Clenbuterol and formoterol decrease force production in isolated intact mouse skeletal muscle fiber bundles through a β2-adrenoceptor-independent mechanism

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McCormick C, Alexandre L, Thompson J, Mutungi G. Clenbuterol and formoterol decrease force production in isolated intact mouse skeletal muscle fiber bundles through a β2-adrenoceptor-independent mechanism. J Appl Physiol 109: 1716–1727, 2010; doi:10.1152/japplphysiol.00592.2010.—Although the acute actions of short-acting β2-adrenoceptor agonists on force production in isolated mammalian skeletal muscle fibers have been the subject of a number of previous studies, those of long-acting β2-adrenoceptor agonists have never been investigated. Also, little is known about the cellular signal transduction events mediating their actions. Therefore, the primary aim of this study was to investi...
muscle (a predominantly slow-twitch muscle) of adult CD1 mice aged 56 ± 4 days (range: 46–80 days, n = 41) were used in this study. Mice were killed by cervical disarticulation in accordance with United Kingdom legislation [for a summary of the regulations, see Ref. 7], and all experiments conformed with local Animal Welfare Committee guidelines. EDL and soleus muscles were then isolated, and small bundles containing ~10–15 fibers (mean cross-sectional diameter: 320 ± 16.3 μm, n = 51) were dissected. Care was taken to ensure that all the fibers in a bundle were electrically excitable, and only those bundles that showed a decline in force of <5% at the end of the experiment(s) were included in the final analyses.

**Determination of the effects of β2-adrenoceptor agonists on force.** The procedure used in this experiment was basically similar to that previously described in Ref. 13. Briefly, fiber bundles were mounted horizontally between two stainless steel hooks, with one attached to a force transducer (model 400A, Aurora Scientific, Ottawa, ON, Canada) and the other to a servo motor (model 322C, Aurora Scientific) in a muscle chamber with a glass bottom. They were then perfused at a rate of 1 ml/min with standard Ringer solution or standard Ringer solution plus various concentrations of clenbuterol, formoterol, or salbutamol to determine the dose dependence of force. Twitch and tetanic contractions were then recorded in the presence or absence of each of the drugs. After these initial determinations, all subsequent experiments were performed using 50 μM clenbuterol, 100 μM formoterol, and 500 nM salbutamol.

Used at concentrations higher than 1 mM, DMSO can significantly depress both twitch and submaximal tetanic contractions (34). Therefore, to ensure that the vehicles used in this study (700 μM DMSO in the case of formoterol and 500 μM methanol in the case of salbutamol) did not affect force production, some of the fiber bundles were treated for 1 h with Ringer solution containing either 700 μM DMSO or 500 μM methanol. Twitches and tetani were then recorded in the presence or absence of the vehicles. At the concentrations we used, the vehicles had no effect on the characteristics of both twitch and tetanic contractions (Fig. 1).

**Fig. 1.** DMSO and methanol (MET) do not affect isometric force in isolated intact mouse skeletal muscle fiber bundles. A and B: representative twitch (A) and tetanus (B) myographs recorded from a fast-twitch muscle fiber bundle perfused with standard Ringer solution (NR) before and after incubation in standard Ringer solution plus 700 μM DMSO or standard Ringer solution plus 500 μM MET. Fiber bundles were equilibrated in each solution for at least 10 min before traces were recorded. Note that treatment of the fiber bundle with these concentrations of DMSO and MET had no effect on any of the characteristics of the twitch or tetanus.

![Graph A](http://example.com/graphA.png)

![Graph B](http://example.com/graphB.png)
Determination of the cellular signal transduction events mediating the acute effects of clenbuterol, formoterol, and salbutamol on force. To investigate the cellular signal transduction events underlying the acute effects of the β2-adrenoceptor agonists on force, another set of small muscle fiber bundles was treated with standard Ringer solution for at least 30 min. Ringer solution was then replaced with one containing the inhibitors shown in Table 1 for 20 min. It was then replaced with one containing the inhibitor plus the β2-adrenoceptor agonist whose effects were being investigated for at least another 30 min. Finally, the fiber bundles were switched back into standard Ringer solution. During each treatment, twitches and tetani were recorded and stored in a computer for further analysis.

