, whereas gadolinium ions (Gd^{3+}), which inhibit cation channels, inhibited it by only $\sim 25\%$, indicating that NHE is one of the major Na^+ influx pathways in skeletal myotubes (Figure 5A). The EIPA-sensitive $^{22}\text{Na}^+$ uptake

Figure 4. ERK42/44 is constitutively phosphorylated in BIO14.6 myotubes. **A:** Immunoblotting analysis of total and phosphorylated ERK42/44 in normal and BIO14.6 myotubes. Serum-starved (16 hours) myotubes were treated for 30 minutes with 1 μ mol/L PMA or subjected to 20% elongation for 5 minutes (stretch). Myotubes were homogenized and then subjected to SDS-PAGE (10 μ g each) followed by immunoblotting analysis with anti-phospho-ERK42/44 or anti-ERK42/44. **B:** The apparent amount of phosphorylated ERK42/44 was normalized relative to that of total ERK. Data are means $\pm$ SD of three independent experiments. * P < 0.05 versus control, and ** P < 0.05 versus untreated control normal myotubes.

was higher ($\sim$ 1.5-fold) in BIO14.6 compared with normal myotubes (Figure 5B), consistent with the data for pH_i recovery. We measured the resting level of [Na⁺]_i in myotubes by means of ratiometric imaging technique with SBFI fluorescence in the absence or presence of bicarbonate. Consistent with the elevated ²²Na⁺ uptake

Figure 5. ²²Na⁺ uptake activity and intracellular Na⁺ concentration. **A:** ²²Na⁺ uptake by myotubes was measured for 30 minutes as described in Materials and Methods. As indicated, 0.1 mmol/L EIPA or 0.25 mmol/L GdCl₃ was included in the ²²Na⁺ uptake solution. Data are means $\pm$ SD of triplicate determinations. * P < 0.05 versus normal myotubes. **B:** EIPA-sensitive ²²Na⁺ uptake. * P < 0.05. **C:** The resting level of [Na⁺]_i was measured using the SBFI fluorescence in the absence or presence of bicarbonate. For the latter experiment, BSS contained 10 mmol/L NaHCO₃ (pH 7.4) and bubbled for 30 minutes with 5% CO₂ and 95% O₂ immediately before the experiment. Means $\pm$ SD (n = 3). * P < 0.05. **D:** Effect of cariporide on [Na⁺]_i. At the time points indicated, myotubes were exposed to 10 μ mol/L cariporide.

activity, BIO14.6 myotubes had significantly elevated resting [Na⁺]_i in the presence or absence of bicarbonate, when compared with wild-type myotubes, with the highest [Na⁺]_i in the presence of bicarbonate (Figure 5C). Treatment with cariporide caused a gradual reduction in [Na⁺]_i down to the control level (Figure 5D). These observations suggest that in addition to the resting pH_i, the enhanced Na⁺/H⁺ exchange activity causes elevation in [Na⁺]_i in BIO14.6 myotubes.

Elevated [Na⁺]_i may cause intracellular Ca²⁺ overload by altering the activity of sarcolemmal NCX. Therefore,

