Inhibition of muscle carbonic anhydrase slows the Ca$^{2+}$ transient in rat skeletal muscle fibers

PETRA WETZEL, TANJA KLEINKE, SIMON PAPADOPOULOS, AND GEROLF GROS
Zentrum Physiologie, Medizinische Hochschule Hannover, 30623 Hannover, Germany

Received 7 March 2002; accepted in final form 20 May 2002

Inhibition of muscle carbonic anhydrase (CA) slows the Ca$^{2+}$ transient in rat skeletal muscle fibers. Am J Physiol Cell Physiol 283: C1242–C1253, 2002; 10.1152/ajpcell.00106.2002.—A countertransport of H$^{+}$ is coupled to Ca$^{2+}$ transport across the sarcoplasmic reticulum (SR) membrane. We propose that SR carbonic anhydrase (CA) accelerates the CO$_2$-HCO$_3$ reaction so that H$^{+}$ ions, which are exchanged for Ca$^{2+}$ ions, are produced or buffered in the SR at sufficient rates. Inhibition of this SR-CA is expected to reduce the rate of H$^{+}$ fluxes, which then will retard the kinetics of Ca$^{2+}$ transport. Fura 2 signals and isometric force were simultaneously recorded in fiber bundles of the soleus (SOL) and extensor digitorum longus (EDL) from rats in the absence and presence of the lipophilic CA inhibitors L-645151, chlorzolamide (CLZ), and ethoxzolamide (ETZ), as well as the hydrophilic inhibitor acetazolamide (ACTZ). Fura 2 and force signals were analyzed for time to peak (TTP), 50% decay time ($t_{50}$), and their amplitudes. L-645151, CLZ, and ETZ significantly increased TTP of fura 2 by 10–25 ms in SOL and by 5–7 ms in EDL and TTP of force by 6–30 ms in both muscles. L-645151 and ETZ significantly prolonged $t_{50}$ of fura 2 and force by 20–55 and 40–160 ms, respectively, in SOL and EDL. L-645151, CLZ, and ETZ also increased peak force of single twitches and amplitudes of fura fluorescence ratio ($R_{340/380}$) at an excitation wavelength of 340 to 380 nm. All effects of CA inhibitors on fura 2 and force signals could be reversed. ACTZ did not affect TTP, $t_{50}$, and amplitudes of fura 2 signals or force. L-645151, CLZ, and ETZ had no effects on myosin-, Ca$^{2+}$-, and Na$^{-}$-K$^{+}$-ATPase activities, nor did they affect the amplitude and half-width of action potentials. We conclude that inhibition of SR-CA by impairing H$^{+}$ countertransport is responsible for deceleration of intracellular Ca$^{2+}$ transients and contraction times.

AN EXTRACELLULAR, SARCOLEMMAI (SL) carbonic anhydrase (CA), which is GPI anchored, is present in fast- and slow-twitch skeletal muscles, and CAIII occurs in the cytoplasm of slow-twitch muscles (see Ref. 43 for an overview). Several studies have provided evidence for an additional muscle CA bound to the membrane of the sarcoplasmic reticulum (SR). Bruns et al. (3) were the first to report CA activity in isolated SR vesicles from rabbit muscles. By Triton X-114 phase separation experiments, it could be shown that this CA activity originated from a membrane-bound isozyme, rather than from a cytosolic CA. Estimations of inhibition and catalysis constants revealed different properties of the CA of SR vesicle fractions and CA of SL vesicles and confirmed the existence of two membrane-bound CAs in muscle (41). Histochemical studies with the fluorescent CA inhibitor dimethylaminonaphthalene-5-sulfonamide (3, 7) and immunoelectron microscopic studies with ultrathin sections (6) demonstrated an intracellular staining pattern, which is compatible with a CA associated with the SR membrane. In a previous study, we reported that inhibition of this SR-CA leads to significant changes in single twitches of fiber bundles of the soleus (SOL) and extensor digitorum longus (EDL) from rats (45). Inhibition of this enzyme prolonged the rise and relaxation times and slightly increased force production. To investigate whether these changes were mediated by corresponding changes in the intracellular Ca$^{2+}$ transient, fiber bundles of SOL and EDL were loaded with the ester form of fura 2. We recorded simultaneously intracellular Ca$^{2+}$ transients by fura 2 fluorescence measurements and isometric single twitches in the absence and presence of the lipophilic and highly membrane-permeable CA inhibitors L-645151, chlorzolamide (CLZ), and ethoxzolamide (ETZ) (2, 22). Our results indicate that the prolonged rise time of twitches is accompanied by a prolonged release of Ca$^{2+}$ from the SR, the slow muscle relaxation is accompanied by slow kinetics of Ca$^{2+}$ reuptake, and the increase in peak force is associated with elevated intracellular free Ca$^{2+}$ concentrations under SR-CA inhibition. A possible role of the SR-CA in the transport of Ca$^{2+}$ during excitation-contraction coupling is proposed.

METHODS

Preparation of Fiber Bundles

Female Wistar rats (body mass 180–230 g) were killed by an overdose of diethyl ether. The SOL, a slow-twitch muscle, and the EDL, a fast-twitch muscle, were dissected out and kept in oxygenated Krebs-Henseleit solution. From these muscles, fiber bundles were prepared with spring scissors.
under a Wild M8 microscope (Leica). The bundles consisted of 15–30 muscle cells.

Preparation of Skinned Fibers

For determination of myosin-ATPase activity, the freshly prepared fiber bundles of SOL and EDL were incubated in a skinnning solution overnight at 4°C.

