Inotropic Effects of Salbutamol on Rat Diaphragm Contractility are Potentiated by Foreshortening

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The aim of this study was to investigate whether the positive inotropic effects of the β2-adrenoceptor agonist salbutamol are increased by foreshortening of the diaphragm, and to determine the mechanism of action of these effects. Diaphragm strips were studied either at optimal resting length (Lo) or at ~70% Lo. In an initial experiment (Experiment I) salbutamol was added to the tissue baths in concentrations of 10 μg/L or 80 μg/L. In a second experiment (Experiment II), the effect of salbutamol (80 μg/L) was measured in the presence of 1 μM ryanodine, a sarcoplasmatic reticulum (SR) Ca2+-release inhibitor. Each experiment had a time-matched control group. Foreshortening reduced twitch force (Pt), maximal tetanic force (Po), and force–frequency curves. Salbutamol increased Pt and Po both at Lo and ~70% Lo. These inotropic effects were significantly greater after foreshortening. The force–frequency curve was shifted upward by salbutamol at both lengths. Force–frequency curves relative to maximal percent of Po were depressed by salbutamol at stimulation frequencies of 80 to 160 Hz. Ryanodine blocked the inotropic effect of salbutamol at both muscle lengths, indicating that these inotropic effects are probably mediated by increased SR Ca2+ release. van der Heijden HFM, Dekhuijzen PNR, Folgering H, van Herwaarden CLA. Inotropic effects of salbutamol on rat diaphragm contractility are potentiated by foreshortening.

Foreshortening of the diaphragm is present during acute hyperinflation, a condition frequently observed in patients with obstructive airways disease. Increasing lung volume from functional residual capacity (FRC) to total lung capacity (TLC) shortened the canine diaphragm by ~30% (1), and inflation from residual volume (RV) to TLC shortened the human diaphragm by ~40% (2). As a result of this foreshortening, transdiaphragmatic pressure generation in humans decreased (3, 4). Furthermore, this decrease in force caused by shortening of diaphragm fiber lengths in vitro (5) and with increasing lung volumes in vivo (6) was shown to be augmented by fatigue.

Recent studies demonstrated that the inotropic effects of methylxanthines were augmented by foreshortening or hyperinflation. Gayan-Ramirez and colleagues found an increase in animal diaphragm twitch-force (Pt) development in vitro (7, 8) and in vivo (9). Gauthier and associates described similar effects in healthy humans (4). These latter authors reported that aminophylline also prevented the augmented decrease in force generation found after fatigue at high lung volumes (4).

It is unknown whether this increase in inotropic effect at short diaphragm lengths and the protection against fatigue are characteristic of methylxanthines, or whether other inotropic agents, such as β2-adrenoceptor agonists, exert similar effects. Both classes of agents are thought to increase Ca2+ release from the sarcoplasmic reticulum (SR) (10–12), but the inotropic effect of aminophylline was also influenced by extracellular calcium (13). Recently, we found that the β2-adrenoceptor agonist salbutamol improved diaphragm Pt, maximal tetanic force (Po), and submaximal tetanic force in vitro at optimal length (Lo) (14). We hypothesized that as with the findings for aminophylline, the inotropic effect of salbutamol is augmented by foreshortening of the diaphragm, and that improvement of SR Ca2+ release is likely to be involved. To test this hypothesis, we studied the influence of foreshortening on the inotropic effect of salbutamol on in vitro contractile properties of rat diaphragm strips both at Lo and at ~70% Lo. In a second set of experiments, we investigated whether the inotropic effect of salbutamol could be blocked by the SR Ca2+-release inhibitor ryanodine.

METHODS

Animals and Study Design

Adult male outbred Wistar rats (n = 63), aged 18 wk and with a mean weight of 521 ± 6 (mean ± SE) g, were used. The animals were housed under standard conditions and were fed ad libitum. Animals were randomly allocated to treatment groups. Two experiments were performed.

In the first experiment (Experiment I), 39 rats were studied in three treatment groups as follows: (1) time-matched controls (n = 13); (2) animals treated with salbutamol 0.03 μM (~10 μg/L) (n = 13); and (3) animals treated with salbutamol 0.3 μM (~80 μg/L) (n = 13).

The second experiment (Experiment II) also consisted of three treatment groups: (1) time-matched controls (n = 8); (2) animals treated with salbutamol 0.3 μM (~80 μg/L) and 1 μM ryanodine (n = 8); and (3) animals treated with 1 μM ryanodine (n = 8).

