A Novel Thienylhydrazone, (2-Thienylidene)3,4-methylenedioxybenzoylhydrazine, Increases Inotropism and Decreases Fatigue of Skeletal Muscle

HUGO GONZALEZ-SERRATOS, RUZHANG CHANG, EDNA F. R. PEREIRA, NEWTON G. CASTRO, YASCO ARACAVA, PAULO A. MELO, PATRÍCIA C. LIMA, CARLOS A. M. FRAGA, ELIEZER J. BARREIRO, and EDSON X. ALBUQUERQUE

Departments of Physiology (H.G.-S., R.C.) and Pharmacology and Experimental Therapeutics (E.F.R.P., E.X.A.), University of Maryland School of Medicine, Baltimore, Maryland; and Departamento de Farmacologia Básica e Clínica (E.X.A., N.G.C., Y.A., P.A.M.), Instituto de Ciências Biomédicas, and Departamento de Fármacos (P.C.L., C.A.M.F., E.J.B.), Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT
This study was designed to investigate the effects on single skeletal muscle fibers of a novel thienylhydrazone, referred to as LASSBio-294, which is a bioisoster of pyridazinone compounds that inhibit the cyclic AMP-specific phosphodiesterase (PDE) 4. Twitch and fatigue were analyzed in single skeletal muscle fibers isolated from either the semitendinous or the tibialis anterior muscles dissected from the frog Rana pipiens. LASSBio-294 (12.5–100 μM) increased twitch tension, accelerated the maximal rate of tension decay during relaxation, and had very little effect in the maximal rate of tension development of muscle fibers directly stimulated at ≤30 Hz. The positive inotropic effect of LASSBio-294 developed slowly, reaching its maximum at 40 min and was inversely proportional to the frequency of stimulation, becoming negligible at 60 and 90 Hz. The concentration-response relationship for LASSBio-294-induced potentiation of twitch tension was bell-shaped, with maximal effect occurring at 25 μM. In addition, LASSBio-294 reduced development of fatigue induced by tetanic stimulation of the muscle fibers and reduced the time needed for 80% prefatigue tension recovery after fatigue had developed to 50% of the maximal pretetanic force. These effects of LASSBio-294 can be fully explained by stimulation of the sarcoplasmic reticulum Ca^{2+} pump and could be ascribed to an increase in cellular levels of cyclic AMP due to PDE inhibition. The novel thienylhydrazone LASSBio-294 may be useful for treatment of patients suffering from conditions in which muscle fatigue is a debilitating symptom (e.g., chronic heart failure).

Exertional skeletal muscle fatigue is a major debilitating symptom in patients suffering from a number of chronic disorders, including chronic heart failure (CHF) (Wilson, 1995, and references therein). Skeletal muscle fatigue develops gradually during all forms of exercise (for review, see Jones and Killian, 2000); however, it does so more rapidly in patients with CHF than in normal subjects (for review, see Bishop et al., 1998; Lunde et al., 1998). The enhanced skeletal muscle fatigue in patients with CHF is not due to impaired control of motor drive by the central nervous system or to dysfunctions in the neuromuscular transmission; rather, it is caused by intrinsic physiological changes in excitation-contraction (e-c) coupling in the skeletal muscle cells themselves (Perreault et al., 1993; Wilson, 1995; Bishop et al., 1998).

Skeletal and heart muscles share a number of properties, including regulation of contractile function by cyclic AMP (Gonzalez-Serratos et al., 1981), and evidence has been provided to support a unifying hypothesis that changes in heart and skeletal muscle inotropism associated with CHF are related to altered cyclic AMP content and/or responsiveness in the myocyte (Morgan, 1991; Perreault et al., 1993; Bishop et al., 1998). In fact, drugs that increase cytoplasmic levels of cyclic AMP, by either blocking its degradation or increasing its production, are recognized not only for their positive inotropic effects in cardiac and skeletal muscles (Gonzalez-Serratos et al., 1981; Francis et al., 2001) but also for their ability to reduce skeletal muscle fatigue (Bishop et al., 1998; Fujii et al., 1998).

A novel thienylhydrazone compound (2’-thienylidene)3,4-methylenedioxybenzoylhydrazine, herein referred to as LASSBio-294, was initially obtained as part of a program of synthesis of novel anti-inflammatory leads with an N-acylhydrazone skeleton (Figueiredo et al., 2000). Subsequently,

ABBREVIATIONS: CHF, chronic heart failure; e-c, excitation-contraction; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide; FT, fatigue tetanic tension; SR, sarcoplasmic reticulum; T, tetanic tension.
this compound was identified as a bioisoster of a family of pyridazinone compounds that increase cyclic AMP levels by inhibiting selectively the cyclic AMP-specific, low-$K_m$, PDE4 (Piaz et al., 1997). The present study was undertaken to investigate the effects of this novel thienylhydrazone in the contractile properties of single skeletal muscle fibers of the frog.