Preparation of SL and SR Vesicles

Four female Wistar rats (body mass 180–230 g) were killed by an overdose of diethyl ether. The white muscles from the hindlimbs were rapidly dissected out and kept in 0.75 M KCl and 5 mM imidazole, pH 7.4, at 4°C. SL and SR vesicles were prepared according to the method of Seiler and Fleischer (35) modified by Wetzel and Gros (40). SR membrane vesicles were obtained from the gradient fraction banding at the 35% sucrose phase. To obtain a purified vesicle fraction highly enriched in SR membranes almost free of contamination with SR vesicles, we further enriched SL membrane fractions obtained from the sucrose gradient centrifugation by centrifugation on a discontinuous dextran density gradient. From this gradient, the pellet was removed, and all other dextran gradient fractions were pooled to give the SL vesicle fraction.

The SL vesicle fraction displayed high activities in the SL marker enzymes, the ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase and the Mg\(^{2+}\)-ATPase (31), of 91 ± 7 μmol Pi·mg\(^{-1}\)·h\(^{-1}\) and 1.3 ± 0.2 U/mg, respectively. The activity of the Ca\(^{2+}\)-ATPase, a marker enzyme for SR, was 0.8 ± 0.1 U/mg. The SR vesicle fraction was characterized by a high activity of the Ca\(^{2+}\)-ATPase of 2.4 ± 0.2 U/mg and by minor activities of the ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase of 5 ± 1 μmol Pi·mg\(^{-1}\)·h\(^{-1}\) and the Mg\(^{2+}\)-ATPase of 0.09 ± 0.02 U/mg. From the distribution of these marker enzyme activities, we conclude that the SL vesicle fraction is highly enriched in SL membrane vesicles and only slightly contaminated by SR membranes and that the SR vesicle fraction is highly enriched in SR membranes and is contaminated by SL vesicles only to an insignificant degree.

All experiments were carried out in accordance with the guidelines of the Bezirksregierung Hannover.

Simultaneous Recordings of Isometric Single Twitches and Fura 2 Signals

The fiber bundle was transferred to a chamber that was perfused with oxygenated 25 mM HCO\(_3\)\(-5\%\) CO\(_2\)-buffered Krebs-Henseleit solution and was completely immersed in this solution. One end of the fiber bundle was fixed, and the other end was connected to a force transducer (SensNor, Friedberg, Germany). The length of the bundle was adjusted to give maximal isometric twitch tension. Single twitches were triggered by direct stimulation of the muscle cells by pulses of 1-ms duration and supramaximal voltage using platinum wires. After an initial phase of 2.5 h, during which peak force and the rise and relaxation times achieved stable values, the fiber bundle was loaded with 0.7 μmol fura 2-AM in the presence of 0.0004% Pluronic F-127 (Molecular Probes, Eugene, OR) for 2 h. The Krebs-Henseleit superfusion solution was then completely exchanged to remove the extracellular fura 2-AM. After three to four fura 2 and force signals were recorded as control values within the next 45–60 min, the CA inhibitor was added to the superfusion solution for 60 or 90 min, and the fiber bundle was stimulated every 15 min. Incubation with the CA inhibitor was terminated by a complete exchange of the circulating Krebs-Henseleit solution.

Reversibility of inhibitory effects was tested for a further 90 min. The temperature of the superfusion solution was held at 21°C.

Fura 2 was excited by light wavelengths of 340, 360, or 380 nm (ultraviolet filters; Schott, Mainz, Germany). The intensity of the emitted light was measured by a photomultiplier (type HTV R928) attached to a Zeiss fluorescence microscope using a 500- to 530-nm band-pass filter. The time resolution of light intensity data acquisition was 1 ms for each excitation wavelength. Further details concerning the experimental setup have previously been described (42, 44). Recordings at the wavelength of 360 nm, the isosbestic point of the fura 2 excitation spectrum, were used to select an area of the fiber bundle for fura 2 measurements, the light emission of which was not influenced by the movement of the bundle during contraction. The fura 2 signal for one excitation wavelength and the isometric single twitch could be recorded simultaneously with this setup. Figure 1 shows the isometric force recording and the simultaneously recorded fura 2 signal at the excitation wavelength of 380 nm of a SOL fiber. The ratio of fura 2 fluorescence, R\(_{340/380}\) (Fig. 1C), was estimated from separate recordings at 340- and 380-nm excitation wavelength taken immediately after each other according to the following equation (11):

\[ R_{340/380} = (fura \text{ fluorescence at 340 nm} - \text{autofluorescence of the fiber bundle at 340 nm})/(fura \text{ 2 fluorescence at 380 nm} - \text{autofluorescence of the fiber bundle at 380 nm}). \]

R\(_{340/380}\) values were determined only when the two force recordings taken with the fluorescence measurements were identical and were both taken from a stable bundle within a few minutes. The force recordings were analyzed for peak force, time to peak (TTP), and time of 50% decay (t\(_{50}\)). TTP was defined as the time interval between the point where the traces deviated visibly from the baseline and the peak value. In the case of the fura 2 signals, the former point coincided with the electrical stimulus. The t\(_{50}\) was defined as the time interval between the peak value and the time at which the signal had decayed to 50% of peak amplitude. R\(_{340/380}\) values (Fig. 1C) were analyzed for their value under resting conditions and the peak value after electrical stimulation. The difference between the peak value and the value at rest gives the R\(_{340/380}\) amplitude.