Salbutamol (Glaxo-Wellcome BV, Zeist, The Netherlands) and ryanodine (Molecular Probes, Leiden, The Netherlands) were dissolved in Krebs solution immediately prior to each experiment. Two strips of diaphragm from each animal were mounted in tissue baths containing this solution, one strip at optimal resting length (Lo) and one at ~70% Lo.
The various treatments in each experiment were performed in random order; the investigator (H. vd H.) was blinded with regard to the treatments throughout the experiment. The study was approved by the Animal Experiments Committee of the University of Nijmegen.

General Procedure and Treatments
Similar protocols were used in both experiments to assess contractile properties. The rats were anesthetized with pentobarbital sodium (Nembutal [Apharmo, Arnhem, The Netherlands], 70 mg/kg intraperitoneally). A tracheotomy was performed and a polyethylene cannula was inserted. The animals were mechanically ventilated with 100% oxygen. The diaphragm and adherent lower ribs were quickly excised after combined laparotomy and thoracotomy, and were immediately submerged in cooled, oxygenated Krebs solution at pH 7.4. This Krebs solution consisted of (mM): 117 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM KH₂PO₄, 24 mM NaHCO₃, 7 mM glucose, and 25 μM d-tubocurarine (Sigma Chemicals, Bornem, Belgium). From the middle lateral costal region of each hemidiaphragm, a rectangular strip was dissected parallel to the long axis of the muscle fibers. Silk sutures were tied firmly to both ends of the strip. The strips were suspended in two tissue baths containing Krebs solution, maintained at 37°C and perfused with a mixture of 95% O₂ and 5% CO₂. The central tendon end of each strip was connected to an isometric force transducer (Model 31/1437-10; SETsotec, Columbus, OH) mounted on a micrometer. Two oxygen-stimulating electrodes were placed parallel to the muscle strips. Stimuli were applied with a pulse duration of 0.2 ms and a train duration of 250 ms, and were delivered by a stimulator (ID-electronics; University of Nijmegen, Nijmegen, The Netherlands) activated by a personal computer.

To ensure supramaximal stimulation, the strips were stimulated at approximately 20% above the voltage at which maximal forces were obtained (~6 V applied through the stimulating electrodes). Data acquisition and storage of the amplified signal were done with a Dash-16 interface (Twist-trigger software, ID-electronics, University of Nijmegen) on a personal computer.

Both strips were placed at their optimal length (Lo), defined as the length at which peak Pt was obtained, or at 70% Lo. One strip (obtained from either the left or right hemidiaphragm) was selected in random order for foreshortening. The length of this strip was measured in situ at Lo in the tissue bath, using a micrometer (Model 505-128; Mitutoyo, Veenendaal, The Netherlands). Subsequently, this strip was placed at ~70% Lo and both strips were stimulated twice (twitch contraction) to readjust them to their (new) length (7, 8). No evidence of twitch potentiation was found after these two twitch stimulations, nor after one maximal tetanic stimulation in a pilot experiment.

The salbutamol concentration of 10 μg/L in Experiment I was based on the mean human serum concentration reached after a single oral dose of 4 mg, being ~10 to 20 μg/L (15). The actual salbutamol concentrations were measured in five samples at 0.03 μM and in five samples at 0.3 μM with a high-performance liquid chromatography (HPLC) with fluorescence detection (Scotlab Analytical, Costbridge, UK). The salbutamol concentrations were 10.1 ± 0.8 μg/L and 78.3 ± 4.0 μg/L, respectively. In a pilot study we found no effects of these salbutamol concentrations on the pH of the oxygenated Krebs solution (pH 7.4 in all groups). A maximal increase in Pt was reached within 15 min for salbutamol. In these pilot experiments ryano­dine (in a concentration of 1 μM) produced a peak Pt of 48.5 ± 1.4 mg. No significant differences were found between the separate treatment groups at this length.

In the ~70% Lo group, the length at Lo measured before shortening was similar to the values found for the Lo group. After foreshortening, the average strip dimensions were 17.66 ± 0.29 mm, 3.34 ± 0.05 mm, and 0.58 ± 0.01 mm (length, width and thickness, respectively). The average weight of these strips was 48.5 ± 1.4 mg. No significant differences were found between the separate treatment groups at this length.

In these experiments we sought to limit the influence of time-related fatigue on in vitro diaphragm force output. We therefore used a study design in which the two diaphragm strips obtained from each rat were studied simultaneously (one at Lo and one at ~70% Lo). In this way, consecutive series of measurements on one strip, which might induce fatigue, were prevented. Additionally, at 37°C, there is a time-related decrease of in vitro force production. Therefore, we used separate time-matched control groups in the two experimental protocols.

Measurement of Contractile Properties
In both Experiments I and II, the following protocols were performed: Twitch characteristics. Two twitches were recorded at Lo to determine maximal Pt, contraction time (CT), and half relaxation time (½ RT). Maximal Pt and the corresponding time characteristics were used for further analysis.

