Depression of Peak Force Without Altering Calcium Sensitivity by the Superoxide Anion in Chemically Skinned Cardiac Muscle of Rat

N.G. MacFarlane and D.J. Miller

Among the mechanisms postulated to contribute to myocardial “stunning” is a depression of contractility by oxygen-derived free radicals. It has been suggested that these radicals might depress the calcium sensitivity of the contractile proteins. We have exposed the myofilaments (in chemically “skinned” rat cardiac muscle) to the superoxide anion and measured isometric force at controlled degrees of activation. Superoxide was generated by the xanthine/xanthine oxidase system: the effects to be described were shown to be specifically attributable to superoxide. Maximum calcium-activated force is reduced, or even completely abolished, in a dose-dependent fashion and without any alteration in calcium sensitivity. The myofilaments are highly sensitive to superoxide: significant force reduction has been shown to be caused by enzyme concentrations as low as 2 microunits/ml xanthine oxidase and with exposures of less than 1 minute to the generating system (at higher enzyme concentrations). Once force has been depressed, it cannot be recovered within the duration of the experiments described. When xanthine oxidase is applied during the calcium-induced contracture, tension falls steadily. However, a similar concentration is without immediate effect on the rigor contracture (evoked by applying ATP-free solutions). To account for the depression of maximum calcium-activated force, we conclude that some aspect of crossbridge behavior is particularly vulnerable to superoxide rather than that the radical has a nonspecific “proteolytic” effect. This action on the fundamental units of force production could contribute to myocardial stunning since the effects we report are consistent with many aspects of this phenomenon. (Circulation Research 1992;70:1217-1224)

KEY WORDS • free radicals • calcium sensitivity • myocardial stunning • chemically skinned muscle • force depression

There are a number of clinical situations in which the myocardium is subject to a transient episode of ischemia followed by reperfusion. Examples include percutaneous transluminal coronary angioplasty, myocardial infarction with rapid reperfusion, and cardiac surgery or transplantation with cardioplegic arrest. These situations have been associated with a form of contractile dysfunction that persists after the ischemic episode and is fully reversible. This dysfunction has been termed myocardial “stunning.” Stunning has been suggested to result from a decrease in contractile protein activation because regionally stunned hearts show a contractile reserve when stimulated by positive inotropic interventions.® Such a decrease in activation has been further suggested to occur by a decrease either in the calcium transient or in the calcium sensitivity of regulatory proteins. Since it has been demonstrated that in such situations there is a normal or even supranormal calcium transient, decreased calcium sensitivity has been implicated in the mechanism of stunning.®

One factor concerned with the genesis of posts ischemic contractile dysfunction is the generation of oxygen-derived free radicals. Myocardial production of radicals begins during coronary occlusion and increases after reperfusion, with a peak at approximately 3 minutes, and may persist for a number of hours after this initial burst.® We have investigated the action of one such radical, the superoxide anion (O₂⁻·), in chemically “skinned” trabecular preparations isolated from the right ventricle of rat. This technique leaves the contractile structures of the myofilaments intact and exposed to the bathing medium. The controlled generation of O₂⁻· in the bathing medium allows determination of its effect on maximum calcium-activated force (Cmax) and the calcium sensitivity of the contractile proteins and whether it acts on crossbridges in rigor.

Materials and Methods

The rationale for solution composition, the method of calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, precautions for EGTA purity, calcium contamination determination, the measurement of pH, and other details of our experimental protocol are described elsewhere.®

