Effect of clenbuterol on sarcoplasmic reticulum function in single skinned mammalian skeletal muscle fibers

ANTHONY J. BAKKER,1 STEWART I. HEAD,2 ANTHONY C. WAREHAM,3 AND D. GEORGE STEPHENSON4

School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

1Department of Physiology, University of Western Australia, Nedlands 6907; 2School of Physiology and Pharmacology, University of New South Wales, Sydney 2052; 3School of Zoology, La Trobe University, Bundoora 3083, Australia; and 4Division of Neuroscience, School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Bakker, Anthony J., Stewart I. Head, Anthony C. Wareham, and D. George Stephenson. Effect of clenbuterol on sarcoplasmic reticulum function in single skinned mammalian skeletal muscle fibers. Am. J. Physiol. 274 (Cell Physiol. 43): C1718–C1726, 1998.—We examined the effect of the β2-agonist clenbuterol (50 µM) on depolarization-induced force responses and sarcoplasmic reticulum (SR) function in muscle fibers of the rat (Rattus norvegicus; killed by halothane overdose) that had been mechanically skinned, rendering the β2-agonist pathway inoperable. Clenbuterol decreased the peak of depolarization-induced force responses in the extensor digitorum longus (EDL) and soleus fibers to 77.2 ± 9.0 and 55.6 ± 5.4%, respectively, of controls. The soleus fibers did not recover. Clenbuterol significantly and reversibly reduced SR Ca2⁺ loading in EDL and soleus fibers to 81.5 ± 2.8 and 78.7 ± 4.0%, respectively, of controls. Clenbuterol also produced an approximately 25% increase in passive leak of Ca2⁺ from the SR of the EDL and soleus fibers. These results indicate that clenbuterol has direct effects on fast- and slow-twitch skeletal muscle, in the absence of the β2-agonist pathway. The increased Ca2⁺ leak in the triad region may lead to excitation-contraction coupling damage in the soleus fibers and could also contribute to the anabolic effect of clenbuterol in vivo.

calium uptake; calcium leak; calcium release; β-agonist; excitation-contraction coupling; anabolism

RECENT STUDIES HAVE SHOWN that clenbuterol, a β2-agonist, has different effects in fast- and slow-twitch muscle. Clenbuterol has been shown to potentiate electrical field stimulation-induced contractions of isolated extensor digitorum longus (EDL; fast twitch) muscles of the rabbit (25) but seems to have detrimental effects on soleus (slow twitch) muscle. Clenbuterol treatment was shown to increase the fatigability of soleus muscles of mice by 30-40% (11) and to induce single muscle fiber injuries and significantly increase serum creatine kinase (muscle isoenzyme CK-MM) levels (a marker of muscle damage) in the soleus muscle of clenbuterol-treated rats (34). This difference in sensitivity to damage may reflect higher β2-receptor density in slow-twitch compared with fast-twitch muscle (16). However, other β2-agonists, such as terbutaline, have been shown to potentiate force responses in fast- and slow-twitch muscles of the rat (4) and to slow high-frequency fatigue in rat soleus muscle fibers (5).

Clenbuterol is also a powerful anabolic agent that significantly increases the amount of protein synthesis in skeletal muscle and simultaneously reduces subcutaneous body fat, and it is thought to act via β2-receptors because the anabolic effect is inhibited by high doses of the β2-antagonist propranolol (20) and the selective β2-antagonist ICI-118551 (7). However, other groups have reported that high doses of propranolol have no effect on clenbuterol-induced anabolism (21, 29) but do reduce β2-mediated fat deposition (29).

These reports suggest that clenbuterol may elicit other, direct effects on cell function in skeletal muscle, which are not related to the β2-activation pathway and which disproportionately affect slow-twitch skeletal muscle, making it more susceptible than fast-twitch skeletal muscle to damage. Clenbuterol is membrane permeable, due to its high lipid solubility (30) and is one of the few β2-agonists that readily passes the blood-brain barrier (14) and will therefore readily enter the sarcoplasm in vivo and accumulate within muscle fibers (23). In this study, we used the skinned fiber technique to examine the effect of clenbuterol on fast- and slow-twitch skeletal muscle fibers of the rat in the absence of the β2-activation pathway. In the skinned fiber technique, the plasma membrane of skeletal muscle fibers is mechanically removed (9, 33). This compromises the β2-activation pathway by preventing the accumulation of soluble second messengers, as the cytosol of the fiber is washed out with a practically infinite pool of bath solution. Moreover, the dissection of the sarcolemma removes all surface membrane β2-receptors.