In addition to demonstrating that LASSBio-294 has positive inotropic effects in single fibers of phasic muscles of the frog, the results presented herein also show that the compound reduces fatigue development and accelerates recovery of maximal tetanic force after fatigue has developed. Some lines of evidence suggest that the effects of LASSBio-294 on skeletal muscle inotropism and fatigue can be accounted for by PDE inhibition. Thus, LASSBio-294, by virtue of its ability to increase force of contraction and decrease fatigue of skeletal muscles, can become part of the therapeutic interventions designed to decrease exertional fatigue, and, consequently, improve the quality of life of patients suffering from CHF (LeJemtel et al., 1986) and other physiopathological conditions in which skeletal muscle dysfunctions are associated with decreased levels of cytosolic cyclic AMP.

Materials and Methods

Preparations. Single skeletal muscle fibers were freshly isolated from either the semitendinous or the tibialis anterior muscles dissected from the frog *Rana pipiens* as previously described (Gonzalez-Serratos et al., 1981). After isolation, muscle cells remained in the dissecting dish for at least 30 min in Ringer’s solution, which consisted of 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$, 0.2 mM MgCl$_2$ (pH was adjusted with phosphates to 7.2). Next, the fibers were stimulated with single low-voltage electrical shocks. If they responded with brisk twitches and there were no signs of membrane damage, the fibers were used; otherwise, they were discarded. The sartorius muscle was also dissected out from *R. pipiens* for recordings of membrane and action potentials (see below). All experiments were carried out at room temperature (20–22°C).

Muscle Twitch and Fatigue. Each single fiber was transferred to the experimental chamber, which consisted of a 0.3-ml narrow channel where solutions could be changed several times within 5 s. In the chamber, one tendon was gripped with a small clamp while the other tendon was attached to the hook of an Ekhart type force transducer (Senso Nor, Horten, Norway). The stimulating electrode consisted of platinum wires placed on each side of the fiber. The muscle fiber was then stretched 1.3 times its slack length to reach an average sarcomere length of approximately 2.6 μm. Subsequently, the fiber was stimulated with single electrical pulses of 0.5-ms duration with variable voltage. The voltage was steadily increased until the threshold for contraction was reached. This voltage was then increased by 50%, and the experimental protocol was started.

The stimulating protocol consisted of a series of single twitches elicited every 3 s until the peak twitch force was the same for five consecutive times. The twitches were followed by different frequencies of tetanic stimulation of 10, 30, 60, and 90 Hz. Each fiber rested for 3 min between each of the tetanic stimulations. When the whole series of stimulations was repeated, the fiber was allowed to rest for 10 min between each series of twitches and tetanic stimulations. Fatigue was induced by repetitive cycles of electrical stimulation. Each cycle consisted of a train of electrical shocks delivered at 60 Hz for 0.8 s followed by a single twitch after 2.2 s and repeated every 4.75 s.

Intracellular Recordings. Resting membrane potential and action potentials were recorded intracellularly during stimulation of frog sartorius muscles. By means of two fine external electrodes, one or two fibers were stimulated on the surface of sartorius muscles that were previously stretched 1.6 times their equilibrium lengths. Under this condition, it was possible to record membrane potentials from a stimulated fiber without damaging it during the contraction by using floating recording microelectrodes (Bezanilla et al., 1972). The recording microelectrode was connected through a Ag-AgCl electrode.

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Fig. 1. A. structural similarity between LASSBio-294 and bioactive pyridazinones. B, schematic representation of the synthetic route to the novel thienylhydrazone LASSBio-294.

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a) KOH, nBuOH; b) O$_2$, MeOH, Zn/AlCl$_3$; c) I$_2$, KOH, MeOH; d) NH$_2$H$_2$O, ETOH; e) ETOH, aq. HCl

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to the input of a cathode follower with negative capacity compensation (World Precision Instruments, Sarasota, FL).

