Effect of lactate on depolarization-induced Ca\(^{2+}\) release in mechanically skinned skeletal muscle fibers

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Effect of lactate on depolarization-induced Ca\(^{2+}\) release in mechanically skinned skeletal muscle fibers. Am. J. Physiol. Cell Physiol. 278: C517–C525, 2000.—It is unclear whether accumulation of lactate in skeletal muscle fibers during intense activity contributes to muscle fatigue. Using mechanically skinned fibers from rat and toad muscle, we were able to examine the effect of L(+)-lactate on excitation-contraction coupling independently of other metabolic changes. We investigated the effects of lactate on the contractile apparatus, caffeine-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum, and depolarization-induced Ca\(^{2+}\) release. Lactate (15 or 30 mM) had only a small inhibitory effect directly on the contractile apparatus and caused appreciable (20–35%) inhibition of caffeine-induced Ca\(^{2+}\) release, seemingly by a direct effect on the Ca\(^{2+}\) release channels. However, 15 mM lactate had no detectable effect on Ca\(^{2+}\) release when it was triggered by the normal voltage sensor mechanism, and 30 mM lactate reduced such release by only <10%. These results indicate that lactate has only a relatively small inhibitory effect on normal excitation-contraction coupling, indicating that lactate accumulation per se is not a major factor in muscle fatigue.

excitation-contraction coupling; fatigue; exercise; calcium release channel; ryanodine receptor

WHEN SKELETAL MUSCLE undergoes intense activity, force production eventually declines; this is commonly referred to as fatigue (1, 15, 39). Repeated short contractions induce a type of fatigue with relatively slow onset and recovery (i.e., over many minutes), which is thought to be due to metabolic changes within the muscle fibers (1, 15, 18, 35). With vigorous exercise, lactate concentrations in fast-twitch skeletal muscles can reach >30 mM (20, 22, 30, 34). In many circumstances, there is a strong inverse correlation between lactate concentration and force production during the onset of fatigue and recovery from fatigue (15, 22). It was thought that this correlation was actually due to the concomitant increase in H\(^+\) concentration ([H\(^+\)]) occurring with lactic acid production rather than to lactate per se (15). However, recent work indicates that an increase in [H\(^+\)] is probably not the primary factor responsible for reductions in Ca\(^{2+}\) release and maximum Ca\(^{2+}\)-activated force in fatigue (5, 7, 27). Moreover, other recent studies indicate that lactate itself may have direct effects on force production and Ca\(^{2+}\) release. For example, a 15% reduction in force production was observed when arterial infusion of L(+)-lactate was used to raise muscle lactate concentration from ~8 to 12 mM without any accompanying pH change (19). Furthermore, experiments on chemically skinned muscle fibers show that, with the pH maintained at 7.0, 20 mM lactate in the cytoplasm causes a small (~5%) reduction in maximum Ca\(^{2+}\)-activated force (2). Finally, it has been found that the presence of 10–20 mM lactate inhibits Ca\(^{2+}\)- and caffeine-activated Ca\(^{2+}\) release and ryanodine binding in sarcoplasmic reticulum (SR) vesicles and activation of isolated ryanodine receptor (RyR) Ca\(^{2+}\) release channels, all by ~30–70% (11, 12).

Nevertheless, it is unclear whether lactate inhibits Ca\(^{2+}\) release in functioning muscle fibers. As pointed out by Favero and colleagues (12), the results with SR vesicles and isolated release channels do not show whether lactate interferes with the normal excitation-contraction (E-C) coupling mechanism in muscle, which involves activation of Ca\(^{2+}\) release channels by the voltage sensor/dihydropyridine receptors in the adjacent transverse tubular (T) system (29). Here, we examine this question by investigating the effect of cytoplasmic lactate on Ca\(^{2+}\) release in mechanically skinned muscle fibers in which the normal voltage sensor coupling mechanism is operating (13, 24, 25, 27, 38). We show that lactate appreciably inhibits the ability of caffeine to activate Ca\(^{2+}\) release in muscle fibers but that voltage sensor activation of Ca\(^{2+}\) release is inhibited to only a comparatively small extent (<10%), even at 30 mM lactate. This indicates that lactate accumulation is probably not a major factor in muscle fatigue, even in fast-twitch fibers.

METHODS

Skinned fibers. Mechanically skinned fibers were obtained and used as described previously (25, 27). Briefly, Long-Evans hooded rats were anesthetized with halothane (2% vol/vol) in a bell jar and killed by asphyxiation, and then the extensor digitorum longus (EDL) muscles were removed. Cane toads (Bufo marinus) were stunned by a blow to the head and killed by pithing, and the iliofibularis muscles were removed. Single muscle fibers were mechanically skinned under paraffin oil, and a segment was attached to a force transducer (model AME875, Hormen) at 120% of resting length. The skinned fiber (30–50 µm diameter) was placed within a 2-ml Perspex bath containing a potassium hexamethylene diamine tetraacetate (K-HDTA) solution (see below) for 2 min, then the bath was rapidly lowered and a bath with a different solution was substituted to stimulate the fiber. In the experiments on depolarization-induced Ca\(^{2+}\) release, fibers were used with...
their endogenous level of SR Ca$^{2+}$ and were not additionally loaded. All experiments were performed at room temperature (23 ± 2°C).