In addition, the skinned fiber technique has a number of other important advantages over intact preparations for the present study. After the sarcolemma of the fibers has been mechanically peeled away, the transverse tubular system seals and regolalizes (9, 18, 33). Replacing the K⁺-based solution bathing the fibers with a solution in which K⁺ has been replaced with Na⁺ depolarizes the sealed transverse tubular system and triggers force responses via the normal excitation-contraction (E-C) coupling pathway. Therefore, this preparation is unique in that one has access to the cytosol but at the same time it is possible to trigger force responses by the normal physiological mechanism (18). Furthermore, this preparation also allows the quantitative examination of subcellular events occurring within the fiber, such as sarcoplasmic reticulum (SR) Ca2⁺ release, the level of Ca2⁺ uptake by the SR, and the passive SR Ca2⁺ leak (1), measurement of which is not presently possible with intact preparations. In this study, we used the skinned fiber technique to examine the effects of clenbuterol on E-C coupling, the Ca2⁺ sensitivity of the contractile apparatus, and...
SR Ca\(^{2+}\) release, Ca\(^{2+}\) loading, and Ca\(^{2+}\) leakage in fast- and slow-twitch skeletal muscle fibers of the rat.

**METHODS**

Muscle fibers were isolated from the EDL and soleus muscle of Wistar rats (Rattus norvegicus) killed by an overdose of halothane. The muscle fibers were dissected and skinned in paraffin oil and mounted on a force transducer to monitor isometric force. The length and diameter of each fiber were measured at slack length, and the fiber was then stretched by 20% to bring the sarcomere length to \(-2.8\) to \(-3.0\) \(\mu m\) to maximize the force responses.

The fibers were then transferred to a 2-ml Perspex bath containing a potassium hexamethylenediamine tetraacetate (K\(^{+}\)-HDTA) solution composed of (in mM) 125 K\(^{+}\), 36 Na\(^{+}\), 50 HDTA\(^{2-}\), 8 ATP (total), 8.6 Mg\(^{2+}\) (total), 10 creatine phosphate, 0.03 EGTA (total), 90 HEPES, and 1 NaN\(_3\) at pH 7.10 ± 0.01 and pCa 7.0 (16). NaN\(_3\) was added to prevent mitochondrial Ca\(^{2+}\) fluxes. The free Mg\(^{2+}\) concentration was 1 mM.

Isolation of soleus type I fibers. In the soleus muscle of the rat, two distinct populations of fibers are present, a predominating population of slow-twitch (type I) fibers and a smaller but significant population of fast-twitch (type IIa) fibers. In this study, slow- and fast-twitch soleus fibers could be distinguished by determining the frequency of myofibrillar oscillations measured in response to exposure to highly Ca\(^{2+}\)-buffered solutions containing a submaximal Ca\(^{2+}\) concentration (12). Type I soleus fibers typically exhibited low-frequency myofibrillar oscillations (\(-0.33\) Hz), whereas type IIa soleus fibers exhibited higher frequency myofibrillar oscillations (\(-1\) Hz). Only soleus type I fibers were used in this study.

Contractile apparatus. The effects of clenbuterol on maximal force and the sensitivity of the contractile apparatus to Ca\(^{2+}\) in the EDL and soleus fibers were determined by exposing fibers to solutions of different free Ca\(^{2+}\) concentrations in the presence and absence of 50 \(\mu\)M clenbuterol. The strongly Ca\(^{2+}\)-buffered solutions were prepared by mixing specific proportions of EGTA-containing solution (solution A) and CaEGTA-containing solution (solution B) (32). Solution A contained (in mM) 117 K\(^{+}\), 36 Na\(^{+}\), 8 ATP (total), 1 free Mg\(^{2+}\), 10 creatine phosphate, 50 EGTA (total), 60 HEPES, and 1 NaN\(_3\) at pH 7.10. Solution B was similar to solution A, with the exception that the EGTA and CaEGTA concentrations of solution B were 0.3 and 49.7 \(\mu M\), respectively. The free Ca\(^{2+}\) concentrations of the solutions were calculated using a K\(_{\text{apparent}}\) for EGTA of 4.78 \(\times 10^6\) M\(^{-1}\). Maximal force was determined by exposure to solution B, containing a free Ca\(^{2+}\) concentration of 3.5 \(\times 10^{-5}\) M. During experiments, force was returned to baseline between force measurements by brief exposure to solution A. The plateau of the force responses elicited by exposure to solutions of increasing free Ca\(^{2+}\) concentration were expressed as a percentage of maximum Ca\(^{2+}\)-activated force and plotted as a function of pCa. The data were fitted with Hill curves using the curve-fitting software package GraphPad Prizm (GraphPad Software). The slopes of the curves (Hill coefficients) and the pCa values corresponding to 50% of maximum force were determined for both clenbuterol and control data, and the values were compared.