Measurement of Action Potentials

The microelectrodes were pulled from borosilicate glass tubing with a filament (KBF-112080, ZAK Products, Markthardenfeld, Germany) and filled with a solution containing 1.5 M KCl and 1.5 M potassium acetate (pH adjusted to 6.6–6.7 with HCl). Two microelectrodes were impaled into the same muscle cell of an SOL fiber bundle: one was used for voltage recording and the other for current injection. The resistance of the microelectrodes for voltage recording varied between 8 and 20 MΩ, and that of the electrodes used for current injection varied between 2 and 5 MΩ. Currents of 40–50 μA were injected into the cell by 1-ms pulses. 2,3-Butanedione monoxime (10 mM) was added to the Krebs-Henseleit solution, while the NaCl concentration was reduced by 10 mM, to minimize the movement of the fiber caused by electrical stimulation.

Protein

Protein contents of SL and SR vesicle fractions, as well as skinneled fibers, were measured according to the method of Lowry et al. (18) modified by Peterson (29) using a protein assay kit from Sigma. Myosin was prepared by extraction from skinneled fibers by incubation in 0.6 M KCl, 10 mM

AJP-Cell Physiol • VOL 283 • OCTOBER 2002 • www.ajpcell.org
**RESULTS**

**Effects of Fura 2 on Contraction Parameters of Single Twitches**

The contraction parameters TTP, t₅₀, and peak force were determined before, during, and after the fiber bundles were loaded with fura 2 to test whether they were affected by fura 2 acting as a Ca²⁺ buffer. Table 1 gives data for SOL and EDL. In SOL, TTP was not affected by fura 2 loading, whereas t₅₀ was reduced from 270 ± 36 ms (control) to 252 ± 11 ms after 120 min of loading with fura 2 and remained stable for a further 2 h. Peak force of SOL significantly decreased from 100% to 88 ± 7% during fura 2 loading, but in the following 2 h the values remained stable. In a separate
Table 1. TTP, \( t_{50} \), and peak force of single twitches before, during, and after fibers were loaded with fura 2-AM

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fura 2-AM</th>
<th>Krebs-Henseleit</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTP, ms</td>
<td>162 ± 11</td>
<td>160 ± 4*</td>
<td>161 ± 8*</td>
<td></td>
</tr>
<tr>
<td>( t_{50} ), ms</td>
<td>270 ± 36</td>
<td>252 ± 11*</td>
<td>257 ± 16*</td>
<td></td>
</tr>
<tr>
<td>Peak force, %</td>
<td>100</td>
<td>88 ± 11*</td>
<td>88 ± 6‡</td>
<td></td>
</tr>
<tr>
<td><strong>EDL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTP, ms</td>
<td>40 ± 7</td>
<td>39 ± 3*</td>
<td>38 ± 3*</td>
<td></td>
</tr>
<tr>
<td>( t_{50} ), ms</td>
<td>34 ± 8</td>
<td>35 ± 4*</td>
<td>33 ± 4*</td>
<td></td>
</tr>
<tr>
<td>Peak force, %</td>
<td>100</td>
<td>88 ± 11†</td>
<td>89 ± 9†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 7 fiber bundles of soleus or extensor digitorum longus (EDL). Because of different size of fiber bundles, peak force of controls varied between 1.5 and 3.5 mN in soleus and between 2 and 6 mN in EDL. Therefore, control values were set at 100%, and values obtained during and after fura 2 loading are expressed as percentage of controls. Significance of differences between control values and after 120 min in the presence of fura 2 and between control values and after fura 2 loading in Krebs-Henseleit solution for 120 min were estimated by Student’s paired \( t \)-test. *not significant; † \( P < 0.025; ‡ P < 0.01; ¶ P < 0.05.

Effects of CA Inhibitors on TTP of Fura 2 and Force Signals

Figure 2A shows the time courses of TTP of fura 2 and force signals from EDL fiber bundles. L-645151 was added to the Krebs-Henseleit superfusion solution at time 0 and was present for 90 min. L-645151 (0.1 mM) prolonged TTP of fura 2 signals as well as TTP of force. After removal of L-645151, these effects were reversed. To obtain the values of Table 2, control data from –60 to 0 min were combined in one group and given as means ± SD. Data obtained in the presence of CA inhibitor during an exposure time of 15 min to 60 min (CLZ) or to 90 min (L-645151 and ETZ) were combined into one group and given as means ± SD (Table 2). Data of the washout phases from 75 to 150 min (CLZ) and from 105 to 180 min (L-645151 and ETZ) were also combined into one group. Means ± SD of the phases before, during, and after exposure to the CA inhibitor are listed in Table 2. Significance of differences between the control values and the values determined in the period of exposure to the CA inhibitor was estimated by Student’s unpaired \( t \)-test. Significance of differences between the values in the presence of inhibitor and the values in the washout phase was also estimated. L-645151, CLZ, and ETZ caused significant increases in TTP of fura 2 as well as of force signals (Table 2). All these effects were significantly reversed on removal of the inhibitors.

In SOL, TTP of fura 2 signals and of force signals were also increased by L-645151, CLZ, and ETZ (Table 3; increases in TTP of fura 2 signal by L-645151 and in TTP of force signal by ETZ were not significant). After removal of the CA inhibitors, all effects showed full or partial reversibility.

