Carbonic anhydrases IV and IX: subcellular localization and functional role in mouse skeletal muscle

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Scheibe RJ, Mundhenk K, Becker T, Hallerdei J, Waheed A, Shah GN, Sly WS, Gros G, Wetzel P. Carbonic anhydrases IV and IX: subcellular localization and functional role in mouse skeletal muscle. Am J Physiol Cell Physiol 294: C402–C412, 2008. First published November 14, 2007; doi:10.1152/ajpcell.00228.2007.—The subcellular localization of carbonic anhydrase (CA) IV and CA IX in mouse skeletal muscle fibers has been studied immunohistochemically by confocal laser scanning microscopy. CA IV has been found to be located on the plasma membrane as well as on the sarcoplasmic reticulum (SR) membrane. CA IX is not localized in the plasma membrane but in the region of the t-tubular (TT)/terminal SR membrane. CA IV contributes 20% and CA IX 60% to the total CA activity of SR membrane vesicles isolated from mouse skeletal muscles. Our aim was to examine whether SR CA IV and TT/SR CA IX affect muscle contraction. Isolated fiber bundles of fast-twitch extensor digitorum longus and slow-twitch soleus muscle from mouse were investigated for isometric twitch and tetanic contractions and by a fatigue test. The muscle functions of CA IV knockout (KO) fibers and of CA IX KO fibers do not differ from the function of wild-type (WT) fibers. Muscle function of CA IV/XIV double knockout (dKO) mice unexpectedly shows a decrease in rise and relaxation time and in force of single twitches. In contrast, the CA inhibitor dorzolamide, whether applied to WT or to double KO muscle fibers, leads to a significant increase in rise time and force of single twitches. It is concluded that the deficiency of one SR CA isoform does not, while inhibition of all three CAs does, affect muscle contraction.

METHODS

Preparation of Sarcolemmal and SR Membrane Vesicle Fractions

WT mice, mice deficient for CA IV, and mice doubly deficient for CA IV and CA XIV were narcotized with diethyl ether. Blood samples were taken from the orbital sinus. Mice were then immediately killed by cervical dislocation. Fifty grams of skeletal muscles consisting predominantly of fast-twitch fibers obtained from 20 mice was rapidly excised and kept in 0.75 M KCl and 5 mM imidazole, pH 7.4, at 4°C. Sarcolemmal (SL) and SR membrane vesicle fractions were prepared as described previously (32, 40). The microsomal fraction was layered on top of a discontinuous sucrose density gradient and centrifuged in a Beckman SW 28 rotor at 35,000 rpm for 16 h. SR membrane vesicles were recovered by sedimentation at 40,000 rpm for 2 h in a Beckman type 70 Ti rotor. The SL and SR pellets were resuspended in 5 mM imidazole, pH 7.4, frozen in liquid nitrogen, and stored at −70°C until use.

Protein concentrations of SL and SR membrane vesicle fractions were measured according to the method of Lowry et al. (18). All animal experiments were reviewed and approved by the Bezirksregierung Hannover.
Enzyme Measurements

\( \text{Na}^{+}\text{K}^{-}\text{ATPase}. \) \( \text{Na}^{+}\text{K}^{-}\text{ATPase} \) was measured as described by Seiler and Fleischer (32). To obtain maximal \( \text{Na}^{+}\text{K}^{-}\text{ATPase} \) activity, SL and SR membrane vesicles were preincubated in a medium containing 40 mM imidazole-HEPES (pH 7.3), 2 mM Tris EDTA, and 0.3 mg/ml SDS for 20 min at room temperature. The sample was diluted 50-fold into the assay medium containing (in mM) 120 NaCl, 20 KCl, 3 MgCl2, 3 Na2ATP, 0.5 EGTA, 5 NaN3, and 30 imidazole-HCl (pH 7.5) with or without 1 mM ouabain. The reaction proceeded for 5 min at 37°C before being stopped by the addition of trichloroacetic acid at a final concentration of 5% (wt/wt) and immediate placement on ice. Inorganic phosphate was measured according to the method of Ottolenghi (28). ATPase activity was calculated by the amount of inorganic phosphate produced during the reaction time. Ouabain-sensitive \( \text{Na}^{+}\text{K}^{-}\text{ATPase} \) was calculated as the difference between total \( \text{Na}^{+}\text{K}^{-}\text{ATPase} \) activity and the activity in the presence of 1 mM ouabain.