Figure 6. Effect of cariporide on intracellular Ca²⁺ increment and abnormal NCX function in BIO14.6 myotubes. **A:** Typical trace of changes in fluo-4 fluorescence intensity observed by confocal microscopy in normal or BIO14.6 myotubes loaded with fluo-4. The addition of 2 mmol/L CaCl₂ (final 2.5 mmol/L) to BSS containing 0.5 mmol/L CaCl₂ induced a marked increase in fluo-4 fluorescence in BIO14.6 myotubes. As indicated, exposure to 10 μ mol/L thapsigargin (TG) induced a large increase in fluo-4 fluorescence in both myotubes. **B:** Effects of cariporide on external Ca²⁺-induced change in fluo-4 fluorescence in BIO14.6 myotubes. Myotubes were pretreated with 10 μ mol/L cariporide. **C:** Extracellular Ca²⁺-induced maximal increments of fluorescence in myotubes pretreated with or without cariporide ($\Delta F/F_0$ is the ratio between the fluorescence increment and the fluorescence before Ca²⁺ addition). Data are means $\pm$ SD (trial numbers are shown in parentheses). * P < 0.05 (versus control for drug effect). **D** and **E:** Effect of external Na⁺ concentration on [Ca²⁺]_i. Extracellular Ca²⁺-induced rise in fluorescence ratio (340/380) was monitored by the Fura-2 fluorescence in BIO14.6 and normal myotubes, respectively. BIO14.6 myotubes were placed in BSS containing 0.5 mmol/L CaCl₂ and then Ca²⁺ mobilization was triggered by perfusing with a solution containing 2 mmol/L CaCl₂ and different concentrations of NaCl. When NaCl concentration was reduced, NaCl was replaced with choline chloride (total concentration, 140 mmol/L). In one experiment, 10 μ mol/L KBR was included in the solution. **F:** Extracellular Na⁺ concentration dependence of Ca²⁺-induced increase in fluorescence ratio (340/380). Myotubes were loaded with Fura-2, perfused with BSS containing 0.5 mmol/L CaCl₂, and then Ca²⁺ mobilization was triggered with BSS containing 2 mmol/L CaCl₂ and different concentrations of NaCl (see **D** and **E**). Peak amplitude of the relative fluorescence ratio (340/380) was plotted against external Na⁺ concentration. In some experiments, 10 μ mol/L KBR was included in BSS. Data are means $\pm$ SD (n = 3).

we tested the effects of cariporide on $[Ca^{2+}]_i$. Figure 6A shows typical traces of extracellular Ca^{2+} -induced Ca^{2+} mobilization measured by fluo-4 fluorescence. The addition of 2 mmol/L $CaCl_2$ triggered a marked increase in $[Ca^{2+}]_i$ in BIO14.6 myotubes but very little change in normal myotubes, as described previously.^{10,11} Pretreatment of BIO14.6 myotubes with 10 μ mol/L cariporide for 30 minutes inhibited the Ca^{2+} -induced rise in $[Ca^{2+}]_i$ almost completely (Figure 6, B and C, for summary data). These results suggest that the Na^+/H^+ exchange activity contributes greatly to the increase in $[Ca^{2+}]_i$ observed in BIO14.6 myotubes. To test the possible involvement of NCX, we examined the effect of reduced Na^+ concentration on the Ca^{2+} -induced $[Ca^{2+}]_i$ rise. Exposure of myotubes to solutions with low Na^+ concentrations (20 or 70 mmol/L) induced larger $[Ca^{2+}]_i$ increases than normal Na^+ solution (140 mmol/L NaCl). (Figure 6D). In contrast, exposure of normal myotubes to 20 mmol/L Na^+ had only a marginal effect on $[Ca^{2+}]_i$, whereas exposure to the nominally Na^+ -free solution resulted in a large $[Ca^{2+}]_i$ increase (Figure 6E). The peak $[Ca^{2+}]_i$ amplitude was plotted against external Na^+ concentration (Figure 6F). The $[Ca^{2+}]_i$ amplitude was highly Na^+ -sensitive particularly in BIO14.6 myotubes. Decrease in the Na^+ concentration leads to the reduced transmembrane Na^+ gradient, which in turn causes increased Ca^{2+} influx via the reverse reaction of NCX. In fact, $[Ca^{2+}]_i$ rise in the low Na^+ solution was mostly inhibited by the NCX inhibitor KBR (Figure 6, D and F), which was reported to inhibit the reverse mode of NCX.²⁹ These findings suggest that the NCX contributes to increased $[Ca^{2+}]_i$ in BIO14.6 myotubes, which is at least partly caused by enhanced $[Na^+]_i$.