Effects of CA Inhibitors on \( t_{50} \) of Fura 2 and Force Signals

Figure 2B shows the time courses of \( t_{50} \) of fura 2 and force signals from EDL fibers. L-645151 led to a distinct increase in \( t_{50} \) of fura 2 and of force signals. The observation that in EDL the absolute values of \( t_{50} \) of fura 2 signals are higher than those of force signals has been discussed in a previous study (42). Table 2 gives the data for EDL before, during, and after exposure to the inhibitors. All three membrane-permeable inhibi-
tors caused a significant prolongation of \( t_{50} \), which was significantly reversed on their removal. Table 3 summarizes the effects of L-645151, CLZ, and ETZ in SOL fibers. In SOL, as in EDL, \( t_{50} \) values of fura 2 and of force signals were prolonged by the inhibitors (prolongation of \( t_{50} \) of fura 2 signals by L-645151 and, not significantly, by ETZ), and their effects were reversible. Figure 3B illustrates that \( t_{50} \) of the force signal of SOL also varies with CLZ concentration in a dose-dependent manner, reaching a plateau at \( \sim 5 \times 10^{-4} \) M CLZ.

**Effects of CA Inhibitors on Peak Force of Single Twitches and \( R_{340/380} \)**

In Fig. 2C, peak forces are given as percentages. Because it was not possible to prepare fiber bundles of identical size, the values of force varied: in SOL between 1.5 and 3.5 mN and in EDL between 2 and 6 mN. Therefore, the mean value derived from the values of the control phase of −60 to 0 min (Fig. 2C) has been set at 100%, and the values of peak force during and after exposure to the CA inhibitor have been expressed as a
Table 2. TTP, t½b, and amplitudes of fura 2 and force signals before, during, and after exposure of EDL fiber bundles to CA inhibitors

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Fura 2 signal</th>
<th>Force signal</th>
<th>Fura 2 signal</th>
<th>Force signal</th>
<th>R_{340/380}</th>
<th>Force signal,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-645151 (0.1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>15</td>
<td>13.8 ± 2.0</td>
<td>33.3 ± 2.9</td>
<td>76 ± 15</td>
<td>30 ± 3</td>
<td>100 ± 4</td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>18</td>
<td>20.5 ± 3.4</td>
<td>43.3 ± 3.6</td>
<td>131 ± 30</td>
<td>58 ± 17</td>
<td>179 ± 17</td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>18</td>
<td>12.7 ± 4.1</td>
<td>36.6 ± 1.8</td>
<td>83 ± 15</td>
<td>36 ± 8</td>
<td>110 ± 5</td>
<td></td>
</tr>
<tr>
<td>CLZ (0.5 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>25</td>
<td>17.7 ± 9.3</td>
<td>28.6 ± 2.5</td>
<td>57 ± 14</td>
<td>26 ± 7</td>
<td>0.93 ± 0.61</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>During</td>
<td>20</td>
<td>25.8 ± 10.1‡</td>
<td>49.1 ± 9.6‡</td>
<td>124 ± 24‡</td>
<td>58 ± 21‡</td>
<td>0.81 ± 0.73‡</td>
<td>109 ± 11‡</td>
</tr>
<tr>
<td>After</td>
<td>30</td>
<td>21.4 ± 8.3*</td>
<td>35.5 ± 6.0*</td>
<td>69 ± 19*</td>
<td>36 ± 15*</td>
<td>0.60 ± 0.37*</td>
<td>79 ± 15*</td>
</tr>
<tr>
<td>ETZ (0.1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>8</td>
<td>8.7 ± 5.5</td>
<td>27.3 ± 2.0</td>
<td>50 ± 7</td>
<td>28 ± 5</td>
<td>0.50 ± 0.11</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>During</td>
<td>12</td>
<td>14.5 ± 3.6†</td>
<td>33.1 ± 2.1†</td>
<td>91 ± 16†</td>
<td>35 ± 5†</td>
<td>0.80 ± 0.17†</td>
<td>141 ± 17†</td>
</tr>
<tr>
<td>After</td>
<td>12</td>
<td>9.8 ± 2.6‡</td>
<td>28.7 ± 2.5‡</td>
<td>64 ± 19‡</td>
<td>24 ± 7‡</td>
<td>0.52 ± 0.11‡</td>
<td>92 ± 6‡</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of measurements. Before, −60 to 0 min; during, 15 to 60 min [chlorzolamide (CLZ)] or 90 min [L-645151 and ethoxzolamide (ETZ); see Fig. 2]; after (washout), 75 to 150 min (CLZ) and 105 to 180 min (L-645151 and ETZ). CA, carbonic anhydrase. Significance of differences between control values and values obtained in the presence of inhibitor and between values obtained in the presence of inhibitor and those obtained after removal of inhibitor were estimated by Student’s unpaired t-test: *not significant; †P < 0.05; ‡P < 0.025; §P < 0.01; ††P < 0.001.

percentage of this mean value. ETZ at 0.1 mM increased peak force and amplitude of R_{340/380} (Fig. 2C). The three lipophilic and membrane-permeable CA inhibitors, L-645151, CLZ, and ETZ, significantly increased peak force of twitches in EDL by 10–80% and in SOL by 10–30% (Tables 2 and 3). To determine whether these increases in peak force were accompanied by an increase in Ca^{2+} release from the SR, R_{340/380} values were calculated and analyzed for their peak values and amplitudes. In the case of L-645151, this was not possible, because this inhibitor displayed an autofluorescence at 340- and 380-nm excitation wavelengths. In EDL, CLZ led to a small increase in peak force by 9%, which was not paralleled by an increase in the amplitude of R_{340/380} (Table 2). ETZ caused a significant increase in peak force (−40%), which was accompanied by a significant increase in the amplitudes of R_{340/380} from 0.5 ± 0.1 to 0.8 ± 0.2 (Table 2, Fig. 2C). In SOL, CLZ increased peak force of single twitches by −20% and the amplitudes of R_{340/380} from 0.99 ± 0.30 to 1.27 ± 0.04 (Table 3). ETZ led to an increase in peak force by 12%, which coincided with a slight, not significant, increase in the amplitudes of R_{340/380} (Table 3). In some, but not all, cases, the increases in peak force were accompanied by increased amplitudes of R_{340/380}. It appears possible that, in the presence of permeable inhibitors, more Ca^{2+} is released on muscle activation. The values of R_{340/380} under resting conditions were not influenced by the CA inhibitors.