Depolarization-induced force responses. The skinned muscle fibers used in this study retain normal E-C coupling due to rescaling of the transverse tubular system after mechanical skinning. The sealed transverse tubular system can be normally polarized by exposing the preparation to the K\(^{+}\)-HDTA solution and normal activation of the voltage sensor, and ensuring SR Ca\(^{2+}\) release and contraction can be induced by depolarization of the sealed transverse tubular system through exposure to an HDTA solution in which K\(^{+}\) has been replaced by Na\(^{+}\) (18).

Depolarization of the transverse tubular system between each consecutive depolarization-induced force response was achieved by a 1-min incubation of the fiber in a K\(^{+}\)-HDTA solution. The K\(^{+}\) and Na\(^{+}\) solutions used with rat fibers were isosmotic (295 mosmol/kg) (18). The pCa in the K\(^{+}\)-HDTA and Na\(^{+}\)-HDTA solutions was weakly buffered to about pCa 7.0.

Depolarization-induced force responses measured in the presence of 50 \(\mu\)M clenbuterol were compared with control responses elicited both before and after exposure to the drug. Before depolarization in the presence of clenbuterol, the fibers were first incubated in a K\(^{+}\)-HDTA solution containing the drug for 30 s to allow time for the drug to diffuse into the fiber. The depolarization-induced force responses elicited in the fast-twitch fibers were found to be considerably larger than those elicited in the slow-twitch fibers when expressed as a percentage of maximum Ca\(^{2+}\)-activated force (31).

Caffeine-induced Ca\(^{2+}\)-release experiments. In experiments designed to investigate the effect of clenbuterol on caffeine-induced Ca\(^{2+}\) release from the SR of the EDL and soleus fibers of the rat, the fibers were first depleted of Ca\(^{2+}\) by exposure for 2 min to a K\(^{+}\)-HDTA solution containing low Mg\(^{2+}\) (0.25 mM) and 30 mM caffeine, to maximally release Ca\(^{2+}\) from the SR, and 0.25 mM EGTA, to chelate all released Ca\(^{2+}\) and prevent SR Ca\(^{2+}\) reaccumulation. The fiber was then reloaded with Ca\(^{2+}\) for a particular period of time (7 s for the soleus fibers and 10 s for the EDL fibers) by exposure to a highly Ca\(^{2+}\)-buffered solution (pCa 6.55) made by combining solutions A and B at a ratio of 1:1. Loading was rapidly terminated at the end of each loading period by a brief exposure (1–2 s) to solution A, after which the fiber was washed in a K\(^{+}\)-HDTA solution to remove excess EGTA. The fiber was then reexposed to the caffeine solution (above), and the force response was measured. The time to peak and peak of force responses elicited after reexposure to a caffeine solution containing 50 \(\mu\)M clenbuterol were compared with force responses elicited in an identical caffeine solution without clenbuterol, measured both before and after the clenbuterol response. Before exposure to the caffeine solution, the fibers were incubated for 30 s in a K\(^{+}\)-HDTA solution containing 0.25 mM EGTA to allow time for the EGTA to equilibrate within the fiber. Before exposure to a caffeine release solution containing clenbuterol, the fiber was exposed to a similar K\(^{+}\)-HDTA solution (0.25 mM EGTA) also containing clenbuterol to allow time for clenbuterol to equilibrate within the fiber. The caffeine release solutions were made at double volume and were split.

Experiments to measure the effect of clenbuterol on SR Ca\(^{2+}\) loading in rat EDL and soleus fibers. In experiments to determine the effect of clenbuterol on SR Ca\(^{2+}\) loading, the fibers were depleted and reloaded with Ca\(^{2+}\) in the same way described in the previous section. The fiber was then depleted again, and the time integral of the force response elicited by this depletion was used as an indicator of the amount of Ca\(^{2+}\) loaded during the loading period. Depletion measurements made after loading in the presence 50 \(\mu\)M clenbuterol were compared with control measurements made before and after loading with the drug to minimize errors associated with any deterioration in the size of the control responses. Before exposure to the load solution containing clenbuterol, the fibers were exposed to a K\(^{+}\)-HDTA solution containing clenbuterol for 30 s to allow time for clenbuterol to equilibrate within the fiber.
Experiments to measure the effect of clenbuterol on SR Ca\(^{2+}\) leak in rat EDL and soleus fibers. The experiments used to examine the effect of clenbuterol on leakage of Ca\(^{2+}\) from the SR of rat EDL and soleus fibers were again similar to the SR Ca\(^{2+}\)-release experiments described in the previous section. The SR of the fibers was first depleted of Ca\(^{2+}\), and then the SR was reloaded with Ca\(^{2+}\) for a specific time. In these experiments, the fibers were then exposed to a "Ca\(^{2+}\) leak" solution before being reexposed to the caffeine depletion solution. The Ca\(^{2+}\) leak solution consisted of the K\(^{+}\)-HDTA solution with 0.75 mM EGTA added to sequester all leaked Ca\(^{2+}\). The force responses elicited after a period of exposure to the Ca\(^{2+}\) leak solution were compared with control force responses (no exposure to Ca\(^{2+}\) leak solution) measured before and after the measurement of the force responses designed to measure SR Ca\(^{2+}\) leak. The resulting control leak force responses, which represent the normal leak associated with skeletal muscle SR, were compared with the force responses elicited after exposure to a leak solution containing 50 µM clenbuterol.