Solutions. All chemicals were obtained from Sigma Chemical (St. Louis, MO), unless specified otherwise. The standard K-HDTA solutions used for rat and toad fibers contained (mM) 117 (toad) or 126 (rat) K$^+$, 37 Na$^+$, 50 HDTA$^-$ (Fluka, Buchs, Switzerland), 8 total ATP, 8.6 total Mg$^{2+}$, 10 phosphocreatine, 0.05 total EGTA, 60 (toad) or 90 (rat) HEPES, and 1 N$_2$O, with pH adjusted to 7.10 ± 0.01 and pCa to 7.0. Skinned fibers were depolarized by substitution of an Na-HDTA solution, which was identical to the corresponding K-HDTA solution, except all K$^+$ was replaced with Na$^+$. All solutions had an osmolality of 255 ± 5 (toad) or 295 ± 5 mosmol/kg (rat) and a free Mg$^{2+}$ concentration ([Mg$^{2+}$]) of 1 mM, with the assumption of apparent Mg$^{2+}$ affinity constants of 6.9 × 10$^3$ M$^{-1}$ for ATP, 8 M$^{-1}$ for HDTA, and 15 M$^{-1}$ for phosphocreatine (14, 37). Corresponding solutions with 15 or 30 mM l(+)-lactate were made by mixing the above solutions with an appropriate amount of a corresponding lactate stock solution in which all HDTA (50 mM, K$_2$ or Na$_2$) was replaced with 120 mM lactate (K$_2$ or Na$_2$). The other constituents in the lactate stock solution were unchanged, except the total Mg$^{2+}$ (added as MgO) was increased by an additional 0.7 mM to maintain the free [Mg$^{2+}$] at 1 mM on the basis of an apparent Mg$^{2+}$ affinity constant of 9 M$^{-1}$ for lactate (32). The substitution of a divalent anion (HDTA) with a monovalent anion (lactate) meant that the osmolarity was slightly increased (∼8%) and the ionic strength slightly decreased (∼3.2%) in solutions for rat fibers at the highest lactate concentration used (30 mM).

The net effect of these changes on maximum Ca$^{2+}$-activated force and depolarization-induced force responses would have been very small (∼2% increase) (28) and, furthermore, should be of even less importance when the relative changes of the two parameters are compared (e.g., the percent decline in both parameters in the presence of lactate). In all solutions used for depolarization-induced Ca$^{2+}$ release, total EGTA concentration was 50 µM and pCa was 7.0, as measured with a Ca$^{2+}$-sensitive electrode (Orion Research, Boston, MA).

Solutions used for investigating caffeine-induced Ca$^{2+}$ release (i.e., “7 mM caffeine” solution in Fig. 1) were identical to the above solutions (i.e., K-HDTA, with or without lactate, 50 µM total EGTA, pCa 7.0) except for the addition of 7 mM caffeine. Where required, the SR was totally depleted of releasable Ca$^{2+}$ with use of a K-HDTA solution (“total release” solution) with 30 mM caffeine and 0.05 mM free Mg$^{2+}$ (2.15 total Mg$^{2+}$) and 0.5 mM EGTA (pCa 8) to chelate released Ca$^{2+}$ (3, 17). When the SR was depleted in this way, each fiber was first preequilibrated for 10 s in the standard K-HDTA solution with 0.5 mM EGTA (pCa 8; “preequilibration” solution) to raise the EGTA concentration within the fiber before exposure to the total release solution. After total release, the fiber was washed for 30 s in a solution identical to the preequilibration solution, and then the SR was reloaded with Ca$^{2+}$ in a similar solution with 1 mM total EGTA at pCa 6.7 (“load” solution). Where indicated, all solutions were made with Na-HDTA instead of K-HDTA. In the experiments indicated, 20 mM Cl$^-$ was present in every solution (replacing 10 mM of the HDTA), and all solutions were made with Na-HDTA. Where required, 9-anthracene carboxylic acid (9-AC), a blocker of Cl$^-$ channels of surface and tubular membranes, was added from a 10 mM aqueous stock with NaOH (pH 7.1) to give a final concentration of 100 µM. In the depolarization and caffeine experiments, maximum Ca$^{2+}$-activated force was determined using a solution (“Max”) similar to standard K-HDTA solution, but with 50 mM Ca-EGTA (pCa 4.5) replacing all HDTA, and fibers were subsequently relaxed in a similar solution with 50 mM free EGTA (pCa >10). The total Mg$^{2+}$ was varied to keep the free [Mg$^{2+}$] at 1 mM (37).

Contractile apparatus measurements. In the experiments on the properties of the contractile apparatus, the skinned fiber was exposed to a sequence of solutions, each with 10 mM total EGTA and progressively higher Ca$^{2+}$ concentration ([Ca$^{2+}$]), made by appropriate mixture of the 50 mM Ca-EGTA and 50 mM free EGTA solutions, together with four times the volume of the standard K-HDTA solution or the corresponding K-HDTA solution with lactate (to give a final lactate concentration of 15 or 30 mM). Force measurements in the presence of lactate were always bracketed by similar sequences in the absence of lactate. The force produced by the fiber at each pCa under a given condition was expressed as a percentage of the corresponding maximum Ca$^{2+}$-activated force and plotted against pCa. The scientific analysis program
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GraphPad Prism (GraphPad Software, San Diego, CA) was used to fit Hill curves to the force-pCa data obtained under each condition.