Table 3. TTP, t½b, and amplitudes of fura 2 and force signals before, during, and after exposure of SOL fiber bundles to CA inhibitors

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Fura 2 signal</th>
<th>Force signal</th>
<th>Fura 2 signal</th>
<th>Force signal</th>
<th>R_{340/380}</th>
<th>Force signal,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-645151 (0.1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>8</td>
<td>29 ± 9</td>
<td>127 ± 13</td>
<td>78 ± 24</td>
<td>234 ± 19</td>
<td>100 ± 2</td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>12</td>
<td>39 ± 17</td>
<td>153 ± 23</td>
<td>99 ± 26</td>
<td>375 ± 92</td>
<td>128 ± 16</td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>12</td>
<td>33 ± 16</td>
<td>138 ± 14</td>
<td>79 ± 13</td>
<td>331 ± 67</td>
<td>100 ± 5</td>
<td></td>
</tr>
<tr>
<td>CLZ (0.5 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>12</td>
<td>18 ± 4</td>
<td>110 ± 10</td>
<td>98 ± 7</td>
<td>241 ± 25</td>
<td>0.99 ± 0.30</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>During</td>
<td>12</td>
<td>46 ± 4</td>
<td>129 ± 24</td>
<td>136 ± 18†</td>
<td>382 ± 118‡</td>
<td>1.27 ± 0.04‡</td>
<td>119 ± 3‡</td>
</tr>
<tr>
<td>After</td>
<td>12</td>
<td>41 ± 2</td>
<td>113 ± 8</td>
<td>133 ± 16</td>
<td>223 ± 71</td>
<td>1.11 ± 0.03*</td>
<td>101 ± 5*</td>
</tr>
<tr>
<td>ETZ (0.1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>16</td>
<td>28 ± 13</td>
<td>157 ± 8</td>
<td>129 ± 46</td>
<td>321 ± 24</td>
<td>1.19 ± 0.77</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>During</td>
<td>24</td>
<td>39 ± 14</td>
<td>161 ± 12</td>
<td>150 ± 43</td>
<td>350 ± 109†</td>
<td>1.28 ± 0.49*</td>
<td>112 ± 4*</td>
</tr>
<tr>
<td>After</td>
<td>24</td>
<td>31 ± 10</td>
<td>151 ± 12</td>
<td>126 ± 22</td>
<td>342 ± 44‡</td>
<td>1.15 ± 0.49‡</td>
<td>101 ± 8‡</td>
</tr>
<tr>
<td>ACTZ (1 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>4</td>
<td>30 ± 10</td>
<td>97 ± 7</td>
<td>113 ± 9</td>
<td>190 ± 20</td>
<td>1.04 ± 0.24</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>During</td>
<td>16</td>
<td>32 ± 12</td>
<td>96 ± 6</td>
<td>109 ± 13</td>
<td>201 ± 20‡</td>
<td>1.05 ± 0.20*</td>
<td>89 ± 4‡</td>
</tr>
<tr>
<td>After</td>
<td>8</td>
<td>40 ± 15</td>
<td>95 ± 6</td>
<td>118 ± 20</td>
<td>193 ± 10</td>
<td>1.11 ± 0.35*</td>
<td>82 ± 1b</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of measurements. Before, −60 to 0 min; during, 15 to 60 min (CLZ) or 90 min (L-645151 and ETZ); after, 75 to 150 min (CLZ) and 105 to 180 min (L-645151 and ETZ; see Fig. 2). ACTZ, acetazolamide. Significance of differences between control values and values obtained in the presence of inhibitor and between values obtained in the presence of inhibitor and those obtained after removal of inhibitor were estimated by Student’s unpaired t-test: *not significant; †P < 0.05; ‡P < 0.025; §P < 0.01; ††P < 0.001.

AJPCellPhysiol • VOL 283 • OCTOBER 2002 • www.ajpcell.org
Lack of Effects of ACTZ on Fura 2 and Force Signals of SOL Fibers

ACTZ is a hydrophilic and, therefore, poorly membrane-permeable CA inhibitor (22) that predominantly inhibits the extracellular SL-CA and affects the intracellular CAs only to a minor degree. In SOL, ACTZ at 1 mM, which is 10 times higher than the concentration of L-645151 and ETZ and 2 times higher than that of CLZ, did not lead to a prolongation of TTP in fura 2 or in force signals (Fig. 4A). The $t_{50}$ values of fura 2 signals were not affected by ACTZ (Fig. 4B). The $t_{50}$ of force was only slightly increased by $\sim 10-20$ ms by ACTZ (Fig. 4B), whereas L-645151 and ETZ increased $t_{50}$ of force by $\sim 140$ and 70 ms, respectively (Table 3). Neither the amplitudes of $R_{340/380}$ nor the values of peak force were increased by ACTZ (Fig. 4C, Table 3).