In the clenbuterol solutions, the clenbuterol (Sigma) was dissolved in a K\(^{+}\)-HDTA solution similar to that used in the experiments. In all cases, an equal volume of K\(^{+}\)-HDTA solution was added to the matching control (no clenbuterol) solution. Ascorbate (0.56 mM) was added to all clenbuterol (and control) solutions in this study to minimize oxidation of the drug during the course of the experiments.

All experiments were conducted at room temperature (21–22°C) except those involving depolarization-induced force responses in soleus, which were undertaken at 25°C. Results are expressed as means ± SE. The results were analyzed with t-tests using the statistical software package INSTAT.

RESULTS

The effect of clenbuterol on the contractile apparatus of rat EDL and soleus fibers. Because the main experimental parameter measured in this study was force, we first examined the effect of clenbuterol on the contractile apparatus of the EDL and soleus fibers to ensure that the experimental results in the following sections were not misconstrued due to clenbuterol-induced changes in the sensitivity of the contractile apparatus to Ca\(^{2+}\). The skinned EDL and soleus fibers were exposed to highly buffered Ca\(^{2+}\) solutions of different, known free Ca\(^{2+}\) concentrations, and the resulting force responses were measured. The data were plotted as percentage of maximum force vs. pCa and fitted with Hill curves (Fig. 1, A and B). No significant difference in either maximum force production or the sensitivity of the contractile apparatus to Ca\(^{2+}\) (Table 1) compared with control measurements was found in the presence of 50 µM clenbuterol in the EDL and soleus fibers.

The effect of clenbuterol on the E-C coupling in rat EDL and soleus fibers. In these experiments, we examined the effect of clenbuterol on force response triggered via the normal E-C coupling pathway. The skinned fibers used in this study retain an intact E-C coupling pathway. During the mechanical skinning process used to remove the sarcolemma from the fibers, the transverse tubular system seals and repolarizes after exposure to a K\(^{+}\)-HDTA solution. The transverse tubular system can then be depolarized, and depolarization-induced force responses can be elicited by exposing the preparation to an HDTA solution in which K\(^{+}\) has been replaced with Na\(^{+}\) (18).

Clenbuterol had a marked effect on the peak of depolarization-induced force responses in both skinned EDL and soleus fibers (Table 2). In the EDL fibers, 50 µM clenbuterol decreased the peak of depolarization-induced force responses by 23% compared to initial control responses (Fig. 2A, Table 2), and force responses similar to initial control measurements could be obtained after clenbuterol was removed (t-test, \(P = 0.78\), \(n = 10\)), showing that the effect of clenbuterol on depolarization-induced force responses in rat EDL fibers was reversible (Fig. 2A, Table 2). The presence of 50 µM clenbuterol had no significant effect on the half peak width of the depolarization-induced force responses evoked in the EDL fibers (paired t-test, \(P = 0.48\)).

Depolarization-induced force responses elicited in skinned soleus type I fibers were substantially more sensitive to clenbuterol than were the EDL fibers. The peaks of the depolarization-induced force responses elicited in the soleus type I fibers fell by ~45% in the presence of clenbuterol (Fig. 2B, Table 2) and failed to significantly recover after washout of the drug (Fig. 2B, Table 2). In contrast, in fast-twitch fibers the peak of the depolarization-induced force responses fell by only 23% in the presence of clenbuterol, and the effect was fully reversible. These results suggest that 50 µM clenbuterol causes marked, irreversible damage to E-C coupling in soleus fibers. No significant difference was found between force measurements made in presence of 50 µM clenbuterol and under control conditions, indicating that clenbuterol had no significant effect on maximum force or sensitivity of contractile apparatus to Ca\(^{2+}\) in skinned fibers.
The effect of clenbuterol on caffeine-induced force responses in rat EDL and soleus fibers. To test whether clenbuterol was inhibiting the depolarization-induced force responses in the EDL and soleus fibers by altering the caffeine-induced Ca\(^{2+}\)-release properties of the SR, fibers were initially exposed to an HDTA solution containing 30.0 mM caffeine and 0.75 mM EGTA to completely deplete the SR of Ca\(^{2+}\). The fibers were then reloaded with Ca\(^{2+}\) for a known length of time by exposure to a highly Ca\(^{2+}\)-buffered load solution (pCa 6.55). After this loading period, the fiber was reexposed to the caffeine-EGTA solution in either the presence or absence of 50 µM clenbuterol (Fig. 3, Table 1). These results indicate that 50 µM clenbuterol has no significant effect on caffeine-induced Ca\(^{2+}\) release or rate of force development in EDL and soleus fibers of the rat, and, therefore, the reduction in the peak of depolarization-induced force responses elicited in these fibers in the presence of clenbuterol must be due to inhibition at another stage of the E-C coupling process.