Furthermore, we also examined the effects of NHE inhibitors on stretch-induced release of CK, which is a marker of muscle damage. The application of cyclic stretching up to 20% elongation for 1 hour induced CK release from BIO14.6 myotubes (Supplemental Figure 2, control; see <http://ajp.amjpathol.org>). Treatment of myotubes with 10 μ mol/L EIPA or 0.1 to 10 μ mol/L cariporide 30 minutes before the stretch significantly reduced the CK release (~30 to 40%) (Supplemental Figure 2; see <http://ajp.amjpathol.org>, see also Figure 7F), suggesting that NHE inhibitors are capable of reducing the stretch-induced muscle damage. Thus, increased susceptibility of BIO14.6 myotubes to mechanical stretching may result from the $[Ca^{2+}]_i$ rise, which would be at least partly caused by the $[Na^+]_i$ increase via enhanced NHE activity.

Enhanced ATP Release in Myotubes from Dystrophic Hamsters and Protective Effects of P2 Receptor Antagonists against Muscle Damage

Activation of NHE and ERK as we mentioned above would be caused directly or indirectly by increased mechanical stress in dystrophic muscles. One possible mechanism is that hormonal factors released by stretch-

Figure 7. Enhanced ATP release from BIO14.6 myotubes and effect of ATP on normal and BIO14.6 myotubes. **A:** ATP release from elongated myotubes. Normal and BIO14.6 myotubes were subjected to cyclic 10% elongation for indicated periods in BSS. An aliquot of the supernatant was taken, and ATP concentration was measured with a luciferase-based luminescence kit as described in Materials and Methods. Data are means $\pm$ SD ($n = 3$). **B:** Effects of stretch strength (0 to 20%) on ATP release. Medium ATP contents 5 minutes after stretching are indicated. **C:** Effects of ATP on Na^+ -induced pH_i recovery. Myotubes were subjected to NH_4^+ prepulse followed by Na^+ -induced pH_i recovery. As indicated, myotubes were continuously exposed to 300 μ mol/L ATP throughout the experiment during NH_4^+ prepulse and pH_i recovery phases. **D:** Summary data for maximal pH_i recovery rates. Data are means $\pm$ SD of three determinations. **E:** Effects of some pharmacological agents on the resting level of pH_i . BIO14.6 myotubes were incubated for 1 hour in BSS with and without indicated each drug and pH_i was measured. Apyrase, 0.4 U/ml; PPADS, 50 μ mol/L; suramin, 100 μ mol/L. Data are means $\pm$ SD ($n = 4$). **F:** Effects of various pharmacological agents on CK release from BIO14.6 myotubes. Myotubes were subjected to cyclic 20% elongation for 1 hour. Myotubes were preincubated with each drug for 10 minutes before stretching. Data are means $\pm$ SD ($n = 3$). * $P < 0.05$ (versus control). Cariporide, 10 μ mol/L; KBR, 10 μ mol/L; PPADS, 50 μ mol/L; suramin, 100 μ mol/L; and apyrase, 0.4 U/ml.

ing may stimulate their specific receptors, which in turn results in activation of downstream targets. We focused on ATP release because the expression pattern of purinergic receptors was recently reported to be greatly changed during muscular dysgenesis.^{30,31} We measured the level of ATP released from myotubes into the medium by the luciferin-luciferase assay system before and after mechanical stretching. Stretching induced significant ATP release in both normal and BIO14.6 myotubes (Figure 7, A and B). Interestingly, stretch-induced ATP release was significantly higher in BIO14.6 myotubes compared with those from normal controls, and the ATP level was already high in the medium of cultured BIO14.6 myotubes before the stretch (Figure 7, A and B).

Moreover, ATP was found to accelerate the pH_i recovery rate and increased the resting level of pH_i in normal myotubes but not in those from BIO14.6 (Figure 7, C and D). To test whether the NHE activation in BIO14.6 myotubes is mediated by the release of ATP, we examined effects of several pharmacological agents on the resting level of pH_i . Preincubation of BIO myotubes with ATP-hydrolyzing enzyme apyrase, P2X receptor antagonist pyridoxal-5'-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS) or general P2 receptor antagonist suramin, reduced the elevated resting pH_i in BIO myotubes, suggesting that ATP is an important extracellular driver of constitutive activation of NHE1. As expected, these pharmacological agents significantly reduced the stretch-induced CK efflux from BIO14.6 myotubes as effectively as did cariporide. Simultaneous incubation of myotubes with suramin and cariporide exerted the most effective protection (Figure 7F).