Caffeine-induced Ca\(^{2+}\) release experiments. The Ca\(^{2+}\) content of the SR was assayed (Fig. 1) by preequilibrating the fiber for 10 s in the standard K-HDTA solution with 0.5 mM EGTA (pCa 8; preequilibration solution) and then totally depleting the SR in the 30 mM caffeine-low [Mg\(^{2+}\)] solution with 0.5 mM EGTA (pCa 8; total release solution) (3). It is necessary to have 0.5 mM EGTA present in the 30 mM caffeine-low [Mg\(^{2+}\)] solution to rapidly chelate the released Ca\(^{2+}\) in order that the resulting force response can be related to the amount of Ca\(^{2+}\) released. The area under the force response on first depletion of the SR was indicative of the level of SR Ca\(^{2+}\) present endogenously. (Note that the fiber was skinned under oil and could neither gain nor lose Ca\(^{2+}\) during the skinning procedure.) The fiber was left in the total release solution for 2 min to ensure complete Ca\(^{2+}\) depletion (17), washed for 30 s, and then reloaded for a set time (e.g., 30 or 40 s) in the load solution. The fiber was then immediately depleted again with the above procedure (preequilibration and 30 mM caffeine-low [Mg\(^{2+}\)] total release in Fig. 1) or was first equilibrated for 15 s in a weakly Ca\(^{2+}\)-buffered K-HDTA solution (pCa 7.0, 50 µM EGTA) with or without lactate, exposed to the same solution with 7 mM caffeine for 15 s, and then totally depleted of Ca\(^{2+}\) (Fig. 1). Additional caffeine release experiments were carried out with all solutions containing Na-HDTA (i.e., zero K\(^{+}\)) to keep the T system chronically depolarized and the voltage sensors inactivated.

Force traces and presentation and analysis of results. In all traces showing depolarization-induced force responses, unless indicated otherwise, the skinned muscle fiber was bathed in the standard K-HDTA solution (pCa 7.0, 50 µM EGTA) and depolarized in the matching Na\(^{+}\) solution. Values are means ± SE, and n indicates the number of fibers examined. Statistical significance was determined with Student's t-test (1-tailed, paired or unpaired, as appropriate), with mean values considered significantly different if P < 0.05.

RESULTS

Effect of lactate on the contractile apparatus in rat EDL fibers. We first examined the effect of lactate on the properties of the contractile apparatus in the skinned fibers. Each rat EDL fiber was exposed to sequences of solutions, with and without lactate, in which the free [Ca\(^{2+}\)] was heavily buffered (10 mM total EGTA) at various levels. Force was plotted against pCa, and Hill curves were fitted for each fiber individually. In the absence of lactate, the pCa producing half-maximal force (pCa\(_{50}\)) had a mean of 5.97 ± 0.03, with a mean Hill coefficient of 4.6 ± 0.4 (n = 17). Maximum Ca\(^{2+}\)-activated force was reduced in the presence of 15 and 30 mM lactate to 97.0 ± 1.3% (n = 7, P < 0.05) and 97.9 ± 0.8% (n = 16, P < 0.05), respectively, of the average of the bracketing control level in the absence of lactate (HDTA concentration was reduced when lactate was added; see METHODS). [Normalizing data to the average of the bracketing controls shows the effect of lactate independently of the small decline (2–3%) in maximum force that occurs with each successive sequence irrespective of exposure to lactate. The maximum for the control sequence after lactate exposure in the above experiments was 94.8 ± 0.9% of the initial control (n = 33).] The Ca\(^{2+}\) sensitivity of the contractile apparatus, however, was not significantly altered at either concentration of lactate [mean change in pCa\(_{50}\) = 0.008 ± 0.005 (n = 7) and 0.006 ± 0.006 (n = 12) and mean change in Hill coefficient = 0.10 ± 0.06 and 0.09 ± 0.08 in 15 and 30 mM lactate, respectively]. Thus the effect of lactate on the contractile apparatus was very small.

Caffeine-induced Ca\(^{2+}\) release. Lactate inhibits caffeine-induced Ca\(^{2+}\) release in isolated SR vesicles (11, 12). We wished to examine whether lactate showed a similar inhibitory action on caffeine-induced Ca\(^{2+}\) release in skinned fibers with the normal voltage sensor coupling mechanism still intact and with the cytoplasmic [Mg\(^{2+}\)] and SR luminal [Ca\(^{2+}\)] at close to physiological levels. In these experiments, each skinned fiber was subjected to repeated cycles in which the SR was fully depleted of Ca\(^{2+}\) (by exposure to a solution with 30 mM caffeine-low [Mg\(^{2+}\)] and 0.5 mM EGTA, pCa 8; see METHODS) and then reloaded with Ca\(^{2+}\) to particular levels. The [Mg\(^{2+}\)] was lowered in the depleting solution from the physiological level of 1 mM to 0.05 mM, because this makes caffeine far more effective at eliciting total Ca\(^{2+}\) release in mammalian fibers (17). Such load-release cycles gave highly reproducible results in each fiber. As found previously (3, 10), the "area" (i.e., time integral) of the force response produced on full depletion of the SR (e.g., 1st total release in Fig. 1) was approximately linearly related to the loading time (not shown), which meant that it could be used as an indicator of the amount of Ca\(^{2+}\) present in the SR. Because fibers were skinned under oil (see METHODS), the amount of Ca\(^{2+}\) present in the SR endogenously could be ascertained, and then the SR could be repeatedly depleted of Ca\(^{2+}\) and reloaded to that level or some other level as desired.