The effect of clenbuterol on SR Ca\(^{2+}\) loading in rat EDL and soleus fibers. To test whether clenbuterol was inhibiting the depolarization-induced force responses at the level of SR Ca\(^{2+}\) loading, we examined the effect of 50 µM clenbuterol on SR Ca\(^{2+}\) loading in skinned EDL fibers and soleus fibers. In these experiments, the SR of the fibers was initially depleted of Ca\(^{2+}\) and then reloaded with Ca\(^{2+}\) for a known length of time (see previous section). After this loading period, the fiber was reexposed to the caffeine-EGTA solution to release all releasable Ca\(^{2+}\) from the SR. The level of Ca\(^{2+}\) loading that had occurred during the loading process could then be estimated by measuring the area under the force response resulting from reexposure to the caffeine-EGTA solution (1). The integrals of caffeine-induced force responses elicited after loading in the presence and absence of 50 µM clenbuterol were compared.

In the skinned EDL and soleus fibers, SR Ca\(^{2+}\) loading was significantly reduced to 81.5 (n = 38) and 78.7% (n = 19) of control levels, respectively, in the presence of 50 µM clenbuterol (Fig. 4, A and B). This inhibition of SR Ca\(^{2+}\) loading was significantly reversible in both the EDL and soleus fibers, although the SR loading did not totally recover to original control levels (Table 2).

To provide additional evidence that the effects of clenbuterol on SR function were not related to its activity as a β\(_2\)-agonist, the experiments were repeated in the presence of propranolol (1 µM). In the presence of both clenbuterol and propranolol, SR Ca\(^{2+}\) loading in

### Table 1. Effects of clenbuterol on Ca\(^{2+}\) sensitivity of contractile apparatus and caffeine-induced SR force responses in rat EDL and soleus fibers

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<tr>
<th>Contractile Properties</th>
<th>Caffeine-Induced Responses</th>
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<tr>
<td></td>
<td>pCa(_{50})</td>
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<tr>
<td><strong>EDL</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.840 ± 0.018</td>
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<tr>
<td>Clenbuterol (50 µM)</td>
<td>5.841 ± 0.019(^a)</td>
</tr>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.099 ± 0.034</td>
</tr>
<tr>
<td>Clenbuterol (50 µM)</td>
<td>6.095 ± 0.034(^a)</td>
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</table>

Values are means ± SE. Contractile properties: effect of 50 µM clenbuterol on Ca\(^{2+}\) sensitivity of contractile apparatus in skinned fibers of extensor digitorum longus (EDL) and soleus muscles of rat. No significant difference was found in either pCa corresponding to 50% maximum force (pCa\(_{50}\)) or slope of Hill curves (t-test). \(^a\)P = 0.97, n = 5; \(^b\)P = 0.52, n = 5; \(^c\)P = 0.61, n = 9; \(^d\)P = 0.13, n = 9. Caffeine-induced responses: effect of clenbuterol on time to peak and peak of caffeine-induced sarcoplasmic reticulum (SR) force responses in rat EDL and soleus type I fibers. Clenbuterol did not significantly affect either time to peak or peak of caffeine-induced SR force responses in rat EDL (n = 9) or soleus (n = 8) fibers (t-test). \(^a\)P = 0.46; \(^b\)P = 0.95; \(^c\)P = 0.54; \(^d\)P = 0.37. The rate of SR Ca\(^{2+}\) release and/or rate of force development at the cross-bridge level.

No significant difference in the peak or time to peak of caffeine-induced force responses was found in either EDL or soleus fibers in the presence and absence of 50 µM clenbuterol (Fig. 3, Table 1). These results indicate that 50 µM clenbuterol has no significant effect on caffeine-induced Ca\(^{2+}\) release or rate of force development in EDL and soleus fibers of the rat, and, therefore, the reduction in the peak of depolarization-induced force responses elicited in these fibers in the presence of clenbuterol must be due to inhibition at another stage of the E-C coupling process.