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In summary, Wistar rats (220–270 g) were killed by a blow to the head and cervical dislocation; the heart was rapidly excised and flushed with rat Ringer’s solution containing (mM) NaCl 150, KCl 5, CaCl2 2, MgCl2 1, and HEPES 5 (pH 7.0) at approximately 20°C. The experiments were carried out on small, free-running trabeculae (2–3 mm long and 75–150 μm in diameter) isolated from the right ventricle of the rat. The preparation was mounted for isometric force measurement by being snared at either end (using nylon monofilaments) between two stainless-steel tubes (200 μm o.d. and 100 μm i.d.). The tubes were fixed to the force transducer at one end (model AE 875, Akers, Horten, Norway) and a fixed point at the other. The whole mount was carried on a modified Narashige MM2 micromanipulator, which permitted adjustment of the tube separation (and hence muscle length) and alignment. Since the effect of sarcomere length on calcium sensitivity is well established, it is critical to determine sarcomere length in experiments of this type.6 Sarcomere length was measured by differential interference contrast light microscopy and set to 2.1–2.2 μm for the present study. Once the muscle had been mounted, it was exposed to a “relaxing” solution (solution A, Table 1) including the nonionic detergent Triton X-100 (1% [vol/vol]) for 20–30 minutes. This regimen was sufficient to ensure the complete destruction of surface and intracellular membrane diffusion barriers.7 The chemical skinning agent was then removed by washing in fresh relaxing solution.

The preparation can be activated to produce tension by increasing the free calcium concentration of the bathing medium. A cumulative response was produced by sequentially stepping calcium concentration upward over the appropriate range (solutions made by mixing solutions A and B, Table 1, appropriately). Excess calcium can be added to aliquots of solution B to ensure that the free calcium concentration is high enough for maximal activation. This approach has been widely used in pharmacological studies but has a number of drawbacks. We occasionally find that this approach yields force-pCa curves appreciably different from those derived from making a series of single activations at a test pCa. This difference seems most likely to be due to factors such as increases in phosphate and local reduction in pH, which result when the preparation maintains substantial force for prolonged periods. Therefore, in the present study, we also used a series of single activations at test pCas. To make the activation as rapid and uniform as possible throughout the cross section of the preparation in such experiments, the “calcium-jump” method was used, whereby the calcium buffer was raised at the same time as the free calcium concentration49 (i.e., into the test pCa solution from solution E, Table 1).

We studied the action of O2•− on the preparation during the rigor state (i.e., during the very strong interaction between actin and myosin filaments that develops in the absence of ATP even at a very low calcium concentration). In these experiments, the preparation was initially relaxed in a solution without creatine phosphate (solution C, Table 1) to prevent creatine phosphate contamination of the “rigor” solutions (which would otherwise continue to phosphorylate ADP generated by crossbridges from the residual ATP carried over in the preparation when the solution change was made). The rigor contracture was subsequently induced by exposing the preparation to a similar solution containing no ATP (solution D, Table 1). Each rigor-inducing solution was only applied once to avoid complications due to cumulative ATP contamination. The MgCl2 was reduced in the rigor solution to ensure the same free magnesium concentration as the relaxing solution.

Superoxide was generated by an established system in which xanthine oxidase (EC 1.1.3.22, grade IV [from milk] suspension in 2.3 M ammonium sulfate, Sigma Chemical Co., Poole, UK) catalyzes oxidation of xanthine to uric acid. Xanthine oxidase concentration was varied by dilution from a stock solution with 2 milliunits/ml (1.5 μl commercial stock in 5 ml of the appropriate test solution). Xanthine was 50 μM in all tests. All chemicals were obtained from Sigma unless otherwise stated.

Tension responses are shown calibrated in either absolute force or relative force. For relative force, tension was normalized to the initial C_in, which was determined from a number of control activations before the first exposure of the preparation to the O2•−-generating system. Since the preparations are not always of the same size, they develop different absolute levels of force, so accumulated data are expressed in relative terms.

The relation between steady-state force and calcium concentration obtained from all otherwise satisfactory experiments is well described by the Hill equation, allowing objective definition of the position and steep-

### Table 1. Composition of Solutions at pH 7.0

<table>
<thead>
<tr>
<th>Solution</th>
<th>K** (mM)</th>
<th>Mg† (mM)</th>
<th>Ca_{free} (mM)</th>
<th>pCa</th>
<th>ATP (mM)</th>
<th>CrP (mM)</th>
<th>Na+ (mM)</th>
<th>EGTA (mM)</th>
<th>HEPES (mM)</th>
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<tr>
<td>A</td>
<td>140</td>
<td>7</td>
<td>0.02</td>
<td>9.03</td>
<td>5</td>
<td>15</td>
<td>40</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>B</td>
<td>140</td>
<td>7</td>
<td>10</td>
<td>4.25§</td>
<td>5</td>
<td>15</td>
<td>40</td>
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<td>0.02</td>
<td>9.03</td>
<td>5</td>
<td>...</td>
<td>40</td>
<td>10</td>
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</tr>
<tr>
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<td>0.02</td>
<td>9.03</td>
<td>...</td>
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<td>...</td>
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</tr>
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<td>5</td>
<td>15</td>
<td>40</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