Finally, we examined whether the putative P2 receptor antagonist, suramin, improves muscular dysgenesis in dystrophic animals *in vivo*. Intraperitoneal injection of suramin significantly reduced the serum CK level to a similar extent as oral intake of cariporide (Figure 8A) and muscle damage as evidenced by Masson's trichrome staining (Figure 8B) in BIO14.6 hamsters. Furthermore, simultaneous administration of cariporide and suramin reduced the CK level and fibrosis more extensively as well as other abnormal dystrophic features such as the number of fibers with central nuclei or fiber size variability (Figure 8, A–C). A similar protective effect of suramin was also observed in *mdx* mice, as evidenced by significant reduction of serum CK level (Figure 8D) and improvement of muscle performance measured by the grip test (Figure 8E), suggesting that suramin has a protective effect against muscle dysgenesis in these animals.

Discussion

In the present study, we demonstrated that NHE inhibitors, cariporide and EIPA, have protective effects against muscle degeneration in dystrophic animals: BIO14.6 hamsters and *mdx* mice. NHE inhibitors significantly reduced the serum CK level, reduced the muscle damage, and improved the muscle performance measured by grip test. Furthermore, an *in vitro* study using cultured myotubes revealed that the NHE activity is constantly enhanced in myotubes from BIO14.6 hamsters, which would in turn contribute to the abnormal cytoplasmic Ca^{2+} handling, in part through inhibition of the Ca^{2+} extrusion by NCX or activation of the Ca^{2+} influx by reverse mode of NCX. Finally, we presented evidence that stimulation of P2 receptor with ATP released by stretching is a possible mechanism leading to activation of NHE. The results of the present study represent strong evidence that Na^+ -dependent ion exchangers exert an important pathological impact on skeletal muscle degeneration caused primarily by genetic defects in cytoskeletal proteins. In Figure 9, we summarize a possible pathway leading to muscle degeneration, particularly based on the data from BIO14.6 hamsters. Although genetic

Figure 8. Protective effect of suramin against muscle degeneration. **A:** Effect of suramin and/or cariporide on CK level in serum from BIO14.6 hamsters. Suramin (sur) and/or cariporide (car) were administered by intraperitoneal injection or by oral intake into 60-day-old BIO14.6 hamsters, respectively, and 14 days after drug administration (74-day-old hamsters), serum CK level was measured. Data are means $\pm$ SD ($n = 4$ to 5). * $P < 0.05$, whereas ** $P < 0.05$ versus either cariporide or suramin alone. **B:** Masson's trichrome staining of the quadriceps muscle sections from BIO14.6 hamsters. **C:** Quantitation of fibers containing central nuclei in muscles of BIO14.6 hamsters. Centrally located nuclei (**a**), fibrosis area (blue region) (**b**), and variability (**c**) were measured as described in Materials and Methods. Data are means $\pm$ SD ($n = 3$ to 4). * $P < 0.05$. **D:** Effect of suramin on serum CK level in *mdx* mice. Suramin or PBS (for control) was injected into 14-day-old *mdx* mice and serum CK level was measured 7 or 14 days after the start of drug injection. **E:** Effect of suramin on muscle performance measured by the grip test. Data are means $\pm$ SD ($n = 3$ to 5). * $P < 0.05$ versus *mdx*/control or normal/control, respectively. Scale bar = 100 μm .

background is different, we consider that a similar mechanism may be involved in the pathogenesis of BIO14.6 hamsters and *mdx* mice.