To test the effect of lactate on caffeine-induced Ca\(^{2+}\) release, the SR was loaded with Ca\(^{2+}\) to a particular level, and then the fiber was equilibrated for 15 s in a weakly Ca\(^{2+}\)-buffered solution (50 µM EGTA) at pCa 7.0 with 1 mM free Mg\(^{2+}\) and then exposed to the same solution with 7 mM caffeine (e.g., Fig. 1). Provided that the SR was loaded above some threshold level (between ~25 and 50% of the endogenous level), exposure to the 7 mM caffeine solution invariably elicited Ca\(^{2+}\) release and a force response in every EDL fiber. After a total of 15 s of exposure, the caffeine was washed out and the SR was fully depleted of Ca\(^{2+}\) with the 30 mM caffeine-low [Mg\(^{2+}\)] solution (e.g., 2nd total release in Fig. 1) to ascertain how much Ca\(^{2+}\) had remained in the SR after the 15-s exposure to 7 mM caffeine. The entire procedure was then repeated with lactate in the 7 mM caffeine solution (and during the preceding 15-s equilibration) and then again without lactate. Because there was very little Ca\(^{2+}\) buffer in the 7 mM caffeine solutions, the area of the resulting force response is not simply related to the amount of Ca\(^{2+}\) released from the SR and cannot be directly compared with the area of the force response to the total release solution (30 mM caffeine-low [Mg\(^{2+}\)]-0.5 mM EGTA). It is likely that, during the Ca\(^{2+}\) release in the 7 mM caffeine solution, a substantial amount of Ca\(^{2+}\) was simultaneously being
taken back up by the SR. Consequently, the reduction in the area of the force response on subsequent total release indicates the net loss of Ca\(^2+\) from the fiber over the period in 7 mM caffeine. The finding that the force response to 7 mM caffeine ceased even though the SR evidently still contained substantial Ca\(^2+\) (Fig. 1) appears broadly consistent with the observation that the SR had to be loaded to some minimum level to induce any Ca\(^2+\) release to 7 mM caffeine (see above), with both results possibly suggesting that the SR loading level regulates the responsiveness of the Ca\(^2+\) release channels to caffeine. More studies need to be done on the detailed characteristics and basis of this regulation.

When the SR was loaded with Ca\(^2+\) at approximately the level present endogenously, the force response to 7 mM caffeine was significantly smaller and slower rising in the presence of 30 mM lactate than in the absence of lactate (reduction to 75 and 64%, respectively; Table 1). Less marked effects were observed in the presence of 15 mM lactate (Table 1). These reduced force responses cannot be explained by effects of lactate on the contractile apparatus (see above) and imply that caffeine-induced Ca\(^2+\) release was inhibited to some extent in lactate. The reduction in Ca\(^2+\) release in lactate was further apparent from the extent of depletion of SR Ca\(^2+\) in these experiments. In the absence of lactate, exposure to the 7 mM caffeine solution resulted in a decrease in the area of the force response on subsequent emptying of the SR to 79 ± 9% (n = 11) of that found without any exposure to 7 mM caffeine (cf. 1st and 2nd total release in Fig. 1). In other words, there had been a net loss of ~21% of the total SR Ca\(^2+\) from the fiber over the equilibration and 7 mM caffeine exposure periods in the absence of lactate. When the same procedure was performed with lactate, the force response on emptying the SR was significantly larger than that in the absence of lactate (110 ± 3% of non-lactate case, n = 11, P < 0.05, Student’s paired t-test; cf. 3rd total release with 2nd and 4th in Fig. 1), indicating that there had been less net Ca\(^2+\) loss from the fiber in 7 mM caffeine when lactate was present, which in turn suggests that there had been less Ca\(^2+\) release. All the above experiments were performed using K-HDTA solutions, with the T system polarized and the voltage sensors activatable (see below). Virtually identical results were also obtained (not shown) when all solutions were made with Na-HDTA (see METHODS), so that the T system was kept depolarized and the voltage sensors were inactivated.

The above reduction in caffeine-induced Ca\(^2+\) release is consistent with previous reports that lactate directly inhibits activation of the RyR Ca\(^2+\) release channel (11, 12). Nevertheless, it could also be argued that the results found here are due to a potential difference developing across the SR when lactate is added to the cytoplasmic side (see DISCUSSION). To test this, we performed additional experiments with 20 mM Cl\(^-\) in all the solutions (added ~15 min before the effect of lactate was examined), because the highly permeable Cl\(^-\) should act as an electrical shunt, greatly reducing any effect of lactate on the SR potential. It was found that the inhibitory effect of lactate on caffeine-induced Ca\(^2+\) release was not significantly altered when Cl\(^-\) was present (Table 1), suggesting that lactate exerted its effects directly on the Ca\(^2+\) release channel rather than via some effect on SR potential.