Ca_{free}, total calcium; CrP, creatine phosphate.

*Potassium ions added as KCl and KOH.
†Magnesium added as 1 M MgCl2 (free Mg²⁺ = 2.1–2.5 mM in solutions A and B and mixtures of these solutions).
‡Sodium ions from the salts Na2ATP and Na2CrP.
§An additional 0.1 mM CaCl2 added to aliquots of this solution to yield “full activating” solution (pCa 3.98). Total chloride concentrations varied from −110–120 mM.
||1,6-Diaminohexane-N,N,N',N'-tetraacetic acid (9.8 mM) added to this solution to maintain equivalent ionic strength.
ness of the curve relating calcium concentration and tension. However, although the Hill coefficient of the force pCa curve is useful to describe this curve, it does not permit any inference in terms of the number of calcium binding sites. The curves are fitted by a nonlinear least-squares fitting procedure,¹⁰ and the fact that we can be confident about the use of the Hill equation permits curve fitting even with relatively few test calcium concentrations.

Student’s paired t test was used for comparison of data obtained within an experimental preparation, with differences considered significant at p<0.05.

**Results**

**Maximum Calcium-Activated Force**

Addition of 50 μM xanthine, 70 μM ammonium sulfate (the level achieved with the highest concentration of xanthine oxidase used), or heat-denatured xanthine oxidase to the bathing medium had no substantial effect on Cₘₓₛ. The effect of the xanthine/xanthine oxidase system on Cₘₓₛ is illustrated in Figures 1 and 2. Figure 1 shows the effect of concentrations of xanthine oxidase spanning four orders of magnitude. In other experiments, single doses of xanthine oxidase were applied only once or twice, after which the developed tension stabilized at a reduced level. Initially, three control maximum calcium-induced contractions were evoked. The O₂⁻-generating system was then applied (in relaxing solution) for 2 minutes, and then, after a brief rinse in standard relaxing solution, a maximum calcium-induced contraction was evoked. The effect of each xanthine oxidase concentration was tested for six successive activations, each 10 minutes apart. Figure 1 shows results from a typical experiment. Similar results were found in eight other preparations. Figure 1A shows that control Cₘₓₛ is relatively stable. (In other experiments, we typically found that Cₘₓₛ when repeatedly evoked, fell by less than 0.2% per minute.) Cₘₓₛ fell more steeply as the concentration of xanthine oxidase was increased. In this case, after 22 activation-relaxation cycles (i.e., 220 minutes), Cₘₓₛ was effectively zero. Figure 1B shows that Cₘₓₛ falls linearly on semilog axes (i.e., exponentially and toward zero) at each xanthine oxidase concentration.

In these experiments, it was noted that in conjunction with the depression of Cₘₓₛ, exposure to the O₂⁻-generating system increased the time to half-maximal activation and half relaxation in every case (from 1.95±0.83 to 2.58±1.25 seconds [p<0.05] and from 1.25±0.49 to 2.65±1.26 seconds [p<0.05], respectively; mean±SD; n=6 preparations).

Figure 2 shows results from a similar protocol except that xanthine oxidase concentration was kept constant (at 0.02 milliunits/ml) and the time of exposure to xanthine/xanthine oxidase was varied. As might be
expected, prolonging the exposure time produced a greater relative fall in $C_{\text{max}}$. Similar results were found with three other preparations.