### Table 2. Effects of clenbuterol on depolarization-induced force responses and SR Ca\(^{2+}\) loading in EDL and soleus fibers

<table>
<thead>
<tr>
<th>Depolarization-Induced Force Responses</th>
<th>SR Loading Responses</th>
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<tr>
<td>Peak responses in 50 µM clenbuterol, % of control</td>
<td>Mean area of force response after SR loading in 50 µM clenbuterol, % of initial control</td>
</tr>
<tr>
<td>Control</td>
<td>Recovery after SR loading</td>
</tr>
<tr>
<td>EDL</td>
<td>77.2 ± 9.0</td>
</tr>
<tr>
<td>Soleus</td>
<td>55.6 ± 5.4</td>
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</table>

Values are means ± SE. Depolarization-induced responses: effect of 50 µM clenbuterol on peak of force responses elicited by normal excitation-contraction coupling pathway in skinned EDL and soleus fibers. \(^a\)P = 0.029, n = 11; \(^b\)P < 0.0001, n = 9; \(^c\)P < 0.0001, n = 8 (t-test). SR loading responses: effect of 50 µM clenbuterol on SR Ca\(^{2+}\) loading in EDL and soleus fibers of rat. \(^a\)P < 0.0001, n = 38; \(^b\)P < 0.0001, n = 19 (t-test). SR loading in both EDL (t-test, P < 0.0001) and soleus (t-test, P = 0.0163) fibers significantly recovered. In soleus fibers, mean SR loading value after recovery was not significantly different from initial control values (t-test, P = 0.123). In EDL fibers, mean SR loading value after recovery was similar to that of soleus but was found to be significantly different from initial controls (t-test, P = 0.013).
skinned EDL fibers \((n = 14)\) was also reduced by a similar amount, to 82.8 ± 4.0% of control levels (paired \(t\)-test, \(P = 0.004\)), suggesting that the decrease in SR loading in the presence of clenbuterol is due to a direct effect of clenbuterol on SR function. In the presence of propranolol alone, SR Ca\(^{2+}\) loading in EDL fibers \((n = 14)\) was not significantly different from control measurements (paired \(t\)-test, \(P = 0.068\)).

The effect of clenbuterol on leak of Ca\(^{2+}\) from the SR of rat EDL and soleus fibers. The simplest hypothesis to explain the decrease in SR Ca\(^{2+}\) loading induced by clenbuterol is that this drug slows the SR Ca\(^{2+}\) pump. However, substances that slow or inhibit the SR Ca\(^{2+}\) pump typically produce significantly wider depolarization-induced force responses due to decreased SR Ca\(^{2+}\) uptake (1). In this study (see previous section), no significant difference was found between half peak width of the depolarization-induced force responses induced in EDL and soleus fibers in the presence and absence of 50 µM clenbuterol, which strongly suggests that 50 µM clenbuterol does not slow the SR Ca\(^{2+}\) pump in mammalian EDL and soleus fibers. However, mammalian skeletal muscle has recently been shown to possess a small passive Ca\(^{2+}\) leak from the SR (1). Therefore, it is possible that clenbuterol acts to decrease SR Ca\(^{2+}\) loading by increasing the passive leak of Ca\(^{2+}\) from the SR.

Fig. 2. Effect of 50 µM clenbuterol on peak of force responses elicited by normal excitation-contraction coupling pathway in skinned fibers from EDL (A) and soleus (B) muscles of rat. Clenbuterol decreased peak of depolarization-induced force responses in both EDL and soleus fibers. In all cases, fibers were exposed to a potassium hexamethylenediamine tetraacetate solution for 30 s between responses to allow repolarization of transverse tubular system. In clenbuterol responses, depolarization was preceded by a 30-s exposure to repolarization solution containing clenbuterol. Effect of clenbuterol on peak of depolarization-induced responses was reversible in EDL fibers but not in soleus fibers (last responses; A and B, right). Depol, depolarization; Repol, repolarization.