**Drugs.** LASSBio-294, 2'-thienylidene 3,4-methylenedioxybenzoylhydrazone or 3,4-methylenedioxybenzoyl-2'-thienylhydrazone or N-(1E)-1-aza-2-(2-thienylvinyl)-2H-benzo[3,4,d]1,3-dioxolan-5-yl-carboxamide, was synthesized as part of a program designed to develop a series of new N-acylhydrazone derivatives with anti-inflammatory properties (Figueiredo et al., 2000) in the Departamento de Farmacos, Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. Based on principles of nonclassical bioisosterism, LASSBio-294 was identified as a bioisoster of bioactive 6-aryl-4,5-heterocyclic-fused pyridazine compounds (Fig. 1A) that are potent and selective inhibitors of PDE4 (Piaz et al., 1997). Briefly, according to the procedure described previously (Lima et al., 2000), LASSBio-294 was synthesized from the starting material safrole (4-allyl-1,2-methylenedioxybenzene), which is a readily available natural substance present in sassafras oil (Fig. 1B). The first step in the synthesis consisted of a base-catalyzed isomerization of safrole (1) that yielded to isosafrole (2). By oxidative cleavage, isosafrole was converted into piperonal (3). Then, by using the Yamada’s oxidative procedure, piperonal (3) was “one-pot” converted, with 90% yield, in the methyl 3,4-methylenedioxybenzoate (4). Subsequently, the key 3,4-methylenedioxyhydrazine (5) was obtained with 70% yield by means of nucleophilic substitution of the ester (4) with hydrazine hydrate in ethanol at reflux for 3.5 h. Finally, LASSBio-294 was obtained with 75% yield by condensing the hydrazine derivative with equimolar amounts of thioephene-2-carboxaldehyde in ethanol by using hydrochloric acid as a catalyst.

A stock solution containing LASSBio-294 (50 mM) was obtained by dissolving the compound in DMSO. A solution containing 100 μM LASSBio-294 was prepared by diluting the stock solution in Ringer’s solution under sonication. The final test concentrations were obtained by further diluting the Ringer’s solution containing 100 μM LASSBio-294. Because DMSO was used as a dispersing agent to dissolve LASSBio-294, the Ringer’s solution under control conditions contained the same amount of DMSO as that present in the test solutions with LASSBio-294.

**Results**

**Effects of LASSBio-294 on Force of Contraction of Skeletal Muscle Fibers: Time and Concentration Dependence.** To investigate the effect of LASSBio-294 on muscle twitch tension, single fibers of the frog semitendinosus or tibialis anterior muscles were directly stimulated at 0.1 Hz in the absence and in the presence of different concentrations of the compound. Three minutes after the last control twitch was recorded, the fibers were perfused continuously with Ringer’s solution containing LASSBio-294 (12.5, 25, 75, or 100 μM), and twitch tension was analyzed at various times. LASSBio-294 increased twitch tension (Fig. 2A). The onset of the positive inotropic effect of LASSBio-294 (25 μM) was observed at about 5 min after starting perfusion of the muscle fibers with the drug; the effect was maximal at 40 min after starting perfusion of the fibers (Fig. 2B).

To determine the magnitude of the positive inotropic effect of LASSBio-294, the fibers were stimulated at 0.1 Hz before and after 40-min perfusion with solution containing various concentrations of the drug. The concentration-response relationship for LASSBio-294-induced potentiation of muscle twitch was bell-shaped (Fig. 2C). At 12.5 μM, LASSBio-294 caused a small, albeit significant increase in twitch tension. The magnitude of the positive inotropic effect of LASSBio-294 was significantly enhanced upon increasing the concentration of the drug to 25 μM (Fig. 2C). However, the positive inotropic effect of LASSBio-294 decreased at concentrations ≥50 μM (Fig. 2C).

**Positive Inotropic Effect of LASSBio-294 Depends on Frequency of Stimulation and Is Slowly Reversible.** As indicated above, 40-min perfusion of single muscle fibers with Ringer’s solution containing LASSBio-294 (12.5 μM) caused a small increase in twitch tension. However, when the fibers were stimulated at 10 or 30 Hz 17 min after they had been continuously bathed with LASSBio-294 (12.5 μM), twitch tension was increased further (Fig. 3, A and C). The effect of LASSBio-294 (12.5 μM) on maximal twitch tension decreased as the frequency of stimulation was increased to 60 Hz and became negligible when the fibers were stimulated at 90 Hz. Similar effects were observed when the muscle fibers were exposed to 25 μM LASSBio-294 (Fig. 3, B and C).

