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

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Wetzel, Petra, Tanja Kleinke, Simon Papadopoulos, and Gerolf Gros. Inhibition of muscle carbonic anhydrase 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.

sarcoplasmic reticulum; H\(^{+}\) countertransport; fura 2 transients; single twitches

AN EXTRACELLULAR, SARCOLEMMAL (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 (33) 
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 SL 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 
maker enzymes, the ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase and the 
Mg\(^{2+}\)-ATPase (13), of 91 ± 7 μmol P\(_i\)·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 P\(_i\)· 
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 en-
riched 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 Hanover.

**Measurement of Action Potentials**

The microelectrodes were pulled from borosilicate glass tubing 
with a filament (KBF-112080, ZAK Products, Markt-
heidenfeld, 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 
the simultaneous 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 
40–90 μA were injected into the cell by 1-ms pulses. 2,3-
Butanediene monoxide (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 
causd by electrical stimulation.

**Protein**

Protein contents of SL and SR vesicle fractions, as well as 
skinned 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 skinned fibers by incubation in 0.6 M KCl, 10 mM

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**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 
typically (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 excita-
tion 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 simulta-
aneously 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\(_{340\text{to}380}\) (Fig. 1C), was estimated from 
separate recordings at 340- and 380-nm excitation wave-
lengths taken immediately after each other according to the 
following equation (11):

\[
R_{340\text{to}380} = \frac{\text{fura 2 fluorescence at 340 nm} - \text{autofluorescence of the fiber bundle at 340 nm}}{\text{fura 2 fluorescence at 380 nm} - \text{autofluorescence of the fiber bundle at 380 nm}}
\]

Values were determined only when the two force recordings taken with the fluorescence measure-
ments 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 ampli-

tude. R\(_{340\text{to}380}\) 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\(_{340\text{to}380}\) amplitude.

**Measurement of Action Potentials**

The microelectrodes were pulled from borosilicate glass tubing 
with a filament (KBF-112080, ZAK Products, Markt-
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1.5 M KCl and 1.5 M potassium acetate (pH adjusted to 
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skinned fibers, were measured according to the method of 
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assay kit from Sigma. Myosin was prepared by extraction 
from skinned fibers by incubation in 0.6 M KCl, 10 mM
Cnm. Simultaneously recorded fura 2 signals at an excitation wavelength of 380 nm. Fig. 1. Recordings of single-twitch force, fura 2 fluorescence at the 380-nm excitation wavelength and of R 340/380. A: force recordings of a single twitch of an SOL fiber bundle in the absence and presence of ethoxzolamide (ETZ). B: fitted by a sigmoidal equation with 4 parameters (Sigma Plot 5.0, SPSS, Chicago, IL). Values of 50% decay time (t50) with (red) and without (black) ETZ are indicated by lengths of bars. ETZ caused increases in time to peak (TTP), t50, and peak force of single twitches, which were accompanied by increases in TTP, t50, and amplitudes of fura 2 signals at the 380-nm excitation wavelength and of R 340/380.

EGTA, 1 mM tetrasodium diphosphate, 0.1 mM dithiothreitol, 5 mM KH2PO4, 5 mM K2HPO4, and 1 mM phenylmethylsulfonyl fluoride, pH 6.8, and proteins were suspended by an ultrasound scanner.

Myosin-ATPase

Myosin-ATPase of skinned fibers from SOL and EDL was measured in an assay medium containing 50 mM imidazole, pH 7.4, 6 mM KCl, 4 mM MgCl2, 0.5 mM EGTA, 5 mM Na3A, 2.2 mM CaCl2, 4.2 mM Na2ATP, 0.33 mM NADH, 1.2 mM phosphoenolpyruvate, 15 U/ml lactate dehydrogenase, and 14 U/ml pyruvate kinase in a spectrophotometer at 37°C. The formation of ADP by myosin-ATPase activity was coupled to the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. The pyruvate-to-lactate conversion was coupled to the oxidation of NADH, which was continuously monitored by the decrease in absorbance at 340 nm.

Ca2+ - and Mg2+ -ATPases

Ca2+ - and Mg2+ -ATPases were measured as described by Seiler and Fleischer (33). Inorganic phosphate was measured according to the method of Ottolenghi (27). The Ca2+ -dependent ATPase activity was calculated as the difference between the activity of the total ATPase and the activity of the Mg2+ -ATPase.

Na+ -K+ -ATPase

Na+ -K+ -ATPase was measured as described by Seiler and Fleischer (33). Ouabain-sensitive Na+ -K+ -ATPase was calculated as the difference between the total Na+ -K+ -ATPase and the Na+ -K+ -ATPase measured in the presence of 1 mM ouabain.