Fig. 3. Effect of 50 µM clenbuterol on caffeine-induced Ca\(^{2+}\) responses in EDL (A) and soleus (B) fibers of rat. The fibers were loaded with Ca\(^{2+}\) for fixed periods of time (EDL, 10 s; soleus, 7 s) in a highly buffered Ca\(^{2+}\) solution (pCa 6.55; note that all Ca\(^{2+}\) loading was undertaken in absence of clenbuterol in this experiment) before exposure to caffeine Ca\(^{2+}\)-release solution. Caffeine-induced responses in presence of clenbuterol were compared with control responses measured before and afterward. Note that in soleus fibers (B) the baseline after caffeine contracture did not return to original level shown before caffeine contracture. This was due to effect of caffeine increasing the sensitivity of contractile apparatus to Ca\(^{2+}\). Effect is visible in soleus fibers because the contractile apparatus of soleus is more sensitive to Ca\(^{2+}\) than is that of EDL (see control values of pCa corresponding to 50% of maximum force in Table 1). After removal of caffeine, baseline always quickly returned to original level, indicating that no damage to contractile apparatus had occurred.
leak of Ca$^{2+}$ from the SR of rat EDL and soleus fibers. To test this hypothesis, rat EDL and soleus fibers, previously depleted of Ca$^{2+}$, were loaded under control conditions and then reexposed to the caffeine release solution after first being exposed for 3.5 min to a Ca$^{2+}$ leak solution with or without 50 µM clenbuterol. The Ca$^{2+}$ leak solution contained 0.75 mM EGTA to chelate leaked Ca$^{2+}$ and to prevent its reuptake by the SR.

The normal passive SR Ca$^{2+}$ leak present in EDL and soleus skeletal muscle fibers reduced Ca$^{2+}$ loading (measured as the area under the force response) to 45.3 ± 8.9% (n = 6) and 56.8 ± 4.7% (n = 6), respectively, of initial controls under the conditions of this study. This normal passive SR Ca$^{2+}$ leakage in the EDL and soleus fibers was significantly elevated by the presence of 50 µM clenbuterol in the Ca$^{2+}$ leak solution (Fig. 5).

**DISCUSSION**

In this study, we examined the effect of the anabolic drug clenbuterol, a β$_2$-agonist, on single fast- and slow-twitch fibers of rat. The fibers were loaded with Ca$^{2+}$ by exposure to a highly buffered Ca$^{2+}$ solution (pCa 6.55) for fixed periods of time (EDL, 10 s; soleus, 7 s) and then exposed to a leak solution that contained 0.75 mM EGTA to sequester all leaked Ca$^{2+}$. Control Ca$^{2+}$ leak force responses elicited after 3.5 min of exposure to a Ca$^{2+}$ leak solution were compared with control force responses (no exposure to Ca$^{2+}$ leak solution, not shown) measured before and after measurement of force responses designed to measure SR Ca$^{2+}$ leak. Resulting control leak force responses demonstrate normal leak associated with skeletal muscle fibers (left and right). These responses were then compared with force responses elicited after exposure to a leak solution containing 50 µM clenbuterol (middle).
Recent studies examining the anabolic effects of clenbuterol might be found in vivo when the anabolic dose is given. This study showed that in the absence of the β2-agonist pathway, 50 µM clenbuterol still has marked detrimental effects on skeletal muscle depolarization-induced force responses, which, in the case of the soleus, were irreversible. The results of this study are consistent with previous reports of the increased detrimental effects of clenbuterol (administered orally) on slow-twitch muscle compared with fast-twitch muscle (11, 34).

In this study, we used a clenbuterol concentration of 50 µM because it ensured a rapid response (50 µM clenbuterol permanently damaged depolarization-induced force responses in all soleus fibers within 30 s) and because it represented the upper level of what might be found in vivo when the anabolic dose is given. Recent studies examining the anabolic effects of clenbuterol report use of a clenbuterol dose of 1–2 mg·kg

<sup>-1</sup>·day

<sup>-1</sup> injected subcutaneously (8, 26, 28). At 2 mg/kg, the maximum internal clenbuterol concentration expected, considering the volume of the rat, would be ~6 µM. However, clenbuterol is lipophilic and has been shown to accumulate within muscle fibers (23), and clenbuterol has a relatively long half-life of ~35 h (35). Therefore, chronic administration of the anabolic dose of clenbuterol could lead to clenbuterol levels in muscle well above 6 µM and closer to the 50 µM used in this study. It has recently been shown in adult rats that a single subcutaneous injection of only 250 µg/kg of clenbuterol rapidly induces degenerative changes in soleus fibers while having no damaging effect on the fast-twitch plantaris muscle (S. J. P. Damment, Glaxo, personal communication). Furthermore, considering the abuse of this drug within the athletic and bodybuilding communities, where it is common practice to administer anabolic substances in concentrations far in excess of the anabolic dose, the concentrations of clenbuterol occurring may be even higher. For example, within bodybuilding circles, users of anabolic steroids have been reported to commonly use up to 26 times the therapeutic dose (3). In the case of clenbuterol, higher doses are likely to severely reduce athletic performance rather than enhance it. It has recently been shown that clenbuterol significantly decreases exercise performance in mice (15, 19). Duncan (10) found that chronic clenbuterol treatment significantly reduced the ability of rats to exercise at high intensity and that chronic clenbuterol treatment in combination with exercise predisposed the animals to sudden death, presumably as a result of cardiac failure.