\( \text{Ca}^{2+}-\text{Mg}^{2+}\text{ATPases}. \) \( \text{Ca}^{2+}-\text{Mg}^{2+}\text{ATPases} \) were measured as described by Seiler and Fleischer (32). \( \text{Mg}^{2+}\text{ATPase} \) was determined in the presence of (in mM) 100 KCl, 3 MgCl2, 3 Tris-ATP, 30 imidazole (pH 7.2), and 1 Tris-EGTA, with 5 μg/ml ionophore A-23187 and protein sample. Total ATPase activity was determined in the presence of 50 μM CaCl2 instead of 1 mM Tris-EGTA. The reaction proceeded for 5 min at 25°C, and inorganic phosphate was determined. \( \text{Ca}^{2+}\)-dependent ATPase activity is the difference between total and \( \text{Mg}^{2+}\)-dependent ATPase activity.

Carboxic anhydrase. \( \text{CA} \) was measured as described by Bruns et al. (3). \( \text{CO}_2 \) was applied at a flow rate of 360 ml/min. The final assay volume of 0.4 ml contained 200 μmol of phenol red solution (12.5 mg phenol red dissolved in 1 liter of 2.6 mM NaHCO3), 130 μl of sample-H2O, 20 μl of 2% Triton X-100, and 50 μl of buffer solution (64 mM 5,5-diethylbarbituric acid Na-salt, adjusted to pH 9.0 with HCl). The temperature was 0°C. The reaction was started by quickly adding the buffer solution after a 2.5-min period of preincubation. The time interval between the start of the reaction and the color change of the indicator from red to yellow was determined. One unit of \( \text{CA} \) activity is defined as the concentration of enzyme required in the final assay volume to halve the uncatalyzed reaction time. The \( \text{CA} \) activity of membrane vesicles was determined in the presence of Triton X-100 to measure extra- as well as intravesicular \( \text{CA} \) activity. In the presence of 0.1% Triton X-100, \( \text{CA} \) was inhibited by 0.1 mM DZ.

Western Blot Analysis

SL and SR membrane proteins were separated on 10% SDS-polyacrylamide gels under reducing conditions. After SDS-polyacrylamide gel electrophoresis, protein polypeptides were electrophoretically transferred on an Immobilon-P Polyvinylidene difluoride membrane (Millipore, Billerica, MA) with a polyblot assembly (Trans-Blot SD semi-dry transfer cell, Bio-Rad Laboratories, Hercules, CA). After peptide transfer, membranes were subjected to immunostaining as described previously (47). The polypeptides for \( \text{CA IV} \) and \( \text{CA XIV} \) were investigated with primary rabbit anti-human \( \text{CA IX} \) and \( \text{anti-rabbit IgG antibody conjugated with peroxidase} \) was used as secondary antibody (1 ng/ml; Calbiochem). The blots were developed with a luminol-peroxide buffer kit from Pierce Biotechnology (Schaumburg, IL). Low-molecular-mass standard proteins were from Bio-Rad Laboratories.

SL WT and SR WT bands of 39 and 43 kDa were also quantitatively analyzed in order to estimate the distribution of the \( \text{CA IV} \) and \( \text{CA IX} \) proteins, respectively, between SL and SR membranes. Therefore, amounts of SL WT and SR WT protein were chosen so that the SL WT and SR WT contained the same amount of \( \text{CA} \) enzyme units (EU), namely, 0.033 EU in the case of \( \text{CA IV} \) and 0.020 EU in the case of \( \text{CA IX} \). In the case of \( \text{CA IV} \) 6.6 μg of SL WT protein and 71.8 μg of SR WT protein were used to give 0.033 EU, and in the case of \( \text{CA IX} \) 3.8 μg of SL WT protein and 43.0 μg of SR WT protein were used to give 0.020 EU. Quantitative analysis of the \( \text{CA IV} \) and \( \text{IX} \) protein bands was performed with ImageMaster 1D software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunofluorescence Staining

\( \text{EDL} \) muscle fiber bundles of WT, \( \text{CA IV KO}, \) \( \text{CA IX KO} \), and \( \text{CA IV/IX dKO} \) mice were fixed with 3% paraformaldehyde and 100% methanol and permeabilized in 0.1% Triton X-100 for 5 min as described previously (24). Fibers were incubated with primary antibodies for 30 min. The primary rabbit anti-mouse \( \text{CA IV} \) and rabbit anti-human \( \text{CA IX} \) antibodies were diluted 1:400, and the primary goat anti-mouse monocarboxylate transporter (MCT4), goat anti-mouse sarco(endo)plasmic reticulum \( \text{Ca}^{2+}\text{-ATPase} \) (SERCA1), and goat anti-mouse ryanodine receptor (RyR) antibodies were diluted 1:200. Incubation with FITC-labeled anti-rabbit IgG secondary antibody and tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-goat IgG secondary antibody was performed for a further 30 min. The subcellular localization of \( \text{CA IV}, \) \( \text{CA IX}, \) MCT4, SERCA1, and RyR was examined by CLSM (Leica DMIRBE, Wetzlar, Germany) and analyzed with ImageJ software (Leica TCS-NT).