Maximal tetanic force. The bundles were stimulated twice at 160 Hz to obtain a plateau in force generation. The maximal force was defined as the maximal tetanic force (Po).

Force-frequency characteristics. The bundles were stimulated at 2-min intervals at the following frequencies: 25, 50, 80, 120, and 160 Hz. Both the absolute forces and the forces as percentages of Po were analyzed. All of these measurements were conducted within 20 min after the thermoequilibration period. Subsequently, the actual length (i.e., at ~70% Lo and ~70% Lo, respectively), thickness, and width of each diaphragm strip were measured. The strips were weighed and the cross-sectional area (CSA) was calculated by dividing diaphragm strip weight (g) by strip length (cm) times specific density (1.056). Forces were expressed per CSA (N/cm²), and the Pt/Po ratio was calculated for each muscle strip.

Statistical Analysis
The salbutamol treatment effects were tested within each group of diaphragm strip length in both experiments, using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test. To determine differences in salbutamol treatment effects between diaphragm strips at Lo and ~70% Lo, a test criterion, T = Z/SE(Z), was calculated (19). Z was calculated by subtracting the natural logarithms of the ratios of salbutamol to control values at Lo and at ~70% Lo (Z = ln[Pt control/Lo, Pt treatment/Lo] + ln[Pt control/~70% Lo, Pt treatment/~70% Lo]). The SE(Z) was calculated with the A-method (19). Values of p were obtained from standard normal distribution tables. The effect of foreshortening was analyzed with paired t tests within the control group of Experiment I. A value of p < 0.05 was considered significant. Mean ± SE values are presented in the text, tables, and figures.

RESULTS

Experiment I

Diaphragm strip dimensions. In the Lo group, average diaphragm strip length, width, and thickness were 23.38 ± 0.35 mm, 2.97 ± 0.05 mm, and 0.46 ± 0.01 mm, respectively. The average weight of these strips was 48.5 ± 1.4 mg. No significant differences were found between the separate treatment groups at this length.

In the ~70% Lo group, the length at Lo measured before shortening was similar to the values found for the Lo group. After foreshortening, the average strip dimensions were 17.66 ± 0.29 mm, 3.34 ± 0.05 mm, and 0.58 ± 0.01 mm (length, width and thickness, respectively). Diaphragm strip weight in these foreshortened strips was 50.5 ± 1.3 mg. No significant differences between treatment groups were found, but length, width, and thickness were all significantly different when compared with the matching treatment group at Lo (p < 0.05; paired t test).

The mean actual percentage of foreshortening in this subgroup was 75.3 ± 0.5%, and was not significantly different between treatment groups. Although the strips in this subgroup were foreshortened to a level of ~70% Lo, therefore, a period of 15 min for thermoequilibration in a pilot experiment.

Twitch and maximal tetanic contraction. In the time-matched
control group, foreshortening reduced in vitro force generation to ~23% of Pt at Lo, and to ~45% of Po at Lo (p < 0.001; Table 1). Likewise, the Pt/Po ratio, CT, and 1/2 RT were reduced by foreshortening (p < 0.001).

Salbutamol increased Pt and Po at both Lo and ~70% Lo. No significant differences were found between the two salbutamol concentrations. The Pt/Po ratio was not changed at either length. Salbutamol increased CT slightly at ~70% Lo; 1/2 RT was not altered (Table 1).

Salbutamol improved force generation to a greater extent at ~70% Lo than at Lo (Table 1 and Figure 1). Expressed as percentages of control values, salbutamol increased Pt at Lo by ~11% and ~16% at 10 μg/L and 80 μg/L, respectively. At ~70% Lo, these increases were significantly greater than the responses at Lo, being ~34% and 50%, respectively (p < 0.05 at 10 μg/L and p < 0.01 at 80 μg/L, both compared with Lo; Figure 1). Similar differences between Lo and ~70% Lo were found for Po (Table 1 and Figure 1). Salbutamol effects were not significantly different at Lo versus ~70% Lo for CT, 1/2 RT, and Pt/Po (Table 1).