Lastly, we tested whether the level of SR loading influenced the inhibitory effect of lactate on caffeine-induced Ca\(^2+\) release. As might be expected, it was found that when the SR was loaded with only approximately one-half as much Ca\(^2+\) as present in fibers endogenously, the force response to 7 mM caffeine was reduced compared with the case where the SR was loaded at the endogenous level (peak force 31 ± 6% (n = 10) and 60 ± 5% (n = 16) of maximum Ca\(^2+\)-activated force, respectively). It was further found that the presence of lactate caused a greater relative reduction in the peak force response to 7 mM caffeine when the SR was loaded below the level present endogenously (Table 1). Thus the extent of the inhibitory effect of lactate on caffeine-induced Ca\(^2+\) release depends on the level of Ca\(^2+\) loading in the SR.

Depolarization-induced responses in skinned fibers. As described previously with rat and toad muscle (25, 27), the E-C coupling mechanism remains functional after muscle fibers are mechanically skinned under paraffin oil. Importantly, the fibers also retain the endogenous level of SR Ca\(^2+\) and require no additional loading. On skinning, the T system seals over (24) and repolarizes if the fiber segment is bathed in a high [K\(^+\)] solution and can then be rapidly depolarized by substitution of a solution in which all K\(^+\) is replaced with Na\(^+\) (see METHODS). Such stimulation elicits a rapidly rising force response that lasts for only 2–4 s, owing to inactivation of the voltage sensors and repump of released Ca\(^2+\) into the SR (25, 26) (Fig. 2). The voltage dependence of activation and inactivation and modulation by pharmacological agents (e.g., D600) and Cl\(^-\) (9, 25, 26, 27) strongly indicate that the responses in these skinned fibers are controlled by the same basic E-C coupling mechanism as in intact fibers. Although the
precise membrane potentials are not known, the above characteristics indicate that the T system of EDL fibers is polarized to more negative levels than −80 mV in the standard 126 mM K+ solution, and this is largely or fully dissipated by Na+ substitution (9).

Depolarization-induced force responses were not significantly altered when 15 mM lactate was present in the cytoplasm (with 30–45 s of equilibration in lactate in the polarizing K+ solution; Fig. 2A); the mean peak size of the response was 96.3 ± 2.5% of the preceding control response in the absence of lactate (Table 2). This value is, in fact, virtually identical to the reduction in maximum Ca2+-activated force under the same conditions (97.0 ± 1.3% see above), showing that the relative size of the depolarization-induced force response in 15 mM lactate was fully explained by the small effect of lactate directly on the contractile apparatus. Given that we previously showed that any decrease in the amount of Ca2+ released by depolarization can be readily detected by a reduction in the peak size and area of the force response in rat EDL fibers (31), the results here indicate that 15 mM lactate does not appreciably inhibit depolarization-induced Ca2+ release. In the presence of 30 mM lactate, the peak and duration (half-width) of the depolarization-induced force response were reduced by a small, although significant, amount (to 90.8 and 95.7% of that for the preceding control case, respectively, n = 39; Table 2, Fig. 2B). The degree of inhibition with lactate was similar across all fibers, irrespective of the relative size of the control response (as a percentage of maximum force). If the lactate was washed out after a single depolarization (i.e., ~35–50 s of total exposure), the depolarization-induced force response returned to a level not significantly different from the original control (e.g., Fig. 2B; peak after washout = 96.4 ± 3.2% (n = 17); peak response in lactate in this same fiber sample was 90.2 ± 2.6% of control, and response increased significantly after washout (P < 0.05 by paired t-test)). The reduction of the response in 30 mM lactate could not be accounted for by direct effects on the contractile apparatus (maximum Ca2+-activated force reduced to 97.9% of control, with no change in Ca2+ sensitivity; see above), demonstrating that 30 mM lactate inhibited the ability of depolarization to induce Ca2+ release to some extent. We also found that when 100 µM 9-AC was present to block the Cl− channels in the T system (9), 30 mM lactate still had a very similar effect (Table 2).

It was also found that 30 mM lactate significantly reduced the peak size of the depolarization-induced force responses in skinned fibers from the iliofibularis muscle of the toad (to 92.1% of preceding control, n = 10), although the half-width of the response actually increased (to 108.0%, Table 2). This concentration of lactate had no significant effect on the maximum Ca2+-activated force (99.7 ± 1.0% of control, n = 6) or the Ca2+ sensitivity of the contractile apparatus (mean change in pCa25 = 0.034 ± 0.032, mean change in Hill coefficient = 0.80 ± 0.80, n = 5). When lactate was washed out after only a single depolarization (~40–60 s of total lactate exposure), the force response recovered in five of the six cases (to 99.2 ± 5.8% of initial control, with the response in 30 mM lactate being 92.4 ± 4.3% of initial control in those same 5 fibers), but in the remaining case the response was greatly reduced (to ~14%).

Effect of prolonged exposure to lactate. When the total duration of the lactate exposure was increased to 3–5 min, the force response to depolarization after washout of lactate was actually reduced considerably, rather than returned to the original control level (e.g., Fig. 3A). The mean depolarization-induced force response on washout of lactate under such circumstances was 76.2 ± 4.9% of the original control response in rat EDL fibers (n = 23) and 31.7 ± 10.4% in toad iliofibularis fibers (n = 10). This reduction in the response was not simply due to fiber “rundown,” because it was largely reversed if the fiber was returned to the lactate solution.