The positive inotropic effect of LASSBio-294 was not easily

![Fig. 2. Effects of LASSBio-294 on twitch tension of single skeletal muscle fibers. A, twitch tension was recorded from a single skeletal muscle fiber before (control) and 40 min after perfusion with Ringer’s solution containing LASSBio-294 (25 μM). B, time dependence of the positive inotropic effect of LASSBio-294 (25 μM). Maximal twitch tension recorded under control condition (T0) was taken as 1 and used to normalize the maximal twitch tension (Tn) recorded at various times in the presence of LASSBio-294. Symbols and bars represent mean and S.E.M., respectively, of results obtained from three fibers. C, concentration dependence of the positive inotropic effect of LASSBio-294. Peak twitch tension (Tn) measured prior to exposure of the fibers to a given concentration of LASSBio-294 was taken as 1 and used to normalize the peak twitch tension (Tn) measured after 40-min exposure of the fibers to the compound. Symbols and bars represent mean and S.E.M., respectively, of results obtained from four experiments. *p < 0.05 and **p < 0.01, according to the paired Student’s t test.](https://jpet.aspetjournals.org/article/S0022-3514(00)01219-0/DC1/fig2.jpg)
reversed upon washing the preparations with drug-free Ringer’s solution (Fig. 3, A, B, and D). A 40-min exposure of muscle fibers \((n = 3)\) to LASSBio-294 (25 \(\mu M\)) increased by \(35 \pm 10\%\) the twitch tension elicited by 0.1-Hz stimulus (Fig. 3D). After a 17-min wash of the preparation with Ringer’s solution and subsequent exposure to LASSBio-294 (25 \(\mu M\)), twitch tension was 57 \(\pm 14\%\) higher than that recorded under control condition. This could be explained by a cumulative effect of the drug. After further washing the preparations with drug-free solution, there was an additional increase in twitch tension (Fig. 3D). A subsequent exposure of the washed preparations to LASSBio-294 (25 \(\mu M\)) reduced twitch tension; however, twitch tension did not return to control levels. It was still 71 \(\pm 14\%\) larger than that recorded prior to exposure of the fibers to LASSBio-294 (25 \(\mu M\)).

**Effects of LASSBio-294 on Time Course of Twitch Tension.** The time course of development of twitch tension was only slightly altered by LASSBio-294. The total duration of the twitch was not significantly altered after 40-min perfusion of the skeletal muscle fibers with Ringer’s solution containing LASSBio-294 (25 \(\mu M\)). Time to peak tension \(T_p\) and relaxation times at 50\% \(T_{0.5}\) and 80\% \(T_{0.8}\) of peak force were about the same in the absence and in the presence of the drug (Table 1). The maximal rate of tension development \(T_{V_{\text{max}}}\) during the twitch was also not altered by LASSBio-294 (Table 2). Although LASSBio-294 (25 \(\mu M\)) had no significant effect on the total duration of the twitch, it accelerated the maximal rate of tension decay \(-T_{V_{\text{max}}}\). \(-T_{V_{\text{max}}}\) was approximately 1.18 times faster in the presence than in the absence of the drug (Table 2).

**LASSBio-294 Causes No Changes in Resting Membrane Potential or Action Potentials of Frog Sartorius Muscle.** The positive inotropic effect of LASSBio-294 could be explained by changes in the first step of the e-c coupling, i.e., the sarcolemmal action potential. If LASSBio-294 would, for example, prolong the action potential and, thereby the

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**Fig. 3.** Effects of LASSBio-294 on force development of skeletal muscle fibers stimulated at various frequencies and during wash. A and B, effects of LASSBio-294 (12.5 and 25 \(\mu M\)) on force development in single muscle fibers stimulated at 0.1 (single twitch, st), 10, 30, 60, and 90 Hz, before (control), after 40-min perfusion with Ringer’s solution containing a given concentration of LASSBio-294, and 17 min after perfusion with drug-free Ringer’s solution (wash). C, effects of LASSBio-294 (12.5 and 25 \(\mu M\)) on fractional twitch potentiation at the different frequencies of stimulation. Symbols and bars represent mean and S.E.M. of results obtained from four experiments. D, plots of fractional twitch tension calculated from data obtained from muscle fibers during various recording times under the experimental conditions indicated by the dotted and continuous lines. Symbols and bars represent mean and S.E.M., respectively, of results obtained from four fibers. The calibration bars shown in the top traces A and B apply to the lower traces of A and B.
mechanically effective period, it could increase the amount of the contractile activator Ca\(^{2+}\) released. The effect could also be explained by sarcolemmal Ca\(^{2+}\) conductance changes that would be reflected in the shape of the action potential and/or in the value of the resting membrane potential. Therefore, the effects of LASSBio-294 on action and resting potentials were investigated.

Resting membrane potential of sartorius muscle continuously perfused with Ringer’s solution was about −86 mV and was not altered after perfusion of the fibers for 30 to 50 min with solution containing LASSBio-294 (12.5 and 25 μM). Also unaltered were the amplitude and the duration of the action potential (Table 3). Thus, the positive inotropic effect of LASSBio-294 cannot be accounted for by changes in the conductance properties of the muscle fibers.