Table 1: Twitch Characteristics and Maximal Tetanic Force: Experiment I

<table>
<thead>
<tr>
<th></th>
<th>Pt (N/cm²)</th>
<th>Ct (ms)</th>
<th>1/2 RT (ms)</th>
<th>Po (N/cm²)</th>
<th>Pt/Po Ratio</th>
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<tr>
<td>Lo</td>
<td>7.45 ± 0.17</td>
<td>25.6 ± 0.3</td>
<td>23.8 ± 0.6</td>
<td>24.41 ± 0.74</td>
<td>0.31 ± 0.01</td>
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<td>10 μg/L salbutamol</td>
<td>8.27 ± 0.23†</td>
<td>26.3 ± 0.3</td>
<td>23.8 ± 0.6</td>
<td>26.31 ± 0.50</td>
<td>0.32 ± 0.01</td>
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<td>80 μg/L salbutamol</td>
<td>8.62 ± 0.20†</td>
<td>26.5 ± 0.3</td>
<td>25.1 ± 0.7</td>
<td>26.93 ± 0.87†</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>~70% Lo</td>
<td>1.68 ± 0.09‡</td>
<td>20.0 ± 0.3‡</td>
<td>14.3 ± 0.6‡</td>
<td>11.15 ± 0.63‡</td>
<td>0.15 ± 0.01‡</td>
</tr>
<tr>
<td>10 μg/L salbutamol</td>
<td>2.28 ± 0.21†</td>
<td>20.6 ± 0.3</td>
<td>14.3 ± 0.3</td>
<td>14.56 ± 1.01†</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>80 μg/L salbutamol</td>
<td>2.54 ± 0.16†</td>
<td>21.1 ± 0.2†</td>
<td>13.6 ± 0.2</td>
<td>15.74 ± 0.73†</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Pt = peak twitch force; CT = contraction time; 1/2 RT = half-relaxation time; Po = maximal tetanic force; Pt/Po = twitch to tetanic ratio; Lo = optimal length.
† Values are mean ± SE (n = 13).
‡ Values were significantly increased compared with control values at equal strip length (p < 0.05, one-way ANOVA).

Figure 1. Relative increase in twitch and maximal tetanic force generation after salbutamol, compared with control values; Experiment I. Hatched bars represent increase after 10 μg/L salbutamol, solid bars represent relative increase after 80 μg/L salbutamol. Pt = peak twitch force, Po = maximal tetanic force, Lo = optimal length. Compared with Lo, significantly greater increases were found at ~70% Lo (*p < 0.05, **p < 0.01).

Figure 2. Force–frequency characteristics Experiment I. Open symbols represent values at optimal length (Lo), closed symbols represent values at ~70% Lo. Circles represent control values, squares represent 10 μg/L salbutamol, and triangles represent 80 μg/L salbutamol. Significant increases with respect to control values within each length group (indicated by *) were found for both salbutamol concentrations at stimulation frequencies up to 50 Hz. Force output in the control group at ~70% Lo was significantly reduced compared with Lo at all frequencies (p < 0.001, paired t test).
reduced as compared with that at Lo (Figure 3). In the foreshortened diaphragm strips, loss of force generation was observed at the end of the protocol, resulting in a maximal level of 83% of Po. In contrast, diaphragm strips at Lo reached a level of 103% of Po (a significant difference between ~70% Lo and Lo). In contrast to the positive inotropic effects on absolute force generation, salbutamol caused largely negative effects on relative force production (i.e., relative to maximal force generation; Figure 3). Only at Lo was relative force generation significantly increased by salbutamol at a stimulation frequency of 25 Hz, this increase being significantly greater at 80 µg/L than at 10 µg/L (p < 0.05). However, at stimulation frequencies of 80 to 160 Hz, salbutamol reduced the relative output at Lo and augmented the reduction in relative force (%Po) induced by foreshortening (p < 0.05; Figure 3). As a result, the final 160-Hz stimulations at ~70% Lo in the force–frequency curve produced a force output of ~75% of Po at 10 µg/L and of ~65% of Po at 80 µg/L salbutamol, respectively (Figure 3). In comparing the two salbutamol concentrations, significantly greater reductions in relative force generation were found at 80 µg/L at Lo, using a stimulation frequency of 120 Hz, and at ~70% Lo at stimulation frequencies of 80 to 160 Hz, suggesting a dose-dependent relationship. Additionally, the decrease in relative force production induced by salbutamol was significantly greater at ~70% Lo than at Lo (10 µg/L salbutamol: p < 0.05 at 120 and 160 Hz; 80 µg/L salbutamol: p < 0.01 at 80 Hz and p < 0.001 at 120 and 160 Hz, respectively).

Experiment II

Diaphragm strip dimensions. In the Lo group, average diaphragm strip length, width, and thickness were 22.78 ± 0.29 mm, 3.06 ± 0.09 mm, and 0.51 ± 0.01 mm, respectively. The average weight of these strips was 48.6 ± 1.6 mg. No significant differences were found between the separate treatment groups at this length.

In the ~70% Lo group, the length at Lo measured before shortening was similar to the values found for the Lo group. After foreshortening, the average strip length was 16.95 ± 0.34 mm.