Measurements of Isometric Contractions

\( \text{EDL} \) and \( \text{Sol} \) muscles were dissected from killed WT, \( \text{CA IV KO}, \) \( \text{CA IX KO} \), and \( \text{CA IV/IX dKO} \) mice. Small intact muscle fiber bundles were prepared from tendon to tendon consisting of ~50 muscle cells. The muscle fiber bundles were directly stimulated with platinum wires, and isometric contraction signals were measured at 25.0 ± 0.2°C as described previously (1). The fiber bundles were superfused with Krebs-Henseleit solution containing (in mM) 120 NaCl, 3.3 KCl, 1.2 MgSO4, 1.2 KH2PO4, 1.3 CaCl2, and 25 NaHCO3. The Krebs-Henseleit solution was equilibrated with 95% O2-5% CO2 to give a pH of 7.4. Single twitches were stimulated by pulses of 1-ms duration and supramaximal voltage. Tetani of \( \text{EDL} \) fiber bundles were elicited by a 0.4-s train of 1-ms pulses at 100 Hz and tetani of \( \text{Sol} \) fiber bundles by a 1.5-s train of 1-ms pulses at 50 Hz. During the fatigue test, \( \text{EDL} \) fibers were stimulated with 0.2-s tetani at 55 Hz repeated every 12.5 s for 40 min or repeated every 2 s for 16 min, and \( \text{Sol} \) fibers were stimulated with 1.2-s tetani at 15 Hz repeated every 5 s also for 40 min. Single twitches were analyzed for time to peak (TTP), 50% relaxation time (t50) and peak force. TTP was defined as the time interval between initial baseline and peak force. t50 was defined as the time interval between peak force and 50% decayed force. Tetani were analyzed for maximum tetanic force and t50. All forces are given in normalized force (mN/mm²), correcting for the varying sizes of the fiber bundles.

Blood Parameters

Blood samples of WT mice and \( \text{CA IV/IX dKO} \) mice were analyzed with a blood gas and electrolyte system (ABL 505, Radiometer Medical, Copenhagen, Denmark). The values of pH, \( \text{Pco}_2, \) \( \text{Po}_2, \) and base excess (BE) as well as plasma \( \text{HCO}_3^- \) concentrations at \( \text{Pco}_2 \) = 40 mmHg (= standard \( \text{HCO}_3^- \)) were determined at 37°C.

Intracellular pH Measurements

The membrane potential electrodes and the \( \text{H}^+\)-sensitive electrodes were pulled from borosilicate glass tubing (GC 150T-7.5, Harvard Apparatus, Edenbridge, UK) as described previously (41). The membrane potential electrodes were filled with a solution containing 1.5 M KCl and 1.5 M potassium acetate (pH adjusted to 6.6–6.7 with HCl). Their resistance varied between 30 and 50 MΩ. The \( \text{H}^+\)-sensitive electrodes were silanized with \( \text{N,N-dimethyltrimethylsilylammine} \) (Fluka)
vapor and then backfilled with (in mM) 100 NaCl, 200 HEPES, and 100 NaOH. A short column of the H⁺ sensor Hydrogen Ionophore Cocktail A (Fluka) was sucked into the tip. The resistance of the H⁺-sensitive electrodes varied between 15 and 30 GΩ. The average slope of the H⁺-sensitive electrode, which was used to measure intracellular pH (pHi), was 58 mV/pH. The reference electrode was filled with 3 M KCl and 3% agar. The muscle fibers were superfused with Krebs-Henseleit solution containing (in mM) 120 NaCl, 3.3 KCl, 1.2 MgSO₄, 1.3 CaCl₂, and 25 NaHCO₃. The solution was gassed with 95% O₂-5% CO₂. The PCO₂ of the solution in the chamber was 32 ± 3 mmHg, and the pH was 7.37 ± 0.05 (n = 6 measurements). pHi measurements were performed at room temperature under resting conditions for the muscle fibers.

Antibodies and CA Inhibitor Dorzolamide

The polyclonal rabbit anti-mouse CA IV and CA XIV antibodies were described previously (14, 30). Polyclonal rabbit anti-mouse CA XII antibody was characterized by Kyllönen et al. (15), and rabbit anti-human CA IX antibody was described by Scheibe et al. (31). Goat anti-mouse MCT4 antibody (sc-14934), goat anti-mouse SERCA1 antibody (sc-8093), and goat anti-mouse RyR antibody (sc-8170) as well as all secondary anti-rabbit IgG and anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DZ was a kind gift from Merck & Company (Rahway, NJ).