In another experiment, fiber bundles isolated from six mice were divided into two groups. Half (6 fiber bundles/treatment) were treated with standard Ringer solution or standard Ringer solution plus vehicle (DMSO or methanol in the formoterol and salbutamol experiments, respectively) for at least 1 h. These bundles acted as controls. The other half (consisting of 6 fibers bundles/treatment) was treated for the same period of time with standard Ringer solution plus 50 μM clenbuterol, 100 μM formoterol, or 500 nM salbutamol for at least 30 min. In another experiment, fiber bundles were preincubated in standard Ringer solution plus the inhibitors shown in Table 1 for 20 min. They were then incubated in Ringer solution containing the inhibitor plus the β2-adrenoceptor agonist whose effects were being investigated for 1 h. At the end of the experiments, muscle fiber bundles were processed for immunoblot analysis as described below.

Immunoblot analysis. At the end of the experiments outlined above, fiber bundles were snap frozen in liquid nitrogen and pulverized, and proteins were extracted using Nonidet P-40 lysis buffer as described in Ref. 31. Equal amounts of the proteins were then immunoblotted using primary antibodies against the phosphorylated forms of the β2-adrenoceptor (LS-C34905, Autogen Bioclair, Calne, UK) and phospholamban (PLB; ab1500, Abcam, Cambridge, UK) and phosphofuramban (PLB; ab1500, Abcam, Cambridge, UK). Briefly, proteins were resolved in 10% SDS-polyacrylamide gels. They were then transferred onto polyvinylidene difluoride membranes, and membranes were blocked for nonspecific antibody binding with 5% milk for at least 30 min. They were then incubated overnight at 4°C with the primary antibodies mentioned above. The next day, they were incubated in Ringer solution containing the inhibitor plus the β2-adrenoceptor agonist whose effects were being investigated for 1 h. At the end of the experiments, muscle fiber bundles were processed for immunoblot analysis as described below.

Western blots were run in triplicate, and each experiment was repeated at least three times. The intensity of the bands from each experimental condition were averaged and divided by the mean force recorded from the same fiber under control conditions. Data (in %) are presented as means ± SE of the control tension.

All Western blots were run in triplicate, and each experiment was repeated at least three times. The intensity of the bands from each experimental condition were averaged and divided by the mean force recorded from the same fiber under control conditions. Data (in %) are presented as means ± SE of the control tension.

RESULTS

Effects of treatment of fiber bundles with long- and short-acting β2-adrenoceptor agonists on maximum isometric force. Treatment of the fiber bundles with clenbuterol led to a dose-dependent reduction in maximum isometric force (P<0.05; equal to tension: Fig. 2A). Similar changes were observed when the fiber bundles were treated with formoterol, another long-acting β2-adrenoceptor agonist (traces not shown). In contrast, treatment of the fiber bundles with salbutamol led to

Table 1. A list of the compounds and the concentrations used

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Concentration, μM</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clenbuterol</td>
<td>4-Amino-α-(1-butylaminomethyl)-3,5-dichlorobenzyl alcohol hydrochloride hydrochloride</td>
<td>0.1–150</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Formoterol fumarate dehydrate</td>
<td>([P*-P*]-N-[2-Hydroxy-5-(1-hydroxy-2-[2-(4-methoxyphenyl)-1-methylthyl][lamino][ethyl][phenyl][formamidine fumarate])</td>
<td>1–250</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>Albuterol; 1-(tert-butylamino)methyl]-4-hydroxy-m-xylene-α,α’-dil</td>
<td>0.001–1</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Propranolol hydrochloride</td>
<td>(R)-1-isopropylaminol-3-(1-naphthoxy)-2-propanol hydrochloride</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>ICI-118551 hydrochloride</td>
<td>(±)-1-(2,3-Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-(1-methylthylaminol)-2-butanol</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>PKA inhibitor</td>
<td>14–22 amide</td>
<td>0.05</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>
an increase in $P_0$ (Fig. 2B). Although the traces shown in Fig. 2 are from fast-twitch fiber bundles, similar findings were observed when slow-twitch fiber bundles were treated with the compounds. Changes in $P_0$ were observed at all concentrations investigated. They occurred within 10–15 min after the application of the drugs and could be easily reversed by switching the fiber bundles back into standard Ringer solution [traces labeled washout (WO) in Fig. 2, A–C].