Until now, little attention has been paid to involvement of NHE in pathogenesis of muscular dystrophy. In this study, based on several criteria we found that NHE is constitutively activated in dystrophic BIO14.6 myotubes. The activity of NHE is known to be controlled by various extrinsic factors, including growth factors, hormones, and mechanical stimuli.^{12,13} Although molecular mechanism of NHE activation is not well established, a large body of evidence suggested that both phosphorylation-dependent and -independent pathways are involved in the full activation of NHE1.¹² Hormones or growth factors bind to their specific receptors, which in turn stimulate the cell signaling pathways including PKC, G proteins, and various kinases, resulting in the activation of NHE. Indeed, the PKC activator PMA activated the pH_i recovery rate via

Figure 9. A possible pathway leading to muscle dysgenesis in BIO14.6 dystrophic hamsters suggested in this and previous studies.¹⁰ A similar mechanism may underlie the pathogenesis of *mdx* mice.

a marked alkaline shift in the pH_i dependence in normal myotubes; however, in BIO14.6 myotubes the pH_i recovery rate was alkaline shifted even in the absence of PMA, suggesting that NHE is constitutively activated without externally added stimuli. This is compatible with the previous observation with ^{31}P -NMR that the resting muscle pH in *mdx* mice is more alkaline than that in normal muscle.³² Previous studies reported that activation of ERK1/2 is a critical step in receptor-mediated stimulation of the exchanger in several cells.^{25–28} In fact, this putative upstream kinase is phosphorylated in the resting state of BIO14.6 myotubes and is not further stimulated in response to PMA and stretch. This is compatible with recent studies to report that the phosphorylation of ERK1/2 is enhanced in dystrophic muscle fibers of *mdx* mice.^{33,34}

Activation of NHE would result in accumulation of intracellular Na^+ as well as an increase in the resting pH_i , which would be pathologically important as a possible cause leading to muscle dysfunction. $[\text{Na}^+]_i$ is controlled by a balance between Na^+ -extrusion via the Na^+ pump and Na^+ influx via multiple Na^+ -coupled transporters and cation channels. Based on $^{22}\text{Na}^+$ flux experiments, we demonstrated that a major fraction ($>60\%$) of Na^+ influx would be attributable to EIPA-sensitive Na^+/H^+ exchanger rather than Gd^{3+} -sensitive cation channels in myotubes from hamster skeletal muscles and $^{22}\text{Na}^+$ uptake via NHE is significantly higher in BIO14.6 myotubes. An increase in $[\text{Na}^+]_i$ has been reported previously in dystrophin-deficient *mdx* mice and eccentric stimulated muscle fibers.^{35–37} In these previous studies, the $[\text{Na}^+]_i$ rise was suggested to be caused by either the reduced Na^+ pump activity^{35,36} or the Gd^{3+} -inhibitable stretch-activated channel.³⁷ However, in contrast to these studies, our results suggest that NHE is the most likely candidate for the elevated $[\text{Na}^+]_i$, at least in BIO14.6 myotubes, although we cannot exclude the possibility of other Na^+ -dependent pathways.

These observations raise the question of how the NHE inhibitors protect against muscle damage in dystrophic animals. Dystrophic damage has been thought to be