The xanthine/xanthine oxidase system generates both $O_2^{-}$ and singlet oxygen. Superoxide forms $H_2O_2$ in the presence of superoxide dismutase, which in turn can form the hydroxyl radical via the Haber-Weiss reaction when a suitable metallic cation, such as iron, is present. The Fenton reaction gives rise to the hydroxyl radical from $O_2^{-}$ directly, in the presence of a substance capable of reducing $Fe^{3+}$ to $Fe^{2+}$. These conditions might prevail in our experimental solutions but are unlikely because of the strong chelation of cations by EGTA. To demonstrate that the contractile dysfunction associated with exposure to the $O_2^{-}$-generating system can be attributed to $O_2^{-}$ itself, we used a variety of scavenging agents to identify the culprit species. Figure 3 shows that when the preparation was exposed to xanthine/xanthine oxidase in the presence of superoxide dismutase, there was little change in tension compared with control activations. The xanthine oxidase concentration used in this case would normally have caused a fall in force production. To demonstrate this, the sequence was continued in the absence of superoxide dismutase, and as expected, tension fell (to <20% of control) within five exposures to xanthine/xanthine oxidase. The bathing media used in these experiments contained HEPES. This substance is effective as a hydroxyl radical scavenger, which should protect the preparation from the effect of any hydroxyl radical formation occurring downstream from the $O_2^{-}$-generation. As a further check, addition of deferoxamine, another hydroxyl radical scavenger, to the solutions did not confer protection. Glutathione, which will scavenge the hydroxyl radical, singlet oxygen, and organic peroxides, did not confer protection. Addition of catalase (to remove $H_2O_2$) to superoxide dismutase was not necessary to confer protection (data not shown). These findings suggest that the effects of xanthine/xanthine oxidase on $C_{\text{max}}$ are attributable to $O_2^{-}$ alone.

**Calcium Sensitivity**

By studying submaximal calcium activations in the Tritton-skinned preparations, we are able to investigate the hypothesis that free radicals depress calcium sensitivity. The relation between calcium concentration and tension for chemically skinned trabecular preparations from rat (and other species) has been thoroughly characterized in this laboratory (see "Materials and Methods"). Figure 4A shows an example of the effect of $O_2^{-}$ on $C_{\text{max}}$. Before being exposed to $O_2^{-}$ (open circles), $C_{\text{max}}$ is constant. After two exposures to $O_2^{-}$ (filled circles), $C_{\text{max}}$ fell progressively to less than 20% of the initial level. Figure 4B shows the relation between tension and calcium concentration obtained from a range of submaximal calcium activations in the same preparation. Open symbols denote the control data obtained before exposure to $O_2^{-}$, and closed symbols denote the data obtained after exposure to $O_2^{-}$. (The symbols have been displaced slightly for clarity.) The best-fit curves obtained can be superimposed, establishing that $O_2^{-}$ produces no alteration in calcium sensitivity despite a large fall in $C_{\text{max}}$.

To confirm the lack of effect, Figure 5 shows experimental tracings indicative of the calcium sensitivity before and after a single exposure to the $O_2^{-}$-generating system (10 milliunits/ml for 2 minutes). The left panel of this figure shows that $C_{\text{max}}$ is approximately 50 mg/wt, whereas in the right panel $C_{\text{max}}$ has fallen to...
approximately 20 mg/wt. Rescaling the tracing in the right panel so that the maxima are of similar size shows that there is very little difference in the relative submaximal levels of activation. This demonstrates again that calcium sensitivity does not alter significantly despite a very large fall in peak force; similar results of this “cumulative activation” were found in seven other preparations. In the same way, no effect of $O_2^-$ on the tension–calcium concentration relation was noted in eight preparations when force–pCa curves were constructed using the calcium jump method of activation, as is the case in Figure 4.