Summary data from 6 fast-twitch and 6 slow-twitch muscle fiber bundles per experiment are shown in Fig. 2C. Treatment of the muscle fiber bundles with either clenbuterol or formoterol led to a reduction in $P_0$, whose magnitude depended on the
concentration of the drugs used, and no statistical differences were observed when the data from the fast- and slow-twitch fibers were compared ($P > 0.05$). For example, in fast-twitch muscle fiber bundles, 5 μM clenbuterol led to a $10.3 \pm 4.8\%$ ($n = 8$ fibers) reduction in $P_o$, whereas at 50 μM it led to a $55.5 \pm 4.1\%$ ($n = 8$ fibers) drop in $P_o$. The corresponding values in slow-twitch muscle fiber bundles were $13.7 \pm 4.1\%$ ($n = 6$ fibers) and $54.2 \pm 5.4\%$ ($n = 6$ fibers), respectively. Additionally, treatment of the fiber bundles with concentrations of clenbuterol of $>150$ μM and formoterol of $>250$ μM completely abolished $P_o$ (results not shown). It is also noteworthy that although clenbuterol and formoterol had similar effects on $P_o$, higher concentrations of the latter were required to induce similar reductions in $P_o$ as the former.

In contrast, treatment of the fiber bundles with salbutamol, a short-acting $\beta_2$-adrenoceptor agonist, led to an increase in $P_o$ in both fiber types at all concentrations examined, and its effects in fast- and slow-twitch fiber bundles were also not significantly different ($P > 0.05$). The increase in force also reached a plateau at $\sim 100$ nM. For example, 100 nM salbutamol led to a $19.6 \pm 4.3\%$ increase in $P_o$ in both fiber types, and this was not statistically different ($P > 0.05$) from the $21.5 \pm 5.4\%$ increase in $P_o$ observed when fiber bundles were treated with 1 μM salbutamol.

**Effects of treatment of muscle fiber bundles with $\beta_2$-adrenoceptor agonists on twitch tension.** The effects of treatment of the fiber bundles with clenbuterol, formoterol, and salbutamol on twitch contractions were basically similar to those that those compounds had on $P_o$. Thus, treatment of the fiber bundles with clenbuterol and formoterol led to a decrease in twitch force (see Fig. 3, A and B), whereas treatment with salbutamol increased it (Fig. 3C). Like $P_o$, the changes in twitch force increased with the dose of the agonists used and were not statistically different between the two fiber types ($P > 0.05$; Fig. 3D). Thus, 25 μM clenbuterol led to a $69.8 \pm 2.1\%$ ($n = 6$ fiber bundles) decrease in peak twitch force in fast-twitch muscle fiber bundles and to a $69.4 \pm 3.1\%$ ($n = 6$ fiber bundles) decrease in twitch force in slow-twitch fibers (Fig. 3E).
3D). In contrast, 500 nM salbutamol led to a 20.2 ± 1.8% and 20.3 ± 2% increase in twitch force in fast- and slow-twitch fiber bundles, respectively (Fig. 3D). Although all three compounds affected twitch amplitude, they had no significant effect on rise time. However, both clenbuterol and formoterol slightly increased the rate of twitch relaxation. For example, the half-rise times of twitch force in slow-twitch fibers were 21.8 ± 0.8 and 23.4 ± 0.4 ms (n = 6) in the absence and presence of clenbuterol, respectively. On the other hand, the half-relaxation of twitch force in the same fibers decreased from 310.2 ± 6 to 500.5 ± 3 ms (n = 6) in the presence and absence of clenbuterol, respectively.

As the effects on force production of all three β₂-adrenoceptor agonists examined were not statistically different between the two fiber types at all concentrations examined (P > 0.05), in the rest of the study data from both fiber types were analyzed together. Moreover, as their effects on twitch tension were similar to those they had on P₀, only their effects on P₀ were considered in the rest of the study.

Effects of propranolol and ICI-118551 on the changes in force induced by β₂-adrenoceptor agonists. To determine whether the changes in force reported above were mediated through the β₂-adrenoceptor, some of the fiber bundles were pretreated with 10 μM propranolol (a general β-adrenoceptor antagonist) or 10 μM ICI-118551 (a specific β₂-adrenoceptor antagonist) for 20 min. The Ringer solution was then changed to one containing propranolol or ICI-118551 plus the β₂-adrenoceptor agonist whose effects were being investigated for a further 30 min. Force was then recorded in the presence and absence of the inhibitors and/or drugs. Treatment of the fiber bundles with 10 μM propranolol alone led to a 10.3 ± 3.8% (n = 7 fibers) decrease in P₀, and combining it with either clenbuterol or formoterol did not abolish their effects on P₀ (Fig. 4, A and C). In contrast, pretreatment of fiber bundles