Lack of Effects of CA Inhibitors on Myosin-, $Ca^{2+}$-, and Ouabain-Sensitive $Na^{+}-K^{+}$-ATPase Activities

Myosin-ATPase. Myosin-ATPase activities were measured in skinned fibers. First, the $Ca^{2+}$-activated myosin-ATPase activity was monitored for 5–10 min, then the inhibitor was added to the assay medium, and the activity was measured for a further 5–10 min. Because of the different size of fibers, the absolute activities varied between 0.9 and 4.7 μmol ADP·mg$^{-1}$·h$^{-1}$. Therefore, the myosin-ATPase activities in the absence of CA inhibitor were set at 100% (i.e., control values), and the activities in the presence of the inhibitor were expressed as percentage of controls. The myosin-ATPase activities of SOL and EDL were not significantly affected by L-645151, CLZ, or ETZ (Table 4).

$Ca^{2+}$-ATPase. $Ca^{2+}$-ATPase activity was determined in SR vesicles of white hindlimb muscles from rat. Different concentrations of the $Ca^{2+}$ ionophore A-23187 were tested, and 5 μg/ml gave the maximum activation of the $Ca^{2+}$-ATPase. Because the activities were dependent on the lot number of the ionophore used in the assay and more than one lot number of the ionophore was needed for all determinations, it was necessary that $Ca^{2+}$-ATPase activities be given as percentages so that they could be compared with activity values that were determined with different lot numbers of ionophore. The mean value of the total ATPase activity was set at 100%, and the ATPase activities in the absence of $Ca^{2+}$ were expressed as percentage of this mean value. The $Ca^{2+}$-ATPase activity was calculated as the difference between the total and the basal Mg$^{2+}$-dependent ATPase activities. Total and basal ATPase activities were measured in the absence and presence of CA inhibitors. Table 4 shows the $Ca^{2+}$-ATPase activities determined in the absence of CA inhibitor (i.e., control values) and the activities determined in the presence of L-645151, CLZ, and ETZ. None of these inhibitors significantly affected the $Ca^{2+}$-ATPase in a stimulatory or an inhibitory manner.

Ouabain-sensitive $Na^{+}-K^{+}$-ATPase. The ouabain-sensitive $Na^{+}-K^{+}$-ATPase activity of white muscle SL vesicles was not affected by the CA inhibitors L-645151, CLZ, and ETZ (Table 4).

Lack of Effects of CLZ and ETZ on the Action Potentials of SOL Fibers

Action potentials (APs) were measured in single muscle cells of SOL at room temperature. APs were evoked every 3–5 min over a 40- to 100-min period by current injection into a single cell. Fifty-two APs of four muscle cells were measured in the absence of a CA inhibitor and used as controls. Seventy APs of six muscle cells were elicited in the presence of 0.1 mM ETZ and 50 APs of four muscle cells in the presence of 0.22 mM CLZ. Each AP signal was analyzed for its amplitude and its half-width. The time courses of AP amplitudes under control and inhibitory conditions are shown in Fig. 5. The linear regression calculation of AP...
amplitude vs. time gave the intercept $a$ of the linear regression line with the y-axis and the regression coefficient $m$. Under control conditions, $a = 78$ mV and $m = -13$ mV/h. In the presence of ETZ, the linear regression line is given by $a = 88$ mV and $m = -7.9$ mV/h and in the presence of CLZ by $a = 88$ mV and $m = -15$ mV/h (Fig. 5). In the case of the half-width of AP signals (not shown), the linear regression line of control is given by $a = 2.34$ ms and $m = 0.82$ ms/h. In the presence of ETZ, $a = 1.98$ ms and $m = 0.44$ ms/h, and in the presence of CLZ $a = 2.10$ ms and $m = 0.56$ ms/h. The linear regression lines obtained in the presence of ETZ or CLZ are not significantly different from control.

Fig. 4. Time courses of TTP and $t_{50}$ of fura 2 and force signals and of amplitudes of R340/380 and peak force of SOL fiber bundles in the absence and presence of acetazolamide (ACTZ). A: TTP values of fura 2 signals (○) and single twitches (●). B: $t_{50}$ values of fura 2 signals (○) and single twitches (●). C: amplitudes of R340/380 (○) and peak force of single twitches (●). Values are means ± SD; 4 SOL fiber bundles were tested.
DISCUSSION

Hypothesis on the Role of SR-CA for Countertransport of H⁺ During Ca²⁺ Release and Reuptake by the SR

Studies of Somlyo et al. (35), Pape et al. (28), and Kamp et al. (14) postulated or showed a transport of H⁺ into the SR during Ca²⁺ release from the SR. The influx of H⁺ can occur via the extremely high H⁺ permeability of the SR membrane (9, 24, 26). An H⁺ ejection coupled to Ca²⁺ uptake by SR vesicles has been demonstrated by several authors (4, 15, 21, 23, 38, 39). In line with this, it has been reported that the Ca²⁺-ATPase directly effects an exchange of H⁺ for Ca²⁺ (17, 39, 46–48). All these studies have provided unambiguous evidence for a vectorial transport of H⁺ associated with the Ca²⁺ transport across the SR membrane. In addition, fluxes of K⁺, Mg²⁺, and Cl⁻ contribute to counterbalance the charge transfer by the Ca²⁺ (23, 35). The protons required for these proton fluxes may be generated by the CO₂-HCO₃⁻ buffer system. This system can serve as an H⁺ source and sink that produces the required H⁺ on one side of the SR membrane and buffers the transported H⁺ on the other side of the SR membrane (Fig. 6). However, the CO₂-HCO₃⁻ system can fulfill this task only when it is catalyzed by CA, because the half-time of the uncatalyzed reaction of ~7 s is far too slow in view of the fast kinetics of Ca²⁺ fluxes. Ca²⁺ release lasts ~20–50 ms, and Ca²⁺ reuptake lasts ~200–300 ms (Fig. 2, Tables 2 and 3) (44). The need for catalysis may be illustrated by the following rough calculation. Let us consider a Ca²⁺ release lasting for 30 ms and causing a depletion of intra-SR Ca²⁺ concentration by 2 mM (12). Ca²⁺ then moves across the SR membrane at a rate of