**Crossbridges in Rigor**

Exposing the preparation to $O_2^-$ in the rigor state can establish whether $O_2^-$ acts on active or nonactive crossbridges. Figure 6 shows that the amplitude of both $C_{\text{max}}$ and rigor contractions can be maintained at a steady level for relatively long periods of time. The $O_2^-$ was then applied for a period of 2 minutes during a rigor contraction, without a change in the amplitude of that rigor contraction. The subsequent amplitude of $C_{\text{max}}$ was slightly but not significantly smaller than control, given that prolonged activations have a detrimental effect on $C_{\text{max}}$ and that more than 40 minutes had elapsed since the control activation. When the same concentration of xanthine oxidase was applied for a similar period of time during a maximum calcium-activated contraction, however, the amplitude of that contraction fell steadily within seconds. Similar results were found in seven preparations. In five other preparations, a different protocol was followed to establish whether the fall in tension, if any, noted after exposure to the $O_2^-$-generating system during a rigor contraction was significantly different from the fall noted when $C_{\text{max}}$ was repeatedly evoked. Five control activations were evoked, and the rate of fall in $C_{\text{max}}$ was calculated. This was compared with the fall, if any, noted after exposure to $O_2^-$ in the rigor-inducing solution. Rate of fall in the control activation was $0.256 \pm 0.008\% \cdot \text{min}^{-1}$ (mean $\pm$ SD) compared with $0.208 \pm 0.048\% \cdot \text{min}^{-1}$ after exposure to $O_2^-$ ($p>0.2$), whereas the rate of fall in tension after exposure to $O_2^-$ in solutions containing ATP ($8.04 \pm 3.86\% \cdot \text{min}^{-1}$) was significantly different from the control rate ($p<0.02$).

**Discussion**

The sequence of events that can occur upon reperfusion after a transient ischemic episode is now widely recognized by the term myocardial stunning. This state is characterized by reduced contractile function, which reverses over a period of weeks. Although the clinical manifestations of this condition are well established, the

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**Figure 5.** Experimental tracings demonstrating the lack of effect of $O_2^-$ on calcium sensitivity. The left panel shows a "cumulative" series of submaximal activations before exposing the preparation to the $O_2^-$-generating system. The right panel shows a similar series of activations after exposure to the $O_2^-$-generating system (10 milliunits/ml xanthine oxidase for 2 minutes). The scale of the right panel has been expanded to facilitate comparison of the relative forces.

**Figure 6.** Experimental tracings demonstrating the lack of effect of $O_2^-$ during a rigor contraction. The preparation was initially activated by raising calcium concentration in the bathing medium; this contraction was maintained for 5 minutes. A rigor contraction was then induced by removing ATP from the bathing medium. After having allowed the rigor force to develop, the preparation was exposed to the $O_2^-$-generating system (10 milliunits/ml xanthine oxidase [XO] for 2 minutes). Before relaxing the preparation, the $O_2^-$-generating system was removed, which did not change the measured force. Subsequent calcium activations are not diminished beyond the extent that would be expected for the time period elapsed from control (see main text). The preparation was then exposed to the same level of $O_2^-$ generation after calcium activation. The force developed falls during such exposure and continues to fall after the $O_2^-$-generating system is removed. The force developed during the subsequent calcium-activated contraction is below 50% of that obtained immediately before $O_2^-$ exposure during the calcium-activated contraction (and far in excess of that which would be expected). Gaps in the tracing indicate periods in “relaxing” solution of 5–10 minutes.
mechanisms remain a topic of active research. Several contributing factors have been recognized; these include calcium overload, generation of oxygen-derived free radicals, and alterations to excitation–contraction coupling. These processes are, of course, not mutually exclusive. The relative importance of these factors still requires definition.

Much of the existing work on the phenomenon of myocardial stunning, whether at the cellular, isolated organ, or whole animal level, has rested on implicit assumptions about the intrinsic contractile potential of the myocardial cell. Specifically, we note that assumptions are made about the contractile “reserve” available to the heart. An example would be the conclusion that calcium sensitivity is reduced when observing that the calcium transient is normal but contraction is reduced. Actual force is the product of two factors: the relative degree of activation of the contractile proteins and the maximum achievable force ($F_{\text{max}}$) for the tissue. Alterations in sensitivity, or the calcium concentration achieved during activation, will change relative force, whereas alterations in $F_{\text{max}}$ will scale these effects. It is difficult to detect changes in $F_{\text{max}}$ in the intact preparation for several reasons. In cellular preparations, even maximal positive inotropic interventions may only achieve $F_{\text{max}} = 0.85 C_{\text{max}}$. These interventions are difficult or undesirable on other grounds in whole organ or whole animal preparations and are clinically inadmissible. It follows that a change in actual force cannot unequivocally be attributed to either factor. Under the conditions likely to prevail physiologically or even experimentally, the contractile reserve can be several times greater than the actual force developed. It follows that a large change in contractile reserve, either an increase or decrease, could go undetected. The use of skinned fibers, however, gives sufficient experimental control to allow one to make definitive statements about calcium sensitivity and $C_{\text{max}}$ changes. These points are illustrated in Figure 7.