attributable to the increase in $[Ca^{2+}]_i$. Elevated $[Ca^{2+}]_i$ would activate the Ca^{2+} -dependent protease calpain, which degrades various cellular proteins.³⁸ We demonstrated that BIO14.6 myotubes show abnormal cytosolic Ca^{2+} handling, as evidenced by the high resting $[Ca^{2+}]_i$ and frequently occurring Ca^{2+} oscillation.^{10,11} These events have been attributed to the activation of stretch-activated nonspecific cation channels.^{9,10,39} Importantly, preincubation with cariporide effectively reduced $[Ca^{2+}]_i$ and the level of CK released from BIO14.6 myotubes, suggesting that NHE, which is probably increased $[Na^+]_i$ via activated NHE, is involved in the genesis of abnormal Ca^{2+} homeostasis. Furthermore, treatment with drugs that increase $[Na^+]_i$, monensin, significantly enhanced the stretch-induced CK release even in the normal myotubes (Supplemental Figure 2; see <http://ajp.amjpathol.org>), suggesting the pathological importance of intracellular Na^+ . The increased $[Na^+]_i$ via NHE in muscles could lead to elevated $[Ca^{2+}]_i$ via either reduced Ca^{2+} extrusion or increased Ca^{2+} influx on NCX. Previous studies indicated that the NCX activity exists in skeletal muscle membranes⁴⁰ and contributes considerably to the regulation of $[Ca^{2+}]_i$ in muscle fibers.⁴¹ By immunoblotting analysis, we also detected the existence of NCX1, amounts of which were similar in normal and BIO14.6 muscles (Supplemental Figure 1; see <http://ajp.amjpathol.org>). Exposure to the Na^+ -reduced solution but containing millimolar concentrations of $CaCl_2$ induces a rapid increase in $[Ca^{2+}]_i$ and exerts severe damage on myotubes even in the absence of stretch, as evidenced by a massive increase in CK release (data not shown). The high Na^+ sensitivity of $[Ca^{2+}]_i$ and CK release imply the pathological importance of NCX in dystrophic damage. Given the resting membrane potential ~ -90 mV in skeletal muscle cells⁴² and the reversal potential of NCX ~ -50 mV,⁴³ NCX is supposed to operate at the forward mode (Ca^{2+} extrusion) in normal and BIO14.6 myotubes. Therefore, increase in $[Na^+]_i$ would cause the elevation in $[Ca^{2+}]_i$ via reduced Ca^{2+} extrusion by NCX in the presence of normal external Na^+ concentration. However, long-chain fatty acyl CoA esters (acyl CoAs) were recently identified as endogenous regulatory factors that activate the reverse mode of NCX1,⁴⁴ suggesting that the reverse mode of NCX1 may be enhanced when the amount of acyl CoAs are elevated under pathological conditions. Acyl CoAs were reported to increase in muscles from patients with Duchenne dystrophy.⁴⁵ Thus, we do not exclude the possibility that increase in $[Na^+]_i$ might cause the elevation in $[Ca^{2+}]_i$ via enhanced Ca^{2+} influx by NCX in BIO14.6 myotubes.

It is an intriguing question which signaling pathway leads to activation of NHE. NHE is known to be activated in response to mechanical stressors, such as stretching, hyperosmotic, or shear stress.^{12,46,47} It is possible that the NHE activity would increase as a result of autocrine/paracrine action of some hormones induced by stretching. In this study, we presented evidence that P2 receptor stimulation may be a likely mechanism leading to activation of NHE followed by muscle degeneration. We found that higher levels of ATP are released from BIO14.6 myo-

tubes in a stretch-dependent manner, which in turn would activate P2X or P2Y receptors via an autocrine mechanism. In both skeletal muscles and cultured myotubes, P2X2, P2X4, P2X7, and P2Y1 receptors were found to be expressed among P2X1, P2X2, P2X4, P2X7, P2Y1, and P2Y2 purinergic receptors tested by us by means of RT-PCR and immunoblot analysis, and these receptors are functional because ATP (and its analogues)-induced Ca^{2+} mobilization was observed and blocked by P2 antagonists in both myotubes (Y. Iwata, unpublished observations). ATP is considered one of the important nucleotides mediating its effect by activation of P2X and P2Y, which belong to the transmitter-gated cation channels and G protein-coupled receptors, respectively.⁴⁸ Recent studies demonstrated that ATP can regulate myoblast proliferation, differentiation, and regeneration *in vitro*³⁰ and that muscle cells of *mdx* mice show increased susceptibility to ATP.³¹ Our data, together with these findings, suggest that ATP is one of the important mediators in the pathogenesis of dystrophic muscles. Although our data suggest the importance of P2 receptors, we think that our results should be evaluated with great caution because chemicals such as suramin and PPADS may also inhibit other target molecules. Besides ATP, we do not exclude the possibility that other factors are involved. Three growth factors have been so far reported to be related to muscular dystrophy: insulin-like growth factor-1 (IGF-1), fibroblastic growth factor, and transforming growth factor- β 1.⁴⁹ These growth factors would also be important for dystrophic muscle pathology, because they are capable of activating NHE. For example, Perron and colleagues⁵⁰ reported that mechanical stretching induced autocrine secretion of IGF-1 in tissue cultures of differentiated avian pectoralis skeletal muscle cells. In our measurement, however, the IGF-1 concentration in serum was not as high in dystrophic animals (BIO14.6 hamsters and *mdx* mice) compared with normal animals, and in addition, scavenging of IGF-1 by anti-IGF-1 antibody did not block CK release in BIO14.6 myotubes (data not shown), suggesting that at least the contribution of IGF-1 may be rather small.