Table 2. Effect of lactate on depolarization-induced force responses in skinned fibers

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>n</th>
<th>Peak Size, %</th>
<th>Half-Width, %</th>
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<tr>
<td>Rat EDL</td>
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<tr>
<td>Lactate (15 mM)</td>
<td>7</td>
<td>96.3 ± 2.5</td>
<td>102.5 ± 1.9</td>
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<tr>
<td>Lactate (30 mM)</td>
<td>39</td>
<td>90.8 ± 2.0*</td>
<td>95.7 ± 1.8*</td>
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<tr>
<td>Lactate (30 mM) + 9-AC</td>
<td>9</td>
<td>88.2 ± 2.5*</td>
<td>98.3 ± 1.6</td>
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<tr>
<td>Toad iliofibularis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate (30 mM)</td>
<td>10</td>
<td>92.1 ± 2.7*</td>
<td>108.0 ± 3.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE of peak size and half-width of force response to depolarization in the presence of lactate (with 30–45 s of equilibration) expressed as percentage of preceding values in the absence of lactate; n, number of fibers. Rat EDL fibers were also examined in the presence of 100 µM 9-anthracene carboxylic acid (9-AC). *Significantly different from 100% (i.e., value in absence of lactate).
(e.g., end of Fig. 3A). Furthermore, the reduction in the response was simply not due to the Na$^+$ substitution becoming less effective at fully depolarizing the T system, because depolarization by ChCl substitution was similarly reduced in effectiveness (not shown), even though such substitution should be a very potent depolarizing stimulus (9, 25, 27). It was also found that, after prolonged exposure to lactate, fibers frequently gave a spontaneous force response ~10 s after the lactate was washed out (Fig. 3B). This was observed in 6 of 10 toad fibers and 7 of 23 rat fibers, with the response lasting from ~2 to 30 s and the peak size ranging from 1 to 34% (mean 9 ± 5%) of the control depolarization-induced response in the toad fibers and from 1 to 16% (mean 6 ± 3%) in the rat fibers. This force response was due to Ca$^{2+}$ release from the SR, and the resulting partial depletion of SR Ca$^{2+}$ in these cases probably exacerbated the reduction in the response to the following depolarization (Fig. 3B).

The inhibition of the depolarization-induced force response and the spontaneous Ca$^{2+}$ release occurring after washout of lactate are probably due in some way to progressive accumulation of lactate in the T system during the prolonged exposure. Spontaneous force responses were never observed 1) when lactate was washed out with the T system kept chronically depolarized with use of Na$^+$-based solutions, although they occurred in the same fibers if the T system was repolarized in the K$^+$-based solutions, and 2) in fibers in which depolarization-induced responses could not be elicited, which occurs in <10% of all fibers and is probably due to chronic depolarization of the T system resulting from incomplete sealing on skinning. Lactate is transported out of muscle fibers in association with H$^+$ by a specific carrier mechanism, and also probably by free diffusion (4, 21), and presumably this occurs to some extent across the T system membrane. The lactate transporter in skeletal muscle is reportedly half-maximally inhibited by 3 µM quercetin (21). Nevertheless, we were unable to prevent the inhibitory effect or spontaneous responses on lactate washout with 5–15 µM quercetin. Furthermore, blocking the major anion channel in the T system with 100 µM 9-AC (9) was also ineffective at preventing the effects.

DISCUSSION

In this study, mechanically skinned skeletal muscle fibers were used to investigate the effect of L(+)-lactate on the sequence of E-C coupling from depolarization to muscle contraction, as well as on individual aspects within that sequence. With this preparation we were able to rapidly apply and wash out lactate in the cytoplasm and quantify its effects on the contractile apparatus and on SR Ca$^{2+}$ release evoked directly with caffeine or by the physiological coupling mechanism involving the voltage sensors in the T system. Lactic acid is virtually fully dissociated into lactate and H$^+$ at physiological pH, and here we held the pH constant at 7.1 to examine the effects of lactate alone.

Effects of lactate on contractile apparatus and caffeine-induced Ca$^{2+}$ release. Under the conditions used here, lactate had only very small effects on the properties of the contractile apparatus. In rat EDL fibers, Ca$^{2+}$ sensitivity was not significantly affected, and the maximum Ca$^{2+}$-activated force in 15 and 30 mM lactate only decreased to 97.0 and 97.9%, respectively, of that in the absence of lactate. These values are quite comparable to those obtained previously in chemically skinned psoas fibers from rabbit (2), where, depending on the exact ion substitution used, the maximum Ca$^{2+}$-activated force was 92.3–97.8% of control in 15 mM lactate and 95.9–101.6% of control in 30 mM lactate.

We further found that lactate inhibited caffeine-induced Ca$^{2+}$ release in the skinned EDL fibers in a concentration-dependent manner. The relative effects