It has been reported that clenbuterol administration increases the proportion of fast-twitch fibers in the soleus muscle (8, 19, 36). In this study, it is shown that clenbuterol preferentially damages soleus type I fibers. Such damage in vivo could contribute to the increase in the number of fast-twitch fibers by selectively destroying a proportion of the slow-twitch type I soleus fibers. Administration of clenbuterol to mice has been reported to decrease the sensitivity of the contractile apparatus to Ca

<sup>2+</sup> in fast-twitch EDL fibers (19). However, the present study shows that clenbuterol has no effect on the Ca

<sup>2+</sup> sensitivity of the contractile apparatus in EDL fibers of the rat when added acutely to the experimental solutions, suggesting that chronic clenbuterol treatment has some permanent effect on the functional state of the contractile and regulatory proteins themselves.

Experiments examining the effect of clenbuterol on SR function in rat EDL and soleus fibers in the present study showed that the decrease in the size of depolarization-induced force responses in the presence of clenbuterol was due to a clenbuterol-induced increase in the rate of passive Ca

<sup>2+</sup> leak from the SR, which reduced SR Ca

<sup>2+</sup> levels, leaving less Ca

<sup>2+</sup> available for release in response to stimulation. A continuously raised intracellular Ca

<sup>2+</sup> concentration in the spatially restricted region of the triad due to increased SR Ca

<sup>2+</sup> leakage could damage E-C coupling, as elevated intracellular Ca

<sup>2+</sup> levels have recently been shown to damage E-C coupling in rat EDL muscle fibers (17). However, in this study, only the soleus fibers were irreversibly damaged by clenbuterol; the EDL fibers recovered almost fully. Therefore, it is possible that slow-twitch muscle is more susceptible to the damage to E-C coupling caused by raised Ca

<sup>2+</sup> levels than that observed in fast-twitch muscle in the rat, which may be able to cope with the increased level of intracellular Ca

<sup>2+</sup> concentration caused by the clenbuterol-induced increase in passive leak in these fibers. Interestingly, increased SR Ca

<sup>2+</sup> release or leak is thought to be the primary cause of malignant hyperthermia, and clenbuterol has been shown to significantly increase body temperature in rats (6) and induce hyperthermia in rats kept at high ambient temperature (24).

If an increased passive leak is also present in vivo in response to the anabolic dose of clenbuterol, the resulting raised Ca

<sup>2+</sup> levels may also play a role in promoting the anabolic effect of clenbuterol via, for example, the activation of the Ca

<sup>2+</sup>/calmodulin-dependent protein kinase, which has been shown to regulate gene expression in many cells (27). β-Adrenoceptor-mediated hypertrophy in neonatal rat cardiac myocytes has recently been shown to be mediated by a Ca

<sup>2+</sup>-dependent pathway involving the SR rather than by a pathway involving cAMP (2).

It is highly unlikely that the effect of clenbuterol on depolarization-induced force responses shown in the present study is due to clenbuterol-induced changes in membrane excitability, as the depolarization-induced force responses are activated by a mechanism equivalent to K

<sup>-</sup> depolarization of intact skeletal muscle fibers, in which the voltage sensors of the transverse tubular system are depolarized directly. Considering the substantial effects of clenbuterol on SR function, it would seem likely that alterations of SR function are ultimately responsible for the effect of clenbuterol on depolarization-induced force responses in mammalian slow-twitch mammalian skeletal muscle fibers of the rat. The fibers had been mechanically skinned to wash out soluble second messengers and remove β2-adrenergic receptors from the surface membrane and thus render the β2-agonist pathway inoperable. This study showed that in the absence of the β2-agonist pathway, 50 µM clenbuterol still has marked detrimental effects on skeletal muscle depolarization-induced force responses, which, in the case of the soleus, were irreversible. The results of this study are consistent with previous reports of the increased detrimental effects of clenbuterol (administered orally) on slow-twitch muscle compared with fast-twitch muscle (11, 34).
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Skeletal muscle. However, this study does not rule out the possibility that clenbuterol may also alter E-C coupling by affecting the charge movement of the voltage sensors.

In conclusion, this study shows that the anabolic drug clenbuterol has direct effects on SR function in fast- and slow-twitch skeletal muscle that are unrelated to the β2-activation pathway. Clenbuterol causes a net decrease in SR Ca2+ accumulation in skeletal muscle fibers due to a clenbuterol-induced increase in the passive Ca2+ leak from the SR. In slow-twitch soleus fibers, exposure to clenbuterol leads to permanent damage to the E-C coupling process. The results of this study have important implications for the safe use of clenbuterol in clinical applications.

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Address for reprint requests: A. J. Bakker, Dept. of Physiology, University of Western Australia, Nedlands 6007, Australia.

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