Effects of LASSBio-294 on Fatigue Development. Muscle fatigue can be induced by prolonged, direct electrical stimulation that leads to a state during which the contractile force declines to the point where the muscle becomes mechanically refractory to further stimulation. Fatigue is the result solely of contractile failure of the muscle involved (Garcia et al., 1991). The finding that LASSBio-294 has a positive inotropic effect in phasic skeletal muscle cells raised the question of whether the drug could also alter fatigue development. To answer this question, single fibers of the semitendinosus or tibialis anterior muscles were fatigued by repetitive cyclic tetanic stimuli (see Materials and Methods) in the presence and in the absence of LASSBio-294 (12.5, 25, or 50 μM). Recovery after fatigue development, produced with intermittent repetitive tetanic stimulations of the type used here, is not always 100%, and the time it takes for recovery varies from fiber to fiber. Therefore, each fatigue experiment in this series was performed on a different fiber.

LASSBio-294 prolonged the time needed for tetanic force to start declining (Fig. 4). To compare curves of fatigue development from different preparations, a fatigue index was estimated at various times of stimulation by taking the ratio of maximum tetanic tension produced during every third tetanus to the tension output in the first tetanus, i.e., \(T_{p}/T_{o}\) (Perreault et al., 1993). The relationship between fatigue index and different cycles of stimulation obtained from several experiments was significantly altered by LASSBio-294 in a concentration-dependent manner (Fig. 5). In agreement with previous studies (Perreault et al., 1993), the rate at which maximal tetanic tension decayed during development of fatigue changed continuously, becoming slower as fatigue developed in muscles perfused with DMSO-containing Ringer’s solution (Fig. 5). The fatigue indexes decreased more slowly at all times in muscle fibers bathed in LASSBio-294 than in control muscle fibers; the higher the concentration of LASSBio-294, the slower the fatigue index decayed.

The effects of LASSBio-294 were also analyzed in three distinct time segments of fatigue development: \(T_{p}\), \(T_{0.5}\), and \(T_{0.8}\). Segment \(T_{p}\) indicates the time from the beginning of the repetitive stimulation to the beginning of the decrease in fatigue index, i.e., the beginning of fatigue development. Segments \(T_{0.5}\) and \(T_{0.8}\) correspond to the time required to decrease the fatigue index by 50 and 80%, respectively. Table 4 summarizes the results obtained from all the experiments done with fibers perfused with DMSO-containing Ringer’s solution (control) and with Ringer’s solution containing different concentrations of LASSBio-294. All three segments of fatigue development were prolonged by LASSBio-294 (Table 4). \(T_{p}\) was 52 and 94% longer in the presence of 12.5 and 50 μM LASSBio-294, respectively, than in the absence of the drug. In addition, \(T_{0.5}\) and \(T_{0.8}\) were 41 ± 7.3 and 52 ± 9.4% longer in the presence of 12.5 μM LASSBio-294. Likewise, in the presence of 50 μM LASSBio-294, \(T_{0.5}\) and \(T_{0.8}\) were on average 149 and 282%, respectively, longer than in the absence of the drug. In other words, 50 μM LASSBio-294 prolonged by approximately 2.5-fold the time needed for tetanic force to decrease by 80% of the pre-fatigue tetanic force. The drug prolonged the time for fatigue recovery to an even greater extent, the higher the concentration of LASSBio-294 used.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>(T_{p}) (ms)</th>
<th>(T_{0.5}) (ms)</th>
<th>(T_{0.8}) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36 ± 2.9</td>
<td>35 ± 1.5</td>
<td>55 ± 2.5</td>
</tr>
<tr>
<td>LASSBio-294 (25 μM)</td>
<td>41 ± 0.33</td>
<td>33 ± 2.5</td>
<td>52 ± 3.2</td>
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</table>

\(T_{p}\), time to peak tension; \(T_{0.5}\), time to 50% relaxation; \(T_{0.8}\), time to 80% relaxation.

### Results

Ratios express the maximal rate values estimated from recordings obtained 40 min after perfusion of single skeletal muscle fibers with Ringer’s solution containing LASSBio-294 (25 μM) divided by the values estimated from recordings obtained under control condition. Results are presented as mean ± S.E.M. (n = 3).

\[+T_{\text{Vmax}}, \text{maximal rate of twitch tension development}; \quad -T_{\text{Vmax}}, \text{maximal rate of twitch tension decay.}\]

\[\text{Ratio} = \frac{+T_{\text{Vmax}}}{-T_{\text{Vmax}}} \]