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Fig. 3. Effect of washing out lactate after prolonged exposure. A: inhibitory effect of 30 mM lactate on depolarization-induced responses could be fully reversed after a relatively brief exposure to lactate (~30 s, 1st exposure). However, after a much longer exposure (~3 min, middle), washout of lactate actually caused a reduction in depolarization-induced response, with response recovering on reapplication of lactate. B: in another EDL fiber, washout of lactate after a 3-min exposure elicited a spontaneous force response lasting >20 s and depressed response to depolarization. In A and B, maximum Ca$^{2+}$-activated force was determined with 50 mM Ca-EGTA (pCa 4.5; Max). Time scale, 2 s during depolarizations and 30 s elsewhere.
were slightly smaller than those found previously with SR vesicles and isolated RyR Ca\(^{2+}\) release channels (11, 12). In the present experiments, the coupling mechanism between the Ca\(^{2+}\) release channels and the voltage sensors remained intact and functional, and the [Mg\(^{2+}\)] and ATP concentration were at physiological levels. We found that the peak and the rate of rise of the force response to caffeine were reduced in the presence of lactate (Fig. 1) but that the effects were less marked when the SR was loaded with Ca\(^{2+}\) at the physiological level than when the SR was partially depleted of Ca\(^{2+}\) (Table 1). This shows that the modulatory effects of various ligands on the Ca\(^{2+}\) release channels depend on the exact conditions used, and this highlights the importance of keeping conditions as close to physiological as possible. At physiological SR loading, 30 mM lactate caused a 25% reduction in the peak of the force response to caffeine and a 36% reduction in the rate of rise of force (Table 1), with the latter value possibly being the better estimate of the relative reduction in rate of Ca\(^{2+}\) release in lactate.

This inhibitory effect of lactate on caffeine-induced Ca\(^{2+}\) release is probably due to a direct action of lactate on the RyR Ca\(^{2+}\) release channel, as evidently occurs in isolated RyRs (12). Nevertheless, we cannot entirely exclude the possibility that lactate exerted its effects more indirectly in the experiments here. We previously showed that addition of Cl\(^{-}\) to the cytoplasm partially inhibits caffeine-induced Ca\(^{2+}\) release in skinned fibers, and one possible explanation of this was that the presence of the highly permeable Cl\(^{-}\) in the cytoplasm induced a potential difference across the SR (lumen becoming negative), which in some way inhibited channel opening and/or Ca\(^{2+}\) efflux (8). Consequently, inasmuch as the SR is probably highly permeable to lactate ions (given the permeability of similar compounds) (23), it is possible that addition of lactate also may have inhibited Ca\(^{2+}\) release by inducing a potential difference across the SR. However, we believe this is unlikely, because lactate still caused similar inhibition of caffeine-induced Ca\(^{2+}\) release even when 20 mM Cl\(^{-}\) was present in all solutions (Table 1), and as such the Cl\(^{-}\) should have equilibrated across the SR and effectively shunted much of whatever effect lactate had on SR potential. Specifically, even if lactate were as permeable to the SR as Cl\(^{-}\) (and hence 50 times more permeable than K\(^{+}\)) (23), the Goldman-Hodgkin-Katz equation (Ref. 8) indicates that the maximum potential change that would occur across the SR on addition of 30 mM lactate to the cytoplasm would be ~60 mV in the absence of Cl\(^{-}\) and ~20 mV in the presence of Cl\(^{-}\). Because lactate is probably not as permeable as Cl\(^{-}\) and also should have equilibrated across the SR to some extent before the effect of caffeine was tested, these values are almost certainly overestimates. Thus the lack of effect of Cl\(^{-}\) on the responses in lactate (Table 1) make it unlikely that lactate is inhibiting Ca\(^{2+}\) release via such a mechanism. Instead, it seems most probable that lactate directly antagonized activation of the Ca\(^{2+}\) release channel.

Effect of lactate on depolarization-induced Ca\(^{2+}\) release. Although lactate evidently inhibits caffeine-induced Ca\(^{2+}\) release, this does not necessarily mean that it has a similar inhibitory effect on Ca\(^{2+}\) release evoked by the normal voltage sensor coupling mechanism. This latter mechanism is considerably more efficacious at inducing release and is also known to be able to largely overcome or bypass the strong inhibitory effects of other ligands, such as Mg\(^{2+}\) and H\(^{+}\), on the release channels (27). Importantly, we found here that the presence of 15 mM lactate had no significant effect on depolarization-induced force responses in EDL fibers and that 30 mM lactate caused only a small decrease in the response (~9% in peak size and ~4% in half-width; Table 2). Only about one-fifth of the reduction in the peak force response observed with 30 mM lactate was due to the direct effects of lactate on the contractile apparatus (i.e., ~2% decline; see above). We previously used a fiber-lysing technique to quantify the relationship between SR Ca\(^{2+}\) content and the size of the depolarization-induced force response and showed that even small decreases in the total amount of Ca\(^{2+}\) release in rat EDL fibers produce a measurable reduction in the peak of the force response to depolarization and a considerably larger reduction in the area of the response (31). From this we can conclude that 30 mM lactate caused <10% reduction in the total amount of Ca\(^{2+}\) release by depolarization in rat EDL fibers. Similar consideration of the data obtained with toad iliofibularis muscle fibers (Table 2) suggests that the decrease is even smaller in such fibers (0–5%). Here we are assessing the total amount of Ca\(^{2+}\) released by a depolarization, not the rate of release, and we would not detect a change in the initial rate of release unless it was substantial. Thus it is still possible that lactate reduces the initial rate of depolarization-induced Ca\(^{2+}\) release and that this might mean that the twitch response of an intact fiber would be reduced. Nevertheless, the contractures found here should be quite comparable to tetanic responses in a muscle fiber and so should indicate the normal physiological response to lactate.