Calcium sensitivity will be reduced during an ischemic episode, and for a relatively short time after, because of the rise in inorganic phosphate concentration and fall in pH that has been demonstrated to occur in the myocardium. It should be remembered that these factors also reduce $C_{\text{max}}$. Stunning persists long after the metabolic consequences of the antecedent ischemia, whereas the alterations of pH and phosphate diminish during the posts ischemic period. Calcium sensitivity and $C_{\text{max}}$ will return to normal as far as these factors are concerned. However, the oxygen-derived free radicals produced during and after ischemic episodes have been suggested to depress the calcium sensitivity of the contractile proteins.

The results presented here show for the first time that $O_2^-$ acts directly on the contractile proteins and diminishes their force-producing ability. In the Triton-treated fibers used in these experiments, any complications due to the action of $O_2^-$ on the cellular membranes or myoplasmic enzymes can be ignored since the preparation comprises only the contractile filaments. The discussion above and Figure 7 emphasize that a reduction in calcium sensitivity will produce a fall in force at a submaximal level of activation. We explored this possibility, and as Figures 4 and 5 reveal, despite substantial falls in $C_{\text{max}}$, no detectable change in calcium sensitivity occurs. Translated to the intact cell, the fall in $C_{\text{max}}$ with no change in calcium sensitivity would account for the observed fall in contractility despite a normal calcium transient. This is the circumstance represented in the bottom panel of Figure 7.

Our results indicate that the myofilaments are particularly sensitive to $O_2^-$. Force is inhibited faster and at lower enzyme concentrations in the $O_2$-generating system than has previously been reported for intact experimental preparations. Falls in force entirely attributable to $O_2^-$ can be produced by as little as 2 microunits/ml xanthine oxidase (e.g., see Figure 1) within 2 minutes; this represents 0.002% of the levels frequently used in experiments with intact cellular systems. We are forced to conclude that the myofilaments
are very susceptible to damage by \( \text{O}_2^- \). This conclusion is particularly important since, in rat heart, physiological concentrations of xanthine oxidase exceed those used here.\(^{19} \) It remains controversial whether xanthine oxidase is present in human myocardium.\(^{20–23} \)

We have demonstrated here that the effects on \( C_{\text{max}} \) can be attributed to \( \text{O}_2^- \) rather than the generating system itself. In the intact cell, there are other sources for \( \text{O}_2^- \) in addition to xanthine oxidase. Superoxide is produced in the cell as a consequence of normal metabolism. Probably the most important source in vivo is the mitochondrial respiratory chain.\(^{24} \) Since the contractile proteins have been revealed by this study to be particularly sensitive, increases in \( \text{O}_2^- \) production associated with ischemia and reperfusion might overcome the capacity of endogenous scavengers and threaten the performance of the myofilaments. However, the well-documented actions of oxygen-derived free radicals on other cellular systems, as well as other processes provoked by ischemia and reperfusion, will have their place in any comprehensive explanation of the phenomenon of myocardial stunning.