We found that ATP is released from BIO14.6 myotubes even in the absence of stimuli, and ATP concentration in the medium reached ~ 50 nmol/L after stretching. We also found that ATP concentration in the serum of dystrophic animals is significantly higher than that of normal controls (data not shown). In general, ATP concentration in the bulk medium is thought to be much lower than that localized close to the cell surface. For example, a previous study clearly showed that ATP levels in the proximity of the plasma membrane surface can be 10- to 20-fold higher than those in the bulk medium.⁵¹ Recent experiments using the recombinant luciferase technique revealed that cells can release large amounts of ATP (100 to 200 $\mu\text{mol/L}$).⁵² Therefore, it is likely that ATP exists in close proximity to the surface at concentrations sufficient for stimulation of P2 receptors, although further analyses are needed to determine the ATP concentration in the vicinity of the cell surface. ATP release has been reported to occur through several pathways, including exocytotic vesicles,⁵³ anion channels,^{54,55} hemi-gap channels,^{56,57}

and some types of transporter.^{58,59} It is possible that some of these pathways are activated by stretching in dystrophic muscles thereby resulting in enhanced release of ATP. In addition, because α -SG is ecto-ATPase,⁶⁰ the reduced level of α -SG would result in preservation of higher ATP concentration in δ -SG-deficient BIO14.6 hamsters. Clearly, the mechanism of enhanced ATP release in dystrophic animals is an important issue to be addressed in future studies, together with identification of the involving P2 receptor species. Our pharmacological experiments demonstrated that suramin is as effective as cariporide in suppressing muscle degeneration. Simultaneous administration of suramin and cariporide exerted an additive beneficial effect on muscle dysgenesis in BIO14.6 hamsters (Figure 8, A–C), it is also possible that other pathways in addition to P2 receptors may be involved in activation of NHE.

Cariporide and suramin were reported to exert the beneficial effect *in vivo* on tissue injury at least partly through the anti-inflammatory effect.^{61–63} For example, cariporide was reported to attenuate leukocyte-dependent inflammatory responses and subsequent tissue damage in myocardial ischemia/reperfusion injury.⁶¹ Thus, it is possible that these chemicals may prevent muscle damage via reduction in leukocyte-mediated inflammation. However, in addition to protective effects *in vivo*, we observed that cariporide and suramin effectively blocked the CK efflux from BIO14.6 myotubes. This observation suggests that these chemicals can exert the protective effect by directly acting on skeletal muscles, although we do not exclude the possibility for their indirect beneficial effects *in vivo*.

In summary, we demonstrated that the NHE inhibitors cariporide and EIPA attenuate the muscle degeneration and myopathy in two dystrophic animal models, BIO14.6 hamsters and *mdx* mice. Based on detailed data obtained using cultured BIO14.6 myotubes, we propose that the activation of NHE is of primary importance in the pathogenesis of muscular dystrophy. We consider that P2 receptor activation by constantly released ATP would activate NHE and result in increases in $[\text{Na}^+]_i$, thereby increasing the resting level of $[\text{Ca}^{2+}]_i$ via NCX, together with activation of Ca^{2+} influx pathway TRPV2. However, it should be noted that protection by NHE inhibitors is not complete, which is reasonable in view of the underlying complexity of dystrophy. Nevertheless, our results suggest that, in principle, NHE inhibition represents a desirable approach to reduce muscle dysgenesis and may represent an attractive therapeutic approach. The benefits of NHE inhibitors could be accentuated when used in combination with other therapies for the treatment of muscular dystrophy. Although the underlying molecular mechanism is still unknown, our present study would provide a novel framework in signaling model connecting the genetic defect and muscle degeneration, which should be addressed in further studies.

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