Thus the presence of lactate in the cytoplasm had a far smaller inhibitory effect on depolarization-induced Ca\(^{2+}\) release than on caffeine-induced Ca\(^{2+}\) release. This might be the result of voltage sensor stimulation largely overcoming or bypassing the inhibitory effects of lactate on the Ca\(^{2+}\) release channel or might simply be due to lactate only exerting a mild degree of inhibition on the release channels, because this would appear proportionately more important with a comparatively weakly activating stimulus, such as caffeine, than with the far more potent stimulating effect of the voltage sensors. We cannot exclude the possibility that lactate simply interfered with caffeine binding to the Ca\(^{2+}\) release channels, but there is no obvious similarity in chemical structure of lactate and caffeine, and also such an explanation would not account for the ability of lactate to inhibit both Ca\(^{2+}\) - and caffeine-induced Ca\(^{2+}\) release in SR vesicles (11, 12). In line with our conclusions on caffeine-induced Ca\(^{2+}\) release (see above), we
assume that the small inhibitory effect of lactate on depolarization-induced Ca\textsuperscript{2+} release is not due to lactate addition causing a transient change in SR potential. If we are wrong in this assumption, lactate would not exert even this small inhibitory effect on depolarization-induced Ca\textsuperscript{2+} release in vivo, because lactate would equilibrate across the SR as its concentration slowly rose in the cytoplasm.

Our experiments with longer exposures to lactate (Fig. 3) indicated that lactate also gradually accumulated in the sealed T system of the skinned fibers. This was concluded from the fact that when lactate had been present in the cytoplasm for 3–5 min, removal of the lactate actually inhibited the response to depolarization, rather than restored it, and also frequently induced Ca\textsuperscript{2+} release. Because this latter effect only occurred when the T system was depolarized and the voltage sensors were operable, we conclude that, when the lactate was washed out, the T system became sufficiently depolarized to trigger Ca\textsuperscript{2+} release. Such partial depolarization would also explain the reduced response to T system depolarization by Na\textsuperscript{+} substitution (Fig. 3A) and ChCl substitution (not shown), because some voltage sensors would not recover from inactivation, and normal coupling would be hindered (25). Evidently, when lactate was present in the cytoplasm, it gradually accumulated in the sealed T system, probably as a result of a specific transport mechanism and passive diffusion (21). Consequently, when lactate was rapidly removed from the cytoplasm, it would still have been present in the T system at first, and this concentration difference apparently caused depolarization of the T system membrane. This might have occurred because of ion movements directly or indirectly accompanying lactate transport back into the cytoplasm. Whatever the basis of the effect may be, we were unable to prevent it with quercetin, an inhibitor of lactate transport (21), or 9-AC, an inhibitor of the major anion channel in the T system (9).

Clearly then, lactate was moving into the T system during even the brief (30- to 45-s) exposures to lactate, even though it apparently did not reach high levels in the T system over this period, because, on removal of lactate from the cytoplasm, depolarization-induced responses recovered to the control level and spontaneous responses were not observed (Figs. 2A and 3A). In view of this, it is possible that even the small degree of inhibition of the depolarization-induced responses seen in the presence of 30 mM lactate (Fig. 2, Table 2) was caused by chronic partial depolarization of the T system arising in some way from the transport of lactate into the T system. We have no evidence that this was occurring, but if it was, it would again mean that even the small level of inhibition of depolarization-induced Ca\textsuperscript{2+} release found here overestimated the effect occurring in vivo, where lactate concentrations would not change so rapidly and, hence, become so disparate between the inside and the outside of the fiber.

The possible indirect effects of lactate on T system potential observed here (Fig. 3) may have contributed to the substantial level of inhibition of tetanic force production seen by Spangenberg and colleagues (33) with application of up to 50 mM lactate to the extracellular solution bathing whole muscles. They found that the inhibitory effect was maximal within 3 min of lactate exposure, and this seems more easily explained by the transport of lactate across the surface and T system membranes, causing some degree of partial depolarization and action potential failure, than by some effect of cytoplasmic lactate itself, because it seems unlikely that the concentration within the fibers would reach a high level in such a brief time, even in the case of superficial fibers.

Relevance to muscle fatigue. The presence of lactate in the cytoplasm has only a very small inhibitory effect directly on the contractile apparatus (see RESULTS) (2). We further found here that lactate has only a minor inhibitory effect on depolarization-induced Ca\textsuperscript{2+} release, even at 30 mM (Fig. 2, Table 2). Thus it appears that the build-up of lactate per se could contribute only a small degree to the development of fatigue, even in fast-twitch muscles. In view of this, the inverse correlation observed between muscle lactate concentration and force (see the introduction) is likely to be the result of other metabolic changes occurring during heavy exercise, such as increases in P\textsubscript{i}, concentration and [Mg\textsuperscript{2+}] and, to a lesser degree, [H\textsuperscript{+}] (1, 16, 18, 27, 35), and also possibly glycolgen depletion (6, 36). The production of lactic acid and other metabolic changes occurring in these circumstances will increase the number of osmotically active particles in the muscle fiber (15, 30), and the influx of extracellular water will result in swelling of the fiber to maintain osmotic equilibrium (1, 39). In total, there will probably be relatively little change in intracellular ionic strength, and any such change is unlikely to substantially affect E-C coupling (28). It is nevertheless possible that, in some circumstances, fiber swelling might affect action potential propagation in the T system or communication between the T system and the SR and, hence, might contribute to muscle fatigue. In summary, although it is possible that lactate accumulation contributes to muscle fatigue in some indirect manner, the findings of this study show that the presence of a high cytoplasmic concentration of lactate itself has only a relatively minor inhibitory effect on normal E-C coupling.

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