The exact mode of action of \( \text{O}_2^- \) on the myofilaments remains to be elucidated, but our results afford some insights. In our experimental protocols we applied \( \text{O}_2^- \) in either the relaxed or calcium-activated states. The relaxed muscle showed no fall in resting tension (induced by stretching the preparation to set the sarcomere length) when exposed to \( \text{O}_2^- \), giving initial evidence to suggest that the effect was more than generalized damage to the structural integrity of the contractile proteins (either by \( \text{O}_2^- \) directly or by protease contamination of the xanthine oxidase). By studying the action of \( \text{O}_2^- \) on the preparation in the rigor state, we sought to establish whether \( \text{O}_2^- \) affects active or inactive crossbridges. Figure 6 illustrates that even a high level of \( \text{O}_2^- \) has no immediate effect on force previously developed by the muscle in rigor, unlike the rapid decline of calcium-activated tension that can be induced. The insensitivity of rigor bridges to alterations in pH has been reported earlier.\(^{35} \) This result suggests that the \( \text{O}_2^- \) is not acting in the manner of a nonspecific protease to damage the structural integrity of the myofilament lattice; tension would otherwise be lost during the rigor contracture (consolidating our initial evidence for this conclusion in relaxed preparations).

We took care, in the protocol used for experiments like those in Figure 6, to remove the xanthine/xanthine oxidase fully before relaxing the muscle by replacing ATP. Thus, in these experiments, \( \text{O}_2^- \) was not present when crossbridges were actively cycling. The subsequent evidence that no significant damage had occurred implies that \( \text{O}_2^- \) acts without provoking crossbridge detachment or weakening the crossbridge structure. The site of action of \( \text{O}_2^- \) is most probably on a part of the crossbridge that remains inaccessible in the attached state. The action of \( \text{O}_2^- \) can be concluded to be on the active or detached crossbridge for the conditions studied here. This action will compromise one of subsequent attachment, crossbridge kinetics, such as the transition from the weakly to the strongly attached state, and/or ATPase activity. It is, however, perhaps difficult to reconcile these observations with those of Krause\(^{26} \) on myofibrillar ATPase of preparations from stunned rabbit myocardium. He reported no detectable reduction in maximum calcium-activated ATPase, its kinetics, or calcium sensitivity. To interpret our results against this background requires that the rate of crossbridge cycling remain essentially unaltered. This would demand a specific alteration to the crossbridge cycle. Attachment time as a fraction of cycle duration would fall substantially, but cycle frequency (and hence the rate of ATP consumption) would remain unaltered. Such a highly specific action seems unlikely. Krause attributed all the postischemic reduction in contractile function to myocardial stunning. The final degree of dysfunction after only some 30 minutes of reperfusion was only approximately 27% (as closer inspection of the original figures reveals). At this time, although any reduction in pH would probably have reversed fully, raised phosphate might still decrease contractile function. Additionally, these preparations were unpaired, and rate-dependent effects on contractility could have contributed to any such decrease. If, for example, only one third of the decrease was due to stunning and that third was entirely attributable to \( C_{\text{max}} \) reduction, then myofibrillar ATPase would reduce by as little as 10%, a level difficult to detect reliably with these methods. In contrast, a reduction of \( C_{\text{max}} \) by 10% is readily detected in the skinned fibers.

It is generally assumed to be the speed of calcium concentration change that determines speed of tension alteration in skinned fibers. However, changes in the rate of force development and decline, observed when the calcium concentration is raised or lowered, are not readily interpreted. If we assume that the diffusion of the calcium EGTA buffer system is unaffected by \( \text{O}_2^- \) treatment, then the 32% slowing of activation and 112% slowing of relaxation half-times could indicate altered crossbridge kinetics. This would require that crossbridge attachment and detachment, rather than buffer diffusion, become the rate-limiting steps and would imply a reduced ATPase rate.

In the experimental conditions that we have studied, we can readily produce a reduction in \( C_{\text{max}} \) to any degree. We observed a slowing in tension responses accompanying the reduction in \( C_{\text{max}} \). We have established that there is no change in calcium sensitivity nor is some nonspecific damage a major contributor to the altered contractile responses. These findings would be consistent with an alteration to crossbridge kinetics or reduced overall ATPase activity, as noted above. Any effect of the radicals on crossbridge kinetics clearly remains to be investigated. Whatever the mechanism of \( \text{O}_2^- \)-induced \( C_{\text{max}} \) reduction that we have described here, it could be a major contributor to the phenomenon of myocardial stunning.

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