CA IV KO, CA IV/XIV dKO, and CA IX KO Mice

CA IV KO and CA IV/XIV dKO mice were characterized by Shah et al. (34) and CA IX KO mice by Ortova Gut et al. (27).

RESULTS

Biochemical Characterization of SL and SR Membrane Vesicle Fractions

SL membrane vesicle fractions of WT, CA IV KO, and CA IV/XIV dKO skeletal muscles, which were predominantly

Table 1. Activities of marker enzymes of SL and SR membrane vesicle fractions

<table>
<thead>
<tr>
<th>Membrane Fraction</th>
<th>Ouabain-Sensitive Na⁺-K⁺-ATPase, μmol Pi/mg protein·h⁻¹</th>
<th>Mg²⁺-ATPase, μmol Pi/mg protein·min⁻¹</th>
<th>Ca²⁺-ATPase, μmol Pi/mg protein·min⁻¹</th>
<th>Protein, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL WT</td>
<td>59.0±4.0</td>
<td>1.52±0.03</td>
<td>0.08±0.04</td>
<td>4.20</td>
</tr>
<tr>
<td>SL CA IV KO</td>
<td>30.3±3.3</td>
<td>1.61±0.04</td>
<td>0.10±0.05</td>
<td>2.50</td>
</tr>
<tr>
<td>SL CA IV/XIV dKO</td>
<td>45.0±3.7</td>
<td>0.84±0.02</td>
<td>0.01±0.36</td>
<td>7.36</td>
</tr>
<tr>
<td>SR WT</td>
<td>1.6±1.7</td>
<td>0.07±0.01</td>
<td>2.57±0.03</td>
<td>32.4</td>
</tr>
<tr>
<td>SR CA IV KO</td>
<td>2.7±0.9</td>
<td>0.06±0.02</td>
<td>2.08±0.06</td>
<td>26.6</td>
</tr>
<tr>
<td>SR CA IV/XIV dKO</td>
<td>2.5±0.5</td>
<td>0.08±0.03</td>
<td>2.12±0.33</td>
<td>28.4</td>
</tr>
</tbody>
</table>

Values are means ± SD. Number of enzyme activity measurements = 4; protein determinations were in duplicate. SL, sarcolemmal; SR, sarcoplasmic reticulum; WT, wild type; CA, carbonic anhydrase; KO, knockout; dKO, double KO.
derived from fast-twitch muscles, are characterized by high activities of the ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase, a marker enzyme for plasma membranes, and by high activities of Mg\(^{2+}\)-ATPase, a marker enzyme for t-tubular membranes (Ref. 11, Table 1). The activities of Ca\(^{2+}\)-ATPase, a marker enzyme for SR membranes, vary in the SL fractions between 0.5% and 5% of the SR fractions, indicating a minor contamination of the SL fractions with SR membrane vesicles. On the other hand, SR membrane fractions show high activities of Ca\(^{2+}\)-ATPase, and the activities of Na\(^{+}\)-K\(^{+}\)- and Mg\(^{2+}\)-ATPases amount only to 3–9% of the activities determined in the SL fractions (Table 1). Therefore, the contamination of the SR fractions with SL membrane vesicles is <10%.

**CA Isoforms in SL and SR Membranes**

Figure 1 shows that the CA IV protein of 39 kDa is expressed in the SL and SR WT fractions but not in the SL-CA IV KO fraction or the SR-CA IV KO fraction. SL and SR membrane fractions derived from CA IV/XIV dKO skeletal muscles are deficient for CA IV as well as for CA XIV, which has a molecular mass of 54 kDa (Fig. 1). As seen in Fig. 2, SL and SR membrane fractions of WT as well as CA IV/XIV dKO muscles express CA IX, which has a molecular mass of 43 kDa. No doublet 43/44-kDa band of CA XII (38) could be detected either in the WT membranes (blot not shown) or in the CA IV/XIV dKO membranes (Fig. 2).

To estimate the distribution of the CA IV protein between SL and SR membranes and of the CA IX protein between SL and SR membranes, equal amounts of CA enzyme units of SL WT and SR WT membrane fractions were applied. The optical density of the 39-kDa and 43-kDa bands, respectively, from SL WT by imaging of the gels was set to 100%. The density of the 39-kDa CA IV band from SR WT accounted for 12 \(\pm\) 6% of the density of the SL WT band (\(n = 7\) Western blots; blots not shown) and the density of the 43-kDa CA IX band from SR WT for 27 ± 52% of the density of the SL WT band (\(n = 6\) Western blots). A representative blot used for the analysis of CA IX bands is shown in Fig. 2.