Solutions

Krebs-Henseleit solution was composed of (in mM) 120 NaCl, 3.3 KCl, 1.2 MgSO4, 1.3 KH2PO4, 1.3 CaCl2, and 25 NaHCO3. Skinning solution consisted of (in mM) 5 KH2PO4, 3.0 magnesium acetate, 50 creatine phosphate, 5 EGTA, 1 Na2ATP, 1 dithiothreitol, and 0.5% Triton X-100, pH 7.4.

CA Inhibitors

Acetazolamide (ACTZ) and ETZ were purchased from Sigma-Aldrich (Munich, Germany). CLZ and L-645151 were generous gifts of Lederle Laboratories (Pearl River, NY). Stock solutions of ETZ, CLZ, and L-645151 at 10 mM each were prepared in Krebs-Henseleit solution in the presence of 20 mM NaOH. Aliquots of these stock solutions were added to the superfusion solution in 100-fold dilution (20-fold in the case of CLZ), and the amount of added NaOH was compensated by the addition of the equivalent amount of HCl. The final inhibitor concentration in the superfusion solution was always checked photometrically.

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, t50, 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>Extracellular Fura 2-AM</th>
<th>Krebs-Henseleit Solution</th>
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<tbody>
<tr>
<td>TTP, ms</td>
<td>162 ± 11</td>
<td>160 ± 4*</td>
<td>161 ± 8*</td>
</tr>
<tr>
<td>t50, ms</td>
<td>270 ± 36</td>
<td>252 ± 11*</td>
<td>257 ± 16*</td>
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<td>Peak force, %</td>
<td>100</td>
<td>88 ± 7†</td>
<td>88 ± 6‡</td>
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</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 ETZ on Force and Fura 2 Signals of a Fiber Bundle From SOL

Figure 1 shows the single twitch, the simultaneously recorded fluorescence signal at 380-nm excitation wavelength, and the calculated R340/380 before the addition of the CA inhibitor ETZ and after 45 min of exposure to 0.1 mM ETZ. When both force signals of series of experiments in which SOL fibers were not loaded with fura 2, the TTP and t50 did not change in 8 h, and at a point comparable to that after 120 min of fura 2 loading, force was reduced only to 97 ± 6%, and the difference from 88 ± 7% is significant as estimated by Student’s unpaired t-test (P < 0.05). In EDL, fura 2 loading of muscle fibers did not affect TTP and t50 but significantly reduced peak force by 12% (Table 1). When EDL fibers were not loaded with the Ca2+ indicator, force was stable over a comparable period of time at 99 ± 2%, and the difference from 88 ± 11% was significant (P < 0.05, Student’s unpaired t-test). Thus fura 2 loading led to a decrease in peak force by ~12% in SOL and EDL and a reduction in t50 by ~7% in SOL. A fluorescent Ca2+ indicator acting as a Ca2+ buffer may reduce the amplitude of the free intracellular Ca2+ concentration and, consequently, peak force and will prolong the decay of free intracellular Ca2+ and, consequently, force relaxation, as shown by Ashley et al. (1) and Noble and Powell (25). Our results indicate that some Ca2+ buffering effect of fura 2 might be responsible for the slight decrease in peak force of twitches, but it did not lead to a prolongation of the kinetics of twitches. All effects of the CA inhibitors on TTP, t50, and the amplitudes of Ca2+ transients and force recordings were compared with control values obtained after fura 2 loading and were tested in terms of reversibility.

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) of 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.

Figure 3A shows that TTP of the force signal of SOL bundles is affected by CLZ in a dose-dependent manner. TTP begins to increase at ≥10−4 M CLZ.

Effects of CA Inhibitors on t50 of Fura 2 and Force Signals

Figure 2B shows the time courses of t50 of fura 2 and force signals from EDL fibers. L-645151 led to a distinct increase in t50 of fura 2 and of force signals. The observation that in EDL the absolute values of t50 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, t50, and amplitudes of fura 2 and force signals before, during, and after exposure of EDL fiber bundles to CA inhibitors