\[
\frac{d[Ca^{2+}]}{dt} = \frac{0.002 \text{ mol}}{1 \times 0.03 \text{ s}} = 0.07 \text{ mol} \cdot l^{-1} \cdot s^{-1}
\]

where [Ca²⁺] is Ca²⁺ concentration and l represents 1 liter of intra-SR volume. If protons move into the SR during the Ca²⁺ release, they will have to be rapidly buffered inside the SR. We estimate the rate of H⁺ buffering by the uncatalyzed dehydration reaction of carbonic acid by assuming an intra-SR pH of 7, an HCO₃⁻ concentration of 10 mM, and a reaction velocity constant (kₐ) of 130,000 l·mol⁻¹·s⁻¹ as derived from a CO₂ hydration velocity constant of 0.1 s⁻¹ and pKᵢ of 6.1. For further simplification, we neglect the backreaction, which yields an overestimate of the possible rate of H⁺ buffering, (d[H⁺]/dt)

\[
\frac{d[H^+]}{dt} = k_a \times [H^+] \times [HCO_3^-]
\]

\[
= 1.3 \times 10^6 \frac{1}{\text{mol} \cdot s} \times 10^{-7} \text{mol} \times 10^{-2} \text{mol} \cdot l^{-1} = 0.00013 \text{ mol} \cdot l^{-1} \cdot s^{-1}
\]

where [H⁺] is H⁺ concentration.

We conclude from this consideration that the uncatalyzed dehydration reaction can buffer protons at ~1/500th of the rate of Ca²⁺ efflux. Thus, even if equal fluxes of H⁺ and Ca²⁺ were required, which would balance 50% of the charges transferred by Ca²⁺, the carbonic acid dehydration reaction will have to be accelerated by a factor of 500. Indeed, Bruns et al. (3) showed that the SR-CA accelerates the CO₂-HCO₃⁻ reaction ~1,000-fold, which reduces the half-time from 7 s to ~7 ms.

From this hypothesis, it is predicted that inhibition of the SR-CA will impair the fast production of H⁺ on the cytoplasmic side of the SR membrane and impair

![Graph showing time courses of amplitudes of action potentials stimulated in single SOL fibers in the absence of ETZ and CLZ (●), with extracellular 0.1 mM ETZ (○), and with extracellular 0.22 mM CLZ (▲).](image-url)
buffering inside the SR of H\(^+\) that have moved into the SR during Ca\(^{2+}\) release. This will slow the H\(^+\) fluxes into the SR during Ca\(^{2+}\) release, slow the kinetics of Ca\(^{2+}\) release, and prolong the rise time of twitches. On the other hand, during Ca\(^{2+}\) uptake, inhibition of SR-CA will impair the fast production of H\(^+\) on the intraluminal side of the SR membrane. This will reduce the rate of H\(^+\) fluxes that move out of the SR during Ca\(^{2+}\) reuptake, reduce the rate of Ca\(^{2+}\) reuptake, and prolong relaxation of twitches.

The presence of the CO\(_2\)-HCO\(_3\) system may be especially important inside the SR because of the small SR volume compared with the volume of the cytoplasm (5) and because of the absence of major nonbicarbonate buffer systems inside the SR. This is in accordance with the observed distribution of CA activity across the SR membrane. By mass spectrometric measurements (10, 43), about two-thirds of the total CA activity of SR vesicles was found to be intravesicular, and only one-third of the CA activity was located on the outside, i.e., the cytoplasmic side, of the SR vesicles.

**Fura 2 and Force Signals are Not Influenced by Possible Effects of CA Inhibitors on Myosin-, Ca\(^{2+}\)-, and Na\(^+\)-K\(^+\)-ATPases and on AP**

To test whether the changes in TTP, \(t_{50}\), and peak values of fura 2 and force signals were caused by the inhibition of SR-CA as postulated by our hypothesis or whether they were influenced by side effects of the CA inhibitors, the effects of CA inhibitors on the activities of different ATPases and on the AP were investigated; e.g., an inhibition of the myosin-ATPase by the CA inhibitors could have led to a prolongation of TTP of twitches or a possible stimulation to an increase in force production. However, neither L-645151 nor CLZ or ETZ affected the activities of the myosin-ATPase (Table 4). An inhibition of the Ca\(^{2+}\)-ATPase would cause a prolongation of \(t_{50}\) of fura 2 signals and, consequently, a prolongation of \(t_{50}\) of force. However, none of the CA inhibitors inhibited the Ca\(^{2+}\)-ATPase (Table 4). If the CA inhibitors affected the resting potential via changes in Na\(^+\)-K\(^+\)-ATPase activity and the AP, the activation of ryanodine receptors (RyR) might be altered. An increased AP might lead to a longer-lasting release of Ca\(^{2+}\) and a greater amount of released Ca\(^{2+}\). This could result in an increase in TTP of fura 2 signals and greater amplitudes of \(R_{340/380}\) values. L-645151, CLZ, and ETZ did not affect the Na\(^+\)-K\(^+\)-ATPase and did not significantly alter APs (Table 4, Fig. 5). We cannot rule out a direct effect of sulfonamides on the RyR, but they exhibit an effect on \(t_{50}\) that is qualitatively similar to the effect on TTP, although Ca\(^{2+}\)-ATPase is not affected. Therefore, we conclude that the changes in TTP, \(t_{50}\), and peak values of fura 2 and force signals are very likely caused by the inhibition of the SR-CA, rather than by any side effect of the CA inhibitors. This conclusion is strengthened by the dose-dependent effect of CLZ on TTP and \(t_{50}\).