The double immunostaining of EDL WT fibers with anti-CA IV/FITC and anti-MCT4/TRITC is seen in Fig. 3, A–C. The immunohistochemical staining was examined by CLSM, and the light was focused on the plasma membrane. In fast-twitch skeletal muscle fibers, the lactate transporter MCT4 has been described to be located on plasma membranes as well as on t-tubular membranes (2). The distribution of anti-MCT4/TRITC fluorescence as seen in Fig. 3B confirms this subcellular localization of MCT4. CA IV is seen mainly in the form of spots of green fluorescence, which appear regularly in lines and indicate that CA IV is especially localized in the regions of...
t-tubular openings (Fig. 3A). A weaker green FITC fluorescence is seen in the regions between these spots, indicating that CA IV is present in the surface membrane. The merged image in Fig. 3C shows the colocalization of CA IV and MCT4 in the same membrane regions. Figure 3, D–F, show the double immunostaining of EDL WT fibers with anti-CA IX/FITC and anti-MCT4/TRITC. Here again, the light of the confocal microscope was focused on the level of the plasma membrane. It is seen that CA IX is not expressed in the plasma membrane (Fig. 3D). Weak spots of anti-CA IX/FITC fluorescence as seen in Fig. 3D are unspecific, as confirmed by anti-CA IX/FITC staining of EDL-CA IX KO fibers (Fig. 3, G–I).

anti-CA IV and anti-RyR. To study the subcellular localization of CA IV, SERCA, and RyR, the light of the laser was focused on a plane within the intracellular space. As seen in Fig. 4C, the merge of anti-CA IV/FITC and anti-SERCA1/TRITC fluorescence shows that CA IV is partly colocalized with SERCA (regions in yellow) but regions of green fluorescence of anti-CA IV/FITC still remain. The merge of anti-CA IV/FITC and anti-RyR/TRITC in Fig. 4F shows that fluorescence sites of anti-CA IV weakly overlap with fluorescence sites of anti-RyR. It is concluded that CA IV is localized in the region of the longitudinal SR membrane as well as to a minor extent in the region of the terminal SR. Double immunostaining of EDL-CA IV KO fibers with anti-CA IV and anti-RyR resulted in a negative control for CA IV (Fig. 4, G–I).

EDL fiber bundles of CA IV/XIV dKO mice were immunostained with anti-CA IX/FITC and anti-SERCA1/TRITC as well as anti-CA IX/FITC and anti-RyR/TRITC (Fig. 5). The merge of anti-CA IX and anti-SERCA fluorescence staining shows that CA IX and SERCA are located on different subcellular membrane regions (Fig. 5C), whereas the merge of anti-CA IX and anti-RyR immunostaining shows that CA IX is predominantly localized in the region of the RyRs, which indicates that CA IX is localized in the membrane of the terminal SR and/or in the membrane of the t-tubular system (Fig. 5F). Immunostaining of EDL fibers from CA IX KO mice did not show any specific CA IX staining with anti-CA IX/FITC and anti-RyR/TRITC (Fig. 5, G–I). EDL fiber bundles of WT mice were also immunostained. In WT fibers, the subcellular localization of CA IX does not differ from its location in CA IV/XIV dKO fibers (not shown).

CA Activities of SL and SR Membrane Vesicle Fractions

As seen in Fig. 6A, the CA activity of the SL CA IV KO fraction is significantly reduced by ~25% compared with the activity of the SL WT fraction and the activity of the SL CA IV/XIV dKO fraction by 80% compared with the CA activity of SL WT. Because of the positive staining of CA IX in the SL CA IV/XIV dKO fraction as shown in Fig. 2, it is concluded that CA IX contributes to the CA activity found in the SL.
CA IV/XIV dKO fraction. DZ, a CA inhibitor that does not distinguish among the different CA isoforms, causes complete CA inhibition in every SL fraction (Fig. 6A). The SR CA IV KO fraction displays a lower CA activity than the SR WT membrane fraction, but this difference is not significant (Fig. 6B). The CA activity of the SR CA IV/XIV dKO fraction is significantly reduced by 40% compared with the activity of the SR WT fraction. The remaining activity of SR CA IV/XIV dKO is probably caused by the activity of CA IX (see Fig. 2).

DZ (0.1 mM) leads to nearly complete CA inhibition in all SR CA fractions (Fig. 6B).