### Discussion

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### Materials and Methods

Values were estimated from recordings obtained before and 30 to 50 min after perfusion of frog sartorius muscles with Ringer’s solution containing LASSBio-294 (12.5 or 25 μM). Results are presented as mean ± S.E.M. (n = 3 fibers from 2 intact muscles). According to the paired Student’s t test, all results obtained from muscle fibers under control conditions were not significantly different from those obtained under LASSBio-294 exposure.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>(V_{R}) (mV)</th>
<th>(V_{p}) (mV)</th>
<th>(V_{Ap}) (mV)</th>
<th>(\Delta T) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−86.2 ± 0.91</td>
<td>33.0 ± 6.12</td>
<td>119.2 ± 5.39</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>LASSBio-294 (12.5 μM)</td>
<td>−88.2 ± 3.06</td>
<td>27.3 ± 3.76</td>
<td>115.5 ± 6.68</td>
<td>1.29 ± 0.08</td>
</tr>
<tr>
<td>LASSBio-294 (25 μM)</td>
<td>−82.4 ± 0.35</td>
<td>25.8 ± 3.96</td>
<td>108.0 ± 3.73</td>
<td>1.12 ± 0.08</td>
</tr>
</tbody>
</table>

\(V_{R}\), resting membrane potential; \(V_{p}\), magnitude of the peak of the action potential above the reference potential; \(V_{Ap}\), magnitude of the action potential, i.e., \(V_{p} - V_{sc}\); \(\Delta T\), duration of the action potential at 0.5 V_{Ap}.
to develop, because it prolonged $F_T_p$ and slowed down the rate at which tension declined.

As seen in Table 5, the average slope of maximal tetanic tension decline during fatigue development from preparations bathed with 12.5, 25, and 50 $\mu$M LASSBio-294 were 1.22, 1.40, and 1.5 times smaller, respectively, than the corresponding control values. From the average slopes it was estimated that 12, 25, and 50 $\mu$M LASSBio-294 would have incremented by 12, 61, and 208%, respectively, the time needed for maximal tetanic force to reach zero as a consequence of fatigue development.

After the muscle cells were fatigued to 50% of the maximal control tetanic force, the time needed for this tetanic tension to recover to prefatigue tetanic levels was significantly de-
Summary of time parameters of fatigue development in the absence and in the presence of LASSBio-294

<table>
<thead>
<tr>
<th>LASSBio-294 (µM)</th>
<th>n</th>
<th>FT&lt;sub&gt;p&lt;/sub&gt;</th>
<th>FT&lt;sub&gt;0.5&lt;/sub&gt;</th>
<th>FT&lt;sub&gt;0.8&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>Ratio</td>
<td>Average</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>118 ± 6.29</td>
<td>1.57</td>
<td>163 ± 6.49</td>
</tr>
<tr>
<td>12.5</td>
<td>8</td>
<td>179 ± 7.50**</td>
<td>1.50</td>
<td>228 ± 8.37***</td>
</tr>
<tr>
<td>25</td>
<td>8</td>
<td>163 ± 7.60**</td>
<td>1.38</td>
<td>219 ± 5.92***</td>
</tr>
<tr>
<td>50</td>
<td>11</td>
<td>229 ± 12.5***</td>
<td>1.94</td>
<td>405 ± 16.5***</td>
</tr>
</tbody>
</table>

FT<sub>p</sub>, time from the beginning of the repetitive stimulations to the beginning of the decrease in fatigue index; FT<sub>0.5</sub>, time from the beginning of the repetitive stimulations to 50% decrease in fatigue index; FT<sub>0.8</sub>, time from the beginning of the repetitive stimulations to 80% decrease in fatigue index.

**&*** p < 0.001, according to the unpaired Student's t test.

### Discussion

This study demonstrates that the novel thienylhydrazone LASSBio-294 has positive inotropic effects in single fibers of frog phasic skeletal muscles. It also shows that in skeletal muscles the compound reduces development of fatigue and accelerates the recovery of maximal tetanic tension after fatigue is developed. LASSBio-294 can, therefore, be effective to treat skeletal muscle fatigue associated with e-c coupling dysfunctions resulting from decreased cyclic AMP content and/or responsiveness of skeletal myocytes.

LASSBio-294 Increases Force of Contraction of Single Skeletal Muscle Fibers by Altering SR Ca<sup>2+</sup> Uptake and Release. The positive inotropic effect of LASSBio-294 was characterized by an increase in twitch tension, an acceleration of the maximal rate of tension decay during relaxation, and no significant changes in the maximal rate of tension development or twitch duration. In addition, it was inversely proportional to the frequency of stimulation, becoming negligible at 60 and 90 Hz. At these frequencies, Ca<sup>2+</sup> released from the SR maintains cytosolic Ca<sup>2+</sup> levels above the binding capacity of troponin C. Thus, maximal tetanic force induced by stimuli of such frequencies can only be potentiated by a compound that increases phosphorylation of the actomyosin complex, which modulates the Ca<sup>2+</sup> sensitivity of the actomyosin-ATPase complex. The finding that LASSBio-294 does not increase maximal tetanic force at 60 or 90 Hz strongly suggests that this novel thienylhydrazone acts by increasing cellular Ca<sup>2+</sup> levels.