<table>
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<tr>
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<th>TTP, ms</th>
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<tr>
<td></td>
<td>Fura 2 signal</td>
<td>Force signal</td>
<td>Fura 2 signal</td>
<td>Force signal</td>
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<tr>
<td>L-645151 (0.1 mM)</td>
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<tr>
<td>Before</td>
<td>15</td>
<td>13.8 ± 2.0</td>
<td>33.3 ± 2.9</td>
<td>76 ± 15</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>
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<tr>
<td>After</td>
<td>12</td>
<td>12.7 ± 4.1</td>
<td>36.6 ± 1.8§</td>
<td>83 ± 15§</td>
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<tr>
<td>CLZ (0.5 mM)</td>
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<tr>
<td>Before</td>
<td>25</td>
<td>17.7 ± 9.3</td>
<td>28.6 ± 2.5</td>
<td>57 ± 14</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>
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<tr>
<td>After</td>
<td>30</td>
<td>21.4 ± 8.3§</td>
<td>35.5 ± 6.0§</td>
<td>69 ± 19§</td>
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<td>ETZ (0.1 mM)</td>
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<tr>
<td>Before</td>
<td>8</td>
<td>8.7 ± 5.5</td>
<td>27.3 ± 2.0</td>
<td>50 ± 7</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>
</tr>
<tr>
<td>After</td>
<td>12</td>
<td>9.8 ± 2.6‡</td>
<td>28.7 ± 2.5§</td>
<td>64 ± 19§</td>
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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 < 0.001.

percentage of this mean value. ETZ at 0.1 mM increased peak force and amplitude of R340/380 (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 Ca2+ release from the SR, R340/380 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 R340/380 (Table 2). ETZ caused a significant increase in peak force (~40%), which was accompanied by a significant increase in the amplitudes of R340/380 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 R340/380 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 R340/380 (Table 3). In some, but not all, cases, the increases in peak force were accompanied by increased amplitudes of R340/380. It appears possible that, in the presence of permeable inhibitors, more Ca2+ is released on muscle activation. The values of R340/380 under resting conditions were not influenced by the CA inhibitors.

Table 3. TTP, t50, 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>TTP, ms</th>
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<tr>
<td></td>
<td>Fura 2 signal</td>
<td>Force signal</td>
<td>Fura 2 signal</td>
<td>Force signal</td>
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<tr>
<td>L-645151 (0.1 mM)</td>
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<tr>
<td>Before</td>
<td>8</td>
<td>29 ± 9</td>
<td>127 ± 13</td>
<td>78 ± 24</td>
</tr>
<tr>
<td>During</td>
<td>12</td>
<td>39 ± 17*</td>
<td>153 ± 23*b</td>
<td>99 ± 26*a</td>
</tr>
<tr>
<td>After</td>
<td>12</td>
<td>33 ± 16*a</td>
<td>138 ± 14*a</td>
<td>79 ± 13</td>
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<td>CLZ (0.5 mM)</td>
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<tr>
<td>Before</td>
<td>12</td>
<td>18 ± 4</td>
<td>110 ± 10</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>During</td>
<td>12</td>
<td>46 ± 4*</td>
<td>129 ± 24d</td>
<td>136 ± 18*b</td>
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<tr>
<td>After</td>
<td>12</td>
<td>41 ± 2*a</td>
<td>113 ± 8*a</td>
<td>133 ± 16*a</td>
</tr>
<tr>
<td>ETZ (0.1 mM)</td>
<td></td>
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</tr>
<tr>
<td>Before</td>
<td>16</td>
<td>28 ± 13</td>
<td>157 ± 8</td>
<td>129 ± 46</td>
</tr>
<tr>
<td>During</td>
<td>24</td>
<td>39 ± 14b</td>
<td>161 ± 12a*</td>
<td>150 ± 43*b</td>
</tr>
<tr>
<td>After</td>
<td>24</td>
<td>31 ± 10b</td>
<td>151 ± 12a*</td>
<td>126 ± 22*c</td>
</tr>
<tr>
<td>ACTZ (1 mM)</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>
</tr>
<tr>
<td>During</td>
<td>16</td>
<td>32 ± 12a*</td>
<td>96 ± 6*</td>
<td>109 ± 13a*</td>
</tr>
<tr>
<td>After</td>
<td>8</td>
<td>40 ± 15a*</td>
<td>95 ± 6*</td>
<td>118 ± 20a*</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.
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 ~10–20 ms by ACTZ (Fig. 4B), whereas L-645151 and ETZ increased \( t_{50} \) of force by ~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 \( \mu \)mol 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 \( \mu \)g/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 in the presence of 0.1 mM ETZ and 50 APs of four muscle cells in the presence of 0.22 mM ETZ were 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.
This system can serve as an H\(_{\text{fl}}\)/Ca\(^{2+}\) exchanger (4, 15, 21, 23, 38, 39). In line with this, it has been reported that the CO\(_2\)-HCO\(_3\) buffering by the uncatalyzed dehydration reaction of carbonic acid will have to be accelerated by a factor of 500. Indeed, Bruns et al. (3) (44). The need for catalysis may be illustrated by the following rough calculation. Let us consider a Ca\(^{2+}\) release lasting for 30 ms and causing a depletion of intra-SR Ca\(^{2+}\) concentration by 2 mM (12). Ca\(^{2+}\) then moves across the SR membrane at a rate of