**Inhibition of SL CA by ACTZ Does Not Affect Fura 2 and Force Signals**

ACTZ is a hydrophilic and, therefore, poorly membrane-permeable CA inhibitor (22) that predominantly inhibits the extracellular SL-CA but not the SR-CA and the CAIII in SOL. ACTZ did not prolong TTP of fura 2 and force signals or \(t_{50}\) of fura 2 signals and prolonged only to a small degree \(t_{50}\) of force (Fig. 4, Table 3). ACTZ exerted no effects on the amplitudes of \(R_{340/380}\) and had no effect on peak force. From these results, we conclude that inhibition of the SL-CA can-
not be responsible for the effects on TTP, \( t_{50} \), and amplitudes of fluorescence ratios and force signals caused by L-645151, CLZ, and ETZ. These three CA inhibitors are rather lipophilic and, therefore, highly membrane permeable (2, 22) and inhibit the extracellular SL-CA as well as the SR-CA and the CAII of SOL. Because they exerted their effects in SOL as well as in EDL, which has no CAII but the same two membrane-bound CA forms as SOL, CAII can be excluded as the enzyme responsible for these changes. Therefore, we conclude that the inhibitory effects of L-645151, CLZ, and ETZ are very likely caused by inhibition of the SR-CA in SOL as well as in EDL.

**Effects of Membrane-Permeable CA Inhibitors on TTP are Consistent With the Proposed Function of SR-CA**

The prolongations of TTP of fura 2 as well as of force signals by L-645151, CLZ, and ETZ are in full agreement with the model of Fig. 6: CA inhibition reduces the rates of \( H^+ \) influx, \( Ca^{2+} \) release, and force development. Dettbarn and Palade (8) observed that concentrations of \( Ca^{2+} \) can markedly affect the rate of \( Ca^{2+} \) release. They reported that the rate of \( Ca^{2+} \) release from SR vesicles was reduced by 75–90% when the countertransport of \( K^+ \) by choline and the replacement of \( Cl^- \) by gluconate, respectively. However, their experiments were conducted in the absence of CO2. Conversely, when CA is inhibited in the present experiments, the impairment of \( H^+ \) influx may partly be compensated by increased fluxes of the other balancing ions \( K^+ \), \( Mg^{2+} \), and \( Cl^- \). This may be the reason for the moderate effect of SR-CA inhibition on TTP.

**Effects of Membrane-Permeable CA Inhibitors on \( t_{50} \) are Consistent With the Proposed Function of SR-CA**

The \( t_{50} \) values of fura 2 and force signals were significantly prolonged by the highly membrane-permeable CA inhibitors L-645151, CLZ, and ETZ. Again, this is consistent with the prediction from the hypothesis of Fig. 6. Analogously, a stimulating effect of countermovements of \( K^+ \) on the rates of \( Ca^{2+} \) uptake has been reported by several authors (17, 39, 48, 49). Another result, which is consistent with the proposed function of the SR-CA, has been reported by Levy et al. (17), who found that increasing intravesicular buffer capacities by increasing intravesicular concentrations of PIPES increased the rates of \( Ca^{2+} \) uptake. In conclusion, reducing the rates of \( H^+ \) fluxes coupled to \( Ca^{2+} \) uptake by inhibition of SR CA may be responsible for the slow kinetics of \( Ca^{2+} \) reuptake. The latter probably causes the slowdown in muscle relaxation.

**Effects of Inhibition of SR CA on Peak Force and Amplitude of \( R_{340/380} \)**

The CA inhibitors caused significant increases in peak force of single twitches (Tables 2 and 3). The decrease in peak force reported for CLZ in a previous study (44) is only seen with \( \geq 1 \) mM CLZ and is not seen with the other lipophilic inhibitors ETZ and L-645151. In the present study, we used CLZ at \(< 1 \) mM only.

It is possible, as indicated by the increased amplitudes of \( R_{340/380} \) seen in some cases (Tables 2 and 3), that the increase in force was induced by a somewhat greater amount of \( Ca^{2+} \) released from the SR in the presence of inhibitor. It may be speculated that, in the presence of CA inhibitors, local pH disequilibria cause an increased open probability of the RyR (16, 19, 20, 30, 34) and, thus, an enhanced release of \( Ca^{2+} \). There is an alternative explanation for an increase in peak force under CA inhibition that does not involve an increase in the amount of \( Ca^{2+} \) released from the SR. Because of the prolonged \( Ca^{2+} \) transient (Fig. 1), the contractile apparatus is exposed to elevated intracellular free \( Ca^{2+} \) concentrations for a longer period of time than under control conditions (Fig. 1, B and C). This should lead to an increase in force generation (31, 36). Thus, by mechanisms different from those effecting the slowdown of \( Ca^{2+} \) transients, inhibition of the SR-CA appears to lead to an increase in force generation by exposing the contractile apparatus to intracellular \( Ca^{2+} \) concentrations that are either higher or last longer than under control conditions. In conclusion, although the observed increases in peak force are not predicted by our hypothesis and may be secondary effects, the described prolongations of TTP and \( t_{50} \) are in excellent agreement with the proposed function of SR-CA.

This research was supported by Deutsche Forschungsgemeinschaft Grant 489/4.

**REFERENCES**