The positive inotropic effect of LASSBio-294 cannot be attributed to changes in Ca<sup>2+</sup> entry into the muscle cell (Curtis, 1970). First, if LASSBio-294 altered Ca<sup>2+</sup> conductance, it would have affected the action potential and/or the...
resting membrane potential of the single muscle fibers. Second, changes in Ca^{2+} conductance appear to have no important role in e-c coupling or twitch development in phasic skeletal muscles (Gonzalez-Serratos et al., 1982).

Increased activity of the SR Ca^{2+} ATPase is the mechanism that best explains the inotropic effect of LASSBio-294. The increased activity of the pump results not only in larger SR Ca^{2+} accumulation but also in larger efficiency of clearance of cytosolic Ca^{2+}. The extra Ca^{2+} accumulated in the SR, once released during activation, increases twitch tension. On the other hand, faster removal of the extra Ca^{2+} released during activation results in decreased binding of Ca^{2+} to troponin C, thus accelerating the relaxation process. The bell-shaped relationship between LASSBio-294 concentration and twitch tension is likely the result of the effects of increased SR Ca^{2+} uptake partially counteracting those of increased SR Ca^{2+} release.

The slow onset and development of the effect of LASSBio-294 on twitch tension are in agreement with an intracellular mechanism of action. The slow reversibility of the effect of LASSBio-294 on twitch tension can be explained by 1) the high lipophilicity of the compound, 2) the time it takes for the increased SR Ca^{2+} content to return to control levels, and/or 3) slow recovery of metabolic changes that might underlie the effect. Potentiation of twitch tension by substances that increase SR Ca^{2+} content by interfering with the cyclic AMP metabolism in frog skeletal muscles is also slowly reversible (Kirchberger et al., 1974; Gonzalez-Serratos et al., 1981).

**LASSBio-294 Decreases Muscle Fatigue Development: Involvement of Cyclic AMP-Dependent Mechanisms.** LASSBio-294 reduced fatigue development of skeletal muscle fibers and accelerated recovery of maximal tetanic tension after fatigue developed. There are two main theories regarding the cellular mechanisms of fatigue. One theory, referred to as the metabolic hypothesis, suggests that fatigue of type II (fast-twitch) skeletal muscles is caused by alterations in the intracellular concentrations of ATP hydrolysis by-products (P_i, H^+, and Mg^{2+}) that result in decreased force-generating capacity (Godt and Nosek, 1989; Chin and Allen, 1998). According to the other theory, referred to as the e-c coupling hypothesis, during repetitive stimulation, there is substantial K^+ accumulation in the transverse tubular system. The Na^-K^+ ATPase, which is localized in the T-system (Dombrowski et al., 1996), then operates at full capacity and becomes unable to remove the excess of tubular K^+ (Clausen, 1996). The increased tubular K^+ concentration depolarizes the tubular membrane, causing failure in generation and/or propagation of tubular action potentials and reduction of SR Ca^{2+} release. Fatigued muscles also have a swollen T-system (Gonzalez-Serratos et al., 1978; Somlyo et al., 1978) that contributes to uneven conduction of action potentials along the T-system (Juel, 1988) and/or to improper signaling between the T-system and the terminal cisternae of the SR (Garcia et al., 1991). Reduced SR Ca^{2+} release in fatigued muscles, which is accompanied by increased resting levels of cytoplasmic Ca^{2+} (Chin and Allen, 1996) and myofibril inactivation (Garcia et al., 1991; Edman and Lou, 1992), especially during the fast decay of tension development, appears to be a predominant factor in the progression of fatigue. Fatigue development induced by repetitive tetanic stimulations of the type used here involves both metabolic and e-c coupling mechanisms.