The average strip width and thickness were 3.65 ± 0.10 mm and 0.62 ± 0.01 mm, respectively. Diaphragm strip width in these foreshortened strips was 50.4 ± 1.8 mg. No significant differences between treatment groups were found, but length, width, and thickness were all significantly different from those of the matching treatment groups at Lo (p < 0.05, paired t test).

The actual percentage of foreshortening was 73.9 ± 0.9% in the control group; 70.5 ± 0.5% in the salbutamol–ryanodine group and 71.0 ± 1.0% in the ryanodine group. Although the strips in this experiment were foreshortened through the same procedure as used in Experiment I, the degree of foreshortening was different in this experiment, and the overall strip length approximately matched the preset value of ~70%. Furthermore, as compared with the control group, the percentage of foreshortening found in this group at the end of the protocol was significantly different in both the salbutamol–ryanodine group and in the ryanodine group (both p < 0.05; one-way ANOVA).

Twist and maximal tetanic contraction. In the presence of ryanodine, salbutamol did not have any inotropic effect on Pt or Po at either Lo or ~70% Lo (Table 2). As compared with the time-matched controls in this experiment, coadministration of salbutamol and ryanodine significantly reduced Pt at both Lo and ~70% Lo. Po was significantly lower only at ~70% Lo. As compared with the ryanodine group, a significantly higher Pt was found at Lo in the coadministration group, but Po was not affected at either length. The Pt/Po ratio was increased at ~70% Lo in both treatment groups (Table 2). Salbutamol in the presence of ryanodine reduced CT at ~70% Lo as compared with the control group. However, salbutamol plus ryanodine increased CT at both Lo and ~70% Lo as compared with the ryanodine group. The value of ½ RT was decreased in both the ryanodine and salbutamol-plus-ryanodine groups, but did not differ between these two groups (Table 2).

Salbutamol in the presence of ryanodine did not affect Pt, Po, or twitch characteristics different at ~70% Lo than at Lo (Table 2 and Figure 4). Salbutamol plus ryanodine decreased Pt at Lo by ~20%, expressed as a percentage of control values; at ~70% Lo this decrease was ~24% (Figure 4). For Pt these decreases were ~17% and ~65%, respectively, but the differences in this case were not significant. However, in the ryanodine group, a significant difference was found between the relative effect at Lo and at ~70% Lo for Po (p < 0.05; Figure 4).

Force–frequency characteristics: absolute force generation. Both at optimal length and after foreshortening, salbutamol in the presence of ryanodine decreased force production significantly at all stimulation frequencies as compared with control values (Table 3; p < 0.05 one-way ANOVA). Ryanodine alone also depressed force production significantly as compared with the time-matched controls at both lengths. No differences were found between the salbutamol-plus-ryanodine group and the ryanodine group, but forces were mostly lower in the coadministration group. In contrast to the control animals, both treatments produced a decline in the force of subsequent contractions in the force–frequency protocol. This effect was most pronounced at ~70% Lo in the salbutamol-plus-ryanodine group, but was also present in the ryanodine group and at Lo. The relative treatment effect on absolute force production was significantly greater at ~70% Lo than at Lo at stimulation frequencies of 25 to 80 Hz (p < 0.01) and 120 Hz (p < 0.05) for salbutamol plus ryanodine, and at 50 to 120 Hz for ryanodine (p < 0.05).

Force–frequency characteristics: force generation relative to maximal force generation. At both lengths and treatment with salbutamol plus ryanodine and with ryanodine alone largely depressed relative force production as compared with control values (i.e., relative to maximal force generation [%Po]; p < 0.05; Table 4). Furthermore, as compared with ryanodine, salbutamol
plus ryanodine had greater negative effects at ∼70% Lo at all but one stimulation frequency. At this length, force production was reduced to approximately zero at the end of the protocol in the salbutamol-plus-ryanodine treatment group. The treatment effect on relative force production (%Po) was significantly greater at ∼70% Lo than at Lo in the salbutamol-plus-ryanodine group at stimulation frequencies of 25 to 80 Hz (p < 0.001), 120 Hz (p < 0.01), and 160 Hz (p < 0.05). In the ryanodine group, a similar result was found for stimulation frequencies of 50 to 160 Hz (p < 0.05).