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

where [Ca\(^{2+}\)] is Ca\(^{2+}\) concentration and l represents 1 liter of intra-SR volume. If protons move into the SR during the Ca\(^{2+}\) 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 HCO\(_3\) concentration of 10 mM, and a reaction velocity constant (k\(_d\)) of 130,000 l\cdot\text{mol}^{-1}\cdot\text{s}^{-1} as derived from a CO\(_2\) hydration velocity constant of 0.1 s\(^{-1}\) and pK\(_i\) of 6.1. For further simplification, we neglect the backreaction, which yields an overestimate of the possible rate of H\(^+\) buffering, (d[H\(^+\)]/dt)

\[
\frac{d[H^+]}{dt} = k_d \times [H^+] \times [HCO_3^-] = 1.3 \times 10^6 \frac{l}{mol \cdot s} \times 10^{-7} \text{mol} \times 10^{-2} \text{mol} \times \frac{1}{l} = 0.00013 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1} \tag{2}
\]

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 Ca\(^{2+}\) efflux. Thus, even if equal fluxes of H\(^+\) and Ca\(^{2+}\) were required, which would balance 50% of the charges transferred by Ca\(^{2+}\), 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 CO\(_2\)-HCO\(_3\) 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

### DISCUSSION

Hypothesis on the Role of SR-CA for Countertransport of H\(^+\) During Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\)-ATPase directly effects an exchange of H\(^+\)/Ca\(^{2+}\) (17, 39, 46–48). All these studies have provided unambiguous evidence for a vectorial transport of H\(^+\) associated with the Ca\(^{2+}\) transport across the SR membrane. In addition, fluxes of K\(^+\), Mg\(^{2+}\), and Cl\(^-\) contribute to counterbalance the charge transfer by the Ca\(^{2+}\) (23, 35). The protons required for these proton fluxes may be generated by the CO\(_2\)-HCO\(_3\) 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 CO\(_2\)-HCO\(_3\) 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 Ca\(^{2+}\) fluxes. Ca\(^{2+}\) release lasts ~20–50 ms, and Ca\(^{2+}\) 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 Ca\(^{2+}\) release lasting for 30 ms and causing a depletion of intra-SR Ca\(^{2+}\) concentration by 2 mM (12). Ca\(^{2+}\) then moves across the SR membrane at a rate of

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

where [Ca\(^{2+}\)] is Ca\(^{2+}\) concentration and l represents 1 liter of intra-SR volume. If protons move into the SR during the Ca\(^{2+}\) 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 HCO\(_3\) concentration of 10 mM, and a reaction velocity constant (k\(_d\)) of 130,000 l\cdot\text{mol}^{-1}\cdot\text{s}^{-1} as derived from a CO\(_2\) hydration velocity constant of 0.1 s\(^{-1}\) and pK\(_i\) of 6.1. For further simplification, we neglect the backreaction, which yields an overestimate of the possible rate of H\(^+\) buffering, (d[H\(^+\)]/dt)

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\]

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 Ca\(^{2+}\) efflux. Thus, even if equal fluxes of H\(^+\) and Ca\(^{2+}\) were required, which would balance 50% of the charges transferred by Ca\(^{2+}\), 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 CO\(_2\)-HCO\(_3\) 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

<table>
<thead>
<tr>
<th>Activity</th>
<th>SOL</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin-ATPase, %</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase, %</td>
<td>96±7(8)</td>
<td>85±8(3)</td>
</tr>
<tr>
<td>Na(^{+})-K(^{-})-ATPase, %</td>
<td>Control 100 100 96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ETZ (0.1 mM) 101±3(4) 95±16(3)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.D.; number of determinations is shown in parentheses. In the case of myosin-ATPase, significance of differences between control values and values determined in the presence of CA inhibitor were estimated by Student’s paired t-test and in the cases of Ca\(^{2+}\)- and Na\(^{+}\)-K\(^{-}\)-ATPases by Student’s unpaired t-test. Control values and values in the presence of CA inhibitors are not significantly different.
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 CAAIII of SOL. Because they exerted their effects in SOL as well as in EDL, which has no CAAIII but the same two membrane-bound CA forms as SOL, CAAIII 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 counterions 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 \( K^+ \) and \( Cl^- \) was prevented by the replacement of \( K^+ \) by choline and the replacement of \( Cl^- \) by gluconate, respectively. However, their experiments were conducted in the absence of \( CO_2 \). 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 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.

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**REFERENCES**