Contraction Measurements of EDL and Sol Fiber Bundles

Isometric twitches were analyzed for peak force, TTP, and \( t_{50} \). In EDL, peak force of CA IV KO fibers is not different from peak force of CA IV/XIV WT fibers (Fig. 7A). Peak force of EDL CA IV/XIV dKO fibers is significantly reduced by \( \sim 30\% \) compared with force of CA IV/XIV WT fibers. Compared with the forces of CA IX WT fibers from mice that had the same genetic background as the CA IX KO mice, forces of EDL CA IX KO fibers are unaffected. Values of peak force from Sol CA IV KO fiber bundles are comparable to those of Sol CA IV/XIV WT fiber bundles, and values of force from Sol CA IV/XIV dKO fibers are 60% greater than those of CA IV/XIV WT fibers (Fig. 7A). Peak force of Sol CA IX KO fibers does not differ from peak force of their corresponding WT fibers. Figure 7B shows that the values of TTP and \( t_{50} \) are not different between CA IV/XIV WT and CA IV KO fiber bundles. However, values of TTP and \( t_{50} \) from CA IV/XIV dKO fibers are significantly shorter than the values from CA IV/XIV WT fibers. Values of TTP and \( t_{50} \) from CA IX WT and CA IX KO fibers are comparable. This holds for EDL as well as for Sol.

Figure 8 shows maximum tetanic forces (left y-axis) and \( t_{50} \) of tetani (right y-axis). Tetanic forces of EDL CA IV/XIV WT and EDL CA IV KO fiber bundles are comparable, whereas tetanic force of EDL CA IV/XIV dKO fibers is significantly reduced by 30% compared with tetanic force of EDL CA IV/XIV WT fibers. Tetanic force of EDL CA IX KO fibers does not differ from tetanic force of CA IX WT fibers. In Sol, maximum tetanic forces of CA IV/XIV WT, CA IV KO, and CA IV/XIV dKO fibers as well as of CA IX WT and CA IX KO fibers are not significantly different. \( t_{50} \) of EDL CA IV/XIV WT and EDL CA IV KO and \( t_{50} \) of Sol WT and Sol CA IV KO are comparable. As in the case of single twitches, values of \( t_{50} \) of tetani from CA IV/XIV dKO fibers are significantly shorter than those of WT fibers in EDL as well as in Sol. \( t_{50} \) of CA IX KO fiber bundles does not vary from \( t_{50} \) of CA IX WT fiber bundles, either in EDL or in Sol.

The fatigue curves of EDL fiber bundles are shown in Fig. 9A. The decline in fractional tetanic forces of EDL CA IV/XIV WT and EDL CA IV KO fiber bundles are nearly identical during the 40-min protocol, whereas the decline in fractional tetanic forces of EDL CA IV/XIV dKO fibers is greater than the decline in CA IV/XIV WT fibers. Even during the more severe muscle exercise of a 16-min fatigue protocol, tetanic forces of EDL CA IV/XIV dKO fibers decrease faster than tetanic forces of EDL CA IV/XIV WT fibers (Fig. 9A). The decline in fractional tetanic forces of EDL CA IX KO fibers is not significantly different from the decline in fractional forces of EDL CA IV/XIV WT fibers and of EDL CA IX WT fibers. The fatigue curves of Sol CA IV/XIV WT and Sol CA IV KO fibers are comparable (Fig. 9B). However, fractional tetanic forces of Sol CA IV/XIV dKO fibers decline to a lesser extent than forces of Sol CA IV/XIV WT fibers, but the differences are not significant. Fractional tetanic forces of Sol CA IX KO fibers do not differ from fractional tetanic forces of Sol CA IV/XIV WT and of Sol CA IX WT fibers.

As seen in Fig. 10, EDL fiber bundles of CA IV/XIV WT as well as of CA IV/XIV dKO mice were exposed to DZ for 90 min. DZ (0.5 mM) had been added to the superfusing solution at time 0 min, and every 15 min one single twitch was elicited. The values of TTP, \( t_{50} \), and peak force at time 0 min were set to 100% (see Fig. 10). DZ significantly increased the values of TTP by \( \sim 25\% \) (Fig. 10A) and the values of peak force by \( \sim 50\% \) (Fig. 10C); \( t_{50} \) is unaffected by DZ (Fig. 10B). It is seen that the inhibitory effects on EDL CA IV/XIV WT and EDL CA IV/XIV dKO fibers by DZ are comparable. In Sol CA IV/XIV WT and Sol CA IV/XIV dKO fiber bundles, DZ significantly prolongs TTP by \( \sim 20\% \) and \( t_{50} \) by \( \sim 10\% \) and increases peak force.
force by ~20% (data not shown). Also in Sol, the effects of DZ are expressed to the same extent in CA IV/XIV WT and CA IV/XIV dKO fibers.