**DISCUSSION**

The present study shows that foreshortening reduced force generation in strips of rat diaphragm, reducing Pt, Po, and the force–frequency curve. This reduction in force was in part reversed by salbutamol. Indeed, the inotropic effects of salbutamol on *in vitro* contractile properties of rat diaphragm strips were potentiated by foreshortening, as shown by the significantly greater increases in both Pt and Po at ∼70% Lo than at Lo. Foreshortening also reduced force production relative to maximal force production during the force–frequency protocol. Salbutamol augmented this reduction, thus potentiating the "fatiguing" effect of foreshortening. In the presence of the SR Ca2+ release inhibitor ryanodine, the inotropic effects of salbutamol were blocked and force production was reduced. During the force–frequency protocol, force production was progressively decreased. These findings indicate that the inotropic effects of salbutamol on diaphragm contractions are likely to be mediated by an increase in SR Ca2+ release.

Several mechanisms may be involved in decreasing force generation in foreshortened diaphragm. When skeletal muscle fibers were electrically stimulated at sarcomere lengths shorter than 75% of optimal length, a wave configuration of central myofibrils was found (20). Failure of T-tubular propagation of membrane depolarization into core regions of the fibers is thought to be an explanation for this phenomenon and for fatigue. Furthermore, in foreshortened diaphragm muscle strips, force production may also be reduced by an impairment of SR Ca2+ release (21). During high-frequency fatigue, reduced levels of intracellular Ca2+ were found in core regions of single muscle fibers (22). This indicates that impaired T-tubular conduction not only leads to wavy myofibrils in muscle fiber core regions, but may also reduce Ca2+ release from the SR (21, 22). This is supported by findings made by Gauthier and coworkers (5), who showed that foreshortening and high-frequency fatigue had an additive effect in rat diaphragm strips: a larger decrease of *in vitro* tetanic force was found after fatigue at shorter muscle lengths (5). Similar effects were found in human diaphragm *in vivo*. When human lung volume increased, the maximal twitch transdiaphragmatic pressure (Pdi) decreased linearly (3, 4, 6), and during fatigue a larger decrease in Pdi was found at high lung volumes (6). In our study, greater inotropic effects of salbutamol were found at shorter muscle lengths. In other words, a length-dependent reduction in force generation, possibly caused by a reduced SR Ca2+ release, was partially prevented by salbutamol. These effects were absent in the presence of ryanodine.

The mechanism of action of β2-adrenergic agonists on skeletal muscle has not yet been fully clarified. Recent studies have indicated that both methylxanthines and β2-agonists have similar intracellular effects in the diaphragm (10–12, 23). These effects may involve excitation-contraction (EC) coupling of skeletal muscle. The present experiments indicate that the inotropic effect of salbutamol is most probably mediated by an increase in this SR Ca2+ release. In Experiment II, coadministration of ryanodine prevented the inotropic effects of salbutamol, and force was reduced as compared with control values throughout the experiment. At the concentration range used in our experiments, ryanodine reduces SR Ca2+ release by locking the calcium release.
channels in an open subconductance state (16, 17). As compared with the ryanodine treatment group, the salbutamol-plus-ryanodine group generated higher forces at Lo but lower forces at 70% Lo. Furthermore, during the force-frequency protocol, a progressive decrease in force production was found, both at Lo and in foreshortened diaphragm strips. This may suggest a progressive depletion of the SR calcium pool, since ryanodine prevents the accumulation of Ca\(^{2+}\) in the SR (16). It could also indicate that salbutamol and ryanodine have additive effects on the mechanism by which Ca\(^{2+}\) is released from the SR, and supports the hypothesis that the inotropic effects of \(\beta\)-adrenoceptor agonists are mediated by an increase of SR Ca\(^{2+}\) release.

The proposed mechanism of action for the inotropic effect of salbutamol is in agreement with previous findings. Cairns and Dulhunty (24) showed that enhancement of sodium-pump activity, dihydropyridine (DHP)-sensitive Ca\(^{2+}\) currents, glycosylation, and altered action potentials are unlikely to be the mechanisms of action for the inotropic effect of the \(\beta\)-adrenoceptor agonist terbutaline (24). Further studies with terbutaline indicated that the increase in in vitro force induced by \(\beta\)-adrenoceptor agonists could be interpreted as a facilitation of EC coupling via enhanced Ca\(^{2+}\) release from the SR in both slow- and fast-twitch skeletal muscle fibers (11, 23). For example, addition of 1 mM caffeine, which stimulates SR Ca\(^{2+}\) release, prevented an additional effect of terbutaline on force generation (11, 24), suggesting that caffeine and terbutaline have a similar mechanism of action. Caffeine in concentrations below 5 mM also potentiated Pt and Po in both slow- and fast-twitch rat skeletal muscles by enhancing SR Ca\(^{2+}\) release (10). These findings in mammalian skeletal muscle are in accord with the findings in earlier experiments with frog skeletal muscle in which adrenaline treatment potentiated twitch force by modulating calcium channels (25, 26).