Alleviation of fatigue development takes place when cytosolic levels of cyclic AMP are increased in type I, slow-twitch (Juel, 1988; Chen and Alway, 2001) and type II, fast-twitch (Clausen, 1996) skeletal muscle fibers as well as in the diaphragm, which has both types of fibers (Kolbeck and Speir, 1991). Thus, the effects of LASSBio-294 on muscle fatigue can be explained by several mechanisms that have a common start point, i.e., cyclic AMP. These mechanisms include, but are not restricted to 1) cyclic AMP-dependent phosphorylation of ryanodine receptors (Cairns et al., 1993); 2) indirect activation of SR Ca^{2+} ATPase by cyclic AMP-dependent phosphorylation of phospholamban (Kirchberger et al., 1974; Gonzalez-Serratos et al., 1981; Schwinger et al., 1999); and 3) cyclic AMP-dependent stimulation of the Na^-K^+ ATPase, which reduces tubular K^+ accumulation allowing full generation and propagation of tubular action potentials.

**PDEs as Potential Molecular Targets for LASSBio-294.** An increase of cellular cyclic AMP levels by LASSBio-294 could explain the effects of the drug on muscle twitch and fatigability. The pleiotropic nature of the intracellular effects of cyclic AMP makes this an attractive explanation of the drug’s effect, because only the simultaneous interaction with several of the relevant targets could have explained the positive inotropic and antifatigue effects of LASSBio-294.

There are two possible mechanisms by which LASSBio-294 could increase cytosolic cyclic AMP concentration. One encompasses direct stimulation of cyclic AMP production, possibly mediated by the interaction of LASSBio-294 with adenylyl cyclase, G_s proteins, or β-adrenoceptors (Gonzalez-Serratos et al., 1981; Van Der Heijden et al., 1998). Another consists of PDE inhibition, and consequently, reduction of cyclic AMP breakdown. Inhibition of different PDE isoforms has positive inotropic effects in cardiac and skeletal muscles (Gonzalez-Serratos et al., 1982; Alvarez et al., 1986; Morner and Mansson, 1990; Bishop et al., 1998). In addition to the cyclic AMP-specific PDE4, which is bound to myofibrils and is present in the SR of skeletal muscles (Worby et al., 1992; Francis et al., 2001, and references therein), several PDE isoforms have been found in skeletal muscles, including PDE1B, PDE2, PDE3, PDE5A, PDE7B, and the newly characterized PDE11 (Ball et al., 1980; Lobbert et al., 1996; Francis et al., 2001, and references therein).

Although the exact molecular target for LASSBio-294 is yet to be identified, it is tempting to speculate that the effects of the drug are mediated by its interaction with a PDE isoform. First, the effects of LASSBio-294 in frog skeletal muscles resemble those of other PDE inhibitors (Mansson and Edman, 1985; Morner and Mansson, 1990). Second, this thiénylhydrazone is a biosoister of pyridazine compounds that are selective and potent inhibitors of PDE4 (Piaz et al., 1997). Third, LASSBio-294 has cardiotonic properties that can be explained by PDE inhibition in the cardiovascular system (Sudo et al., 1998).

**Potential Therapeutic Applications of LASSBio-294.** Abnormal skeletal muscle contractility, metabolism, and easy fatigability are major debilitating symptoms in patients with CHF (Buller et al., 1991). Phasic skeletal muscles from rats with CHF also have abnormalities in e-c coupling and fatigue faster than muscles from normal rats (Perreault et al., 1993). These changes are very similar to those seen in the myocardium of laboratory animals and humans with CHF (Morgan, 1991) and are most likely due to reduced cyclic...
AMP content and/or responsiveness of the skeletal muscle fibers without atrophy (Perreault et al., 1993; Bishop et al., 1998). Indeed, increasing cyclic AMP levels not only ameliorates cardiac function but also alleviates the depressed twitch tension and reduces fatigue of skeletal muscles in animal models of CHF (Grossman et al., 1996; Bishop et al., 1998). Thus, it is conceivable that, by increasing cyclic AMP, LASSBio-294 can improve skeletal muscle function in CHF patients.

Exercise tolerance is an important predictor of survival and quality of life in CHF patients. In fact, many investigators believe that therapeutic interventions designed to improve skeletal muscle function in CHF patients are superior to those designed exclusively to increase cardiac muscle inotropism (Blackwood et al., 1990, and references therein). Therefore, LASSBio-294, by virtue of its ability to increase inotropism and reduce fatigue of skeletal muscles, is a potential candidate compound for treatment of CHF.

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References


Bezannela F, Caputo C, Gonzalez-Serratos H, and Venosa RA (1972) Sodium depen-
tial candidate compound for treatment of CHF.


Piao VD, Giovannoni MP, and Castellana C (1997) New heterocyclic fused pyridiza-


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Address correspondence to: Edson X. Albuquerue, M.D., Ph.D., Depart-
ment of Pharmacology and Experimental Therapeutics, University of Mary-
land School of Medicine, 655 W. Baltimore St., Baltimore, MD 21201. E-mail:
ealbueke@umaryland.edu