Acid-Base Status of WT and CA IV/XIV dKO Mice

Blood samples were analyzed for the acid-base status of CA IV/XIV WT and CA IV/XIV dKO mice (see Table 2). Values of pH, PCO₂, and PO₂ show that venous blood was obtained from the orbital sinus. Because of the deficiency of CA IV and CA XIV in the proximal tubules of the CA IV/XIV dKO mice (14), these mice might be expected to have renal acidosis. However, the values of BE and of standard HCO₃⁻ clearly indicate that renal acidosis is not present in the CA IV/XIV dKO mice.

| Table 2. Acid-base status of WT and CA IV/XIV dKO mice |
|---------------------------------|---------------|
|                                  | WT            | CA IV/XIV dKO |
| pH                               | 7.30±0.04     | 7.32±0.05     |
| PCO₂, mmHg                       | 55.6±7.8      | 52.1±6.6      |
| PO₂, mmHg                        | 40.5±10.0     | 40.0±9.8      |
| BE, mmol/l                       | −0.9±3.1      | −0.4±2.9      |
| Standard [HCO₃⁻], meq/l          | 23.1±2.4      | 23.2±2.5      |
| n                                | 5             | 7             |

Values are means ± SD; n, no. of blood samples tested. BE, base excess; [HCO₃⁻], HCO₃⁻ concentration at PCO₂ = 40 mmHg.
WT and CA IV/XIV dKO fibers are not significantly different. Thus, under resting conditions for muscle fibers, pHi of fibers from CA IV/XIV dKO mice does not differ from pHi of CA IV/XIV WT fibers.

**DISCUSSION**

**Subcellular Localization of CA IV and CA IX**

The present immunohistochemical studies confirm the localization of CA IV in the plasma membrane as reported by Decker et al. (5). Furthermore, they give clear evidence for an additional localization of CA IV in the SR membrane. Double immunostaining with anti-CA IX and anti-MCT4 antibodies clearly demonstrates that CA IX is not located on the plasma membrane. Immunohistochemical studies using anti-CA IX and anti-RyR antibodies indicate that much of the CA IX is colocalized with the RyRs and thus is localized in the t-tubular membrane and/or in the terminal SR membrane. Localization of CA IX in the t-tubular membrane is supported by the CA activity found in the SL CA IV/XIV dKO fraction, which contains both surface and t-tubular membranes and whose CA activity is too high to be explained solely by contamination with SR-CA IV/XIV dKO membrane vesicles. On the other hand, the SR CA IV/XIV dKO fraction is contaminated with SL by between 5% and 10%, as seen from the Na+/K+-ATPase and Mg2+-ATPase activities of the SR CA IV/XIV dKO fraction compared with those of SL CA IV/XIV dKO (Table 1). This contamination will contribute a SR CA activity of 0.1 U·ml⁻¹·mg⁻¹, but the CA activity of SR-CA IV/XIV dKO is 2.6 times higher. From this observation it is concluded that the SR of CA IV/XIV dKO muscles possesses substantial CA IX activity. This is in agreement with the estimates of CA IX from Western blots that are 2.7 times higher for SR than for SL. In summary, it is likely that CA IX is localized in t-tubular as well as SR membranes.

**SR-Associated CAs and Isometric Contractions of Skeletal Muscle Fibers**

It has been predicted that SR-associated CAs are involved in excitation-contraction coupling (3, 42). Several studies provided evidence that H+ in addition to Mg2+, K+, and Cl⁻ (21, 35) are transported into the SR during Ca2+ release (13, 29, 35). This is possible because of the extremely high H+ permeability of the SR membrane (23, 26). Ca2+ uptake into the SR is also accompanied by H+ transport (4, 20, 21, 36, 37, 45, 46). Bruns et al. (3) postulated that a SR CA catalyzes the hydration of CO2 to form the H+ ions, which are transported across the SR membrane. We previously showed that inhibition of SR CAs by various membrane-permeant CA inhibitors such as chlorzolamide, L-645,151, and DZ prolongs Ca2+ release as well as Ca2+ uptake, probably by slowing down the rate of H+ transport. The prolonged Ca2+ release and the prolonged Ca2+ uptake are accompanied by prolongation of TTP and t50 of twitches and by an increase in peak force (42, 43). In these studies isolated muscle fibers of rat EDL and Sol were used. As seen in Fig. 10, in EDL WT fibers of mouse DZ significantly increases TTP and peak force of twitches but does not affect t50. This might be explained by the higher concentration of parvalbumin in the EDL of mouse compared with the EDL of rat (9). Ca2+ binding to parvalbumin as well as Ca2+ pumping by SERCA accelerate the decay in free cytoplasmic Ca2+ concentration, and by this muscle relaxation. Because of the higher concentration of parvalbumin in mouse compared with rat, it seems possible that in mouse Ca2+ binding to parvalbumin is more important than Ca2+ pumping by SERCA in determining the kinetics of Ca2+ decay. Ca2+ pumping by SERCA requires a H+ antiport, which is dependent on the presence of a SR CA. If the latter mechanism is less important in mouse than in rat, it is plausible that CA inhibitors exert only a minor or no effect on t50 of twitches in mouse EDL.