Several studies suggested that SR Ca\(^{2+}\) release is modulated and regulated by phosphorylation of the complex interacting structure of voltage-dependent DHP receptors in the T-tubules and the SR Ca\(^{2+}\)-release channels (ryanodine receptors) (27–29). The \(\beta\)-adrenoceptor-mediated effects on force generation may also be mediated by an intracellular increase of cAMP (30). Administration of cAMP can mimic the effects of terbutaline on skeletal muscle fibers in vitro (11, 24). In turn, this would suggest a cAMP-mediated enhancement of SR Ca\(^{2+}\) release. Other potential mechanisms for the inotropic effect of \(\beta\)-adrenoceptor agonists include phosphorylation of myosin heavy chains or Ca\(^{2+}\)-osin light chains, possible increasing Ca\(^{2+}\) sensitivity or Ca\(^{2+}\)-activated contractile force generation.

Foreshortening also potentiated the inotropic effects of aminophylline on respiratory muscle contraction. The studies by Gayan-Ramirez and coworkers (7–9) and by Gauthier and colleagues (4) found an increased inotropic effect of aminophylline on foreshortened diaphragm. This inotropic effect of aminophylline on foreshortened canine diaphragm was also prevented by ryanodine, a SR Ca\(^{2+}\)-release blocker (12). Compared with these effects of methylxanthines, the present study showed similar results for salbutamol, and suggests that these drugs have at least in part a similar mechanism of action. However, an important discrepancy between the effects of aminophylline and salbutamol on diaphragm contractile properties is that salbutamol increased Po both at Lo and after foreshortening, whereas aminophylline did not increase Po at Lo at 37°C (31). This finding

### Table 3

**FORCE-FREQUENCY CHARACTERISTICS (SPECIFIC FORCES): EXPERIMENT II***

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<tr>
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<th>25 Hz</th>
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<tr>
<td>Control</td>
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<td>18.53</td>
<td>22.41</td>
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<td>Salbutamol + ryanodine</td>
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<td>Ryanodine</td>
<td>4.65</td>
<td>8.46</td>
<td>9.58</td>
<td>8.68</td>
<td>6.57</td>
</tr>
<tr>
<td>70% Lo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.98</td>
<td>5.65</td>
<td>8.45</td>
<td>8.75</td>
<td>8.32</td>
</tr>
<tr>
<td>Salbutamol + ryanodine</td>
<td>0.55</td>
<td>0.51</td>
<td>0.37</td>
<td>0.23</td>
<td>0.12</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>0.70</td>
<td>0.99</td>
<td>1.13</td>
<td>0.97</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* Values are mean ± SE, expressed in N/cm\(^2\); salbutamol and ryanodine concentrations were 0.3 μM (80 μg/L) and 1 μM, respectively.

† p < 0.05 compared with control (one-way ANOVA).

### Table 4

**FORCE-FREQUENCY CHARACTERISTICS (RELATIVE TO Po): EXPERIMENT II***

<table>
<thead>
<tr>
<th></th>
<th>Twitch</th>
<th>25 Hz</th>
<th>50 Hz</th>
<th>80 Hz</th>
<th>120 Hz</th>
<th>160 Hz</th>
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<tr>
<td>Lo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31.0</td>
<td>45.3</td>
<td>85.6</td>
<td>103.2</td>
<td>106.2</td>
<td>105.0</td>
</tr>
<tr>
<td>Salbutamol + ryanodine</td>
<td>30.2</td>
<td>34.7</td>
<td>51.5</td>
<td>48.2</td>
<td>36.5</td>
<td>23.9</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>27.0</td>
<td>28.7</td>
<td>51.0</td>
<td>57.3</td>
<td>51.4</td>
<td>38.7</td>
</tr>
<tr>
<td>70% Lo</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.8</td>
<td>25.1</td>
<td>47.3</td>
<td>69.7</td>
<td>72.4</td>
<td>68.7</td>
</tr>
<tr>
<td>Salbutamol + ryanodine</td>
<td>50.9</td>
<td>11.4</td>
<td>9.5</td>
<td>6.9</td>
<td>3.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>34.0</td>
<td>15.8</td>
<td>19.6</td>
<td>21.1</td>
<td>18.0</td>
<td>13.3</td>
</tr>
</tbody>
</table>

* Values are mean ± SE, expressed as %Po. Salbutamol and ryanodine concentrations were 0.3 μM (80 μg/L) and 1 μM, respectively.

† p < 0.05 compared with control.

‡ p < 0.05 compared with ryanodine (one-way ANOVA).
may indicate that cytosolic Ca²⁺ availability is limited during supramaximal electrical stimulations at physiologic temperatures.

Salbutamol increased in vitro diaphragm force production but reduced relative force generation in the force–frequency protocol (in Experiment I); in other words, the force generated at the end of the force–frequency protocol was lower than the initial Po, indicating some form of fatigue. This could be the result of a progressive depletion of the SR calcium pool by salbutamol treatment, as was discussed earlier. An alternative explanation could be that salbutamol has its main effect on Type II muscle fibers in the rat diaphragm. Several findings support this contention. First, especially in Type IIX/IIb fibers, which are highly susceptible to fatigue (32), a high binding affinity for β₂-agonists was found (as measured by [³²P]lucanipindolol binding) (33). This higher binding affinity may override the previous finding that slow muscles (containing predominantly Type I muscle fibers) have a higher density of β₂-adrenoceptors than do fast muscles (containing predominantly Type II muscle fibers) (34). Second, in fast- and slow-contracting skeletal muscles, opposing effects of catecholamines and β-agonists have been reported both in vivo and in vitro (30, 35). In muscles containing predominantly Type I (generally slow-contracting) muscle fibers, force production is reduced by catecholamines and β-agonists, whereas in fast muscles, containing predominantly Type II muscle fibers, Pₑ and incomplete tetanic force were increased (30, 35). In the present study, salbutamol increased Pₑ, Po, and submaximal tetanic force, but reduced force generation relative to maximal force generation in the force–frequency protocol, indicating fatigue. These effects of salbutamol could be explained by a main effect on Type IIX/IIb muscle fibers, which account for ~35% of rat diaphragm muscle (36).

Although the strips in the present study were foreshortened to 70% Lo at the start of the protocols, the actual level of foreshortening at the end of all measurements was ~75% Lo in Experiment I. This discrepancy may be explained by actual lengthening of the strips during the protocol, or by the difference in the method of measuring length. The first length measurement at Lo and foreshortening of the diaphragm strips was performed in the tissue bath, whereas the final length measurement was performed under dry conditions. In the second experiment, we therefore measured strip length before and after the stimulation protocol in the Lo group. In this group, the measured length at the end of all protocols did not differ from the measured length at the start of the protocols (98.7 ± 0.6%; not significantly different between treatment groups). It is therefore likely that in foreshortened muscles, lengthening of these strips during the stimulation protocols may have contributed to this difference in the degree of foreshortening. A similar observation was made in the control group of the second experiment. However, in contrast to the first experiment, the degree of foreshortening in the second experiment was close to the preset value of 70% Lo in the two groups treated with ryanodine. This difference can partly explain the force reduction in the presence of ryanodine that was found after foreshortening, but cannot explain the blockade of the inotropic effect of salbutamol found in the first experiment. This reduction in muscle length could indicate the development of contracture due to ryanodine treatment, and is in agreement with earlier observations in rat diaphragm (18).

In order to compare our results with those found for aminophylline, we performed our experiments at ~75% Lo. This length reduction to ~75% Lo approximately resembles acute hyperinflation from FRC to TLC (1, 2). At intermediate muscle lengths, (e.g., 85% Lo), similar (although less pronounced) effects were found in terms of specific force reduction (37). Furthermore, in human studies, a linear relation was found between the percentage of inspiratory capacity and Pdi (4), or between the percentages of Ve and Pdi (3) in healthy subjects. In contrast to the effects of aminophylline, the present study showed a significant inotropic effect of low, clinically relevant doses of salbutamol on Pt and Po at Lo. After foreshortening, a relatively greater inotropic effect was found. Therefore, it is likely that the force increase will also occur at intermediate lengths, and the relative increase will be intermediate to the increases found at Lo and ~75% Lo. Clearly, however, these effects need to be investigated in a clinical situation.

It is uncertain how applicable our in vitro results with healthy animal diaphragm are to clinical conditions in patients with obstructive airways disease (asthma and chronic obstructive pulmonary disease [COPD]). The inotropic effects of salbutamol were present at a concentration of 10 µg/L. This concentration is reached in human serum after a single oral dose of 4 mg (15). Since predominantly slow motor units are recruited during normal ventilatory maneuvers in fresh diaphragm (38), stimulation of Type II muscle fibers by salbutamol would have no additive effect on diaphragm force generation in this situation. During fatigue, however, treatment with a β₂-adrenoceptor agonist may increase diaphragm contractility in vivo, as was shown in animal studies for terbutaline (39), fenoterol (40), and broxaterol (41).

The present study may suggest that a similar positive effect of salbutamol may be expected in case of a foreshortened diaphragm, as may occur during acute hyperinflation in asthma or COPD patients. The increased fatigability observed during the force–frequency protocol in the present study would suggest, however, that salbutamol may be used for acute and short interventions only.

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References