Contrary to these earlier data, values of peak force from mouse EDL CA IV/XIV dKO fibers as well as values of TTP and t50 from both EDL and Sol CA IV/XIV dKO fibers are significantly reduced compared with those of mouse WT fibers (Figs. 7 and 8). Acidosis occurring in the muscle fibers, which would affect the function of actin-myosin filaments, might have caused the effects observed in the CA IV/XIV dKO mice. Because of the lack of CA IV and CA XIV, the CA IV/XIV dKO mice may have a reduced rate of renal HCO3− reabsorption resulting in renal acidosis and consequently in acidosis of the muscle fibers. However, CA IV/XIV dKO mice show normal values of BE and normal concentrations of standard HCO3− (Table 2). It has been shown that SL CA activity improves interstitial buffering in muscle (8, 10, 41) and that impaired interstitial buffering will impair pHi regulation (see, e.g., Ref. 39). Since the total SL CA activity of CA IV/XIV dKO muscle cells is reduced by 80% (Fig. 6A), it might be assumed that the pHi of the CA IV/XIV dKO muscle fibers is more acidic than the pHi of WT fibers. Table 3 shows that under resting conditions for the muscle fibers pHi of neither EDL CA IV/XIV dKO nor Sol CA IV/XIV dKO is altered compared with pHi of WT fibers. Thus the effects on contraction parameters of CA IV/XIV dKO fibers can be explained neither by a more acidic pHi under resting conditions of muscle cells nor by a renal acidosis. It cannot be excluded that the lack of CA IV and XIV activities inside the SR might have caused pH changes within the intra-SR environment. Because of the phosphoinositol-glycan anchor of CA IV, it is predicted that CA IV will be catalytically active inside the SR, whereas the membrane orientation of SR CA IV is not known so far. It can thus be assumed that the fast buffering of the H+ ions, which are transported into the SR during Ca2+ release, is impaired because of the lack of SR CA IV. Therefore, during the release of Ca2+, pH within the SR will become more acidic than under normal conditions. A more acidic intra-SR pH will reduce the open probability of the RyR (16, 19) and by this possibly abbreviate the interval of Ca2+ release and reduces TTP and peak force of twitches.
In CA IV single KO muscles as well as in CA IX single KO muscles, the contraction parameters of twitches and tetani are affected neither in EDL nor in SOL compared with WT fibers. Two possibilities may be discussed as to why CA IV KO and CA IX KO muscle fibers are not affected in the manner in which WT fibers are affected by CA inhibitors. 1) CA IV contributes ∼20% and CA IX ∼60% to the total SR CA activity (Fig. 6B). The remaining SR CA activity of 80% or 40%, respectively, may be still sufficient to maintain a normal SR function and consequently a normal muscle function. 2) On the other hand, it cannot be excluded that the transport rates of Mg2+, K+, and Cl–, which can also function as counterions for Ca2+ at the SR membrane, are upregulated in CA IV KO and CA IX KO mice in order to compensate for the reduced H+ transport rates.

Only the acute inhibition of all CA activity by DZ led to a prolongation of TTP and to an increase in force of twitches in WT fibers as well as in CA IV/XIV dKO fibers (Fig. 10). CA IX is the sole known membrane-associated CA isoform present in the CA IV/XIV dKO fibers. We show here that CA IX is very likely localized in the terminal SR. If we assume that CA IX is catalytically active at the cytoplasmic side of the terminal SR membrane, then on inhibition by DZ pH at the cytoplasmic side of the SR membrane will become more alkaline during the release of Ca2+. This is known to increase Ca2+ release (6, 22, 25) and by this increase TTP and force of twitches (36). Studies with muscle fibers of CA IV, IX, and XIV triple KO mice will be helpful in further exploring this concept.

GRANTS

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