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**Fig. 4.** Propranolol does not reverse the effects of clenbuterol and formoterol on P₀ in isolated intact mouse skeletal muscle fiber bundles. **A:** force records showing the effects of treatment of a fast-twitch muscle fiber bundle with standard Ringer solution at the start (Con) and end of the experiment (WO), standard Ringer solution plus propranolol, standard Ringer solution plus clenbuterol, or standard Ringer solution plus clenbuterol and propranolol (Clen + Prop) on P₀. Note that treatment of the muscle fiber bundle with either propranolol or clenbuterol alone decreased P₀, and the combination of the two did not abolish the effects of clenbuterol on force. **B:** P₀ traces showing the effects of treatment of a fast-twitch muscle fiber bundle with standard Ringer solution (control), standard Ringer solution plus salbutamol, or standard Ringer solution plus salbutamol and propranolol (Sal + Prop) on P₀. Note that treatment of the fiber bundle with salbutamol increased P₀, and the combination of salbutamol and propranolol abolished the increase in force. **C:** summary data showing the effects of treatment of 7 muscle fiber bundles per experiment with standard Ringer solution at the start (Con) and end of the experiment (WO), standard Ringer solution plus clenbuterol, formoterol, or salbutamol (Sal), or standard Ringer solution plus a combination of each agonist plus propranolol (Prop) on P₀. Note that both clenbuterol and formoterol decreased P₀, and the combination of them with propranolol did not reverse their effects on P₀. In contrast, salbutamol increased P₀, and the combination of salbutamol with propranolol completely abolished its effects on force. *P < 0.05 when the data indicated were compared with the control recorded before (Con) and after (WO) the experiments.
with propranolol completely reversed the effects of salbutamol on Po (Fig. 4, B and C).

Similar results were obtained when fiber bundles were treated with the drugs plus the β2-adrenoceptor-specific inhibitor ICI-118551 (Fig. 5). However, unlike propranolol, ICI-118551 alone had no effect on Po (Fig. 5, A and B).

Effects of 14–22 amide on the changes in force induced by β2-adrenoceptor agonists. It is generally believed that β2-adrenoceptor agonists exert their effects through the activation of PKA by cAMP (10, 17, 36, 42). However, a number of studies (10, 39) have recently shown that cAMP can exert effects that are independent of PKA. Therefore, in another experiment, we examined the effects of inhibition of PKA on the changes in force induced by our three β2-adrenoceptor agonists. Treatment of the fiber bundles with 10 nM 14–22 amide, a cell-permeable PKA-specific inhibitor, did not significantly affect force production in both fiber types (P > 0.05). Also, it did not abolish the effects of the β2-adrenoceptor agonists (Fig. 6). Indeed, it further potentiated the effects of salbutamol on Po (Fig. 6, B and C). For example, treatment of the fiber bundles with salbutamol alone led to a 15 ± 2% (n = 5 fibers) increase in Po, and this rose to 21.5 ± 3% when the fiber bundles were treated with both salbutamol and 14–22 amide.

β2-Adreceptor expression in fast- and slow-twitch skeletal muscle fiber bundles. Previously, a number of radioligand binding studies (8, 16, 20) have shown that the β2-adrenoceptor is the main β-receptor present in mammalian skeletal muscle and that its concentration is higher in the soleus muscle than in the EDL muscle (8, 16, 20). However, little is known

Fig. 5. The β2-adrenoceptor (β2AR)-specific inhibitor ICI-118551 (ICI) does not reverse the effects of clenbuterol and formoterol on Po, in isolated intact mouse skeletal muscle fiber bundles. A: maximum isometric tension traces recorded from a slow-twitch muscle fiber bundle treated with standard Ringer solution at the start (Con) and end of the experiment (WO), ICI alone, formoterol alone, and formoterol plus ICI ICI. B: another slow-twitch fiber bundle treated with standard Ringer solution (Con/WO), standard Ringer solution plus ICI alone, salbutamol alone, and salbutamol plus ICI. C: summary data showing the effects of treatment of 8 muscle fiber bundles per experiment with standard Ringer solution (Con), standard Ringer solution plus clenbuterol, formoterol, or salbutamol, or the combination of each drug agonist plus ICI on Po. Note that treatment of the muscle fiber bundles with ICI alone did not significantly affect Po (P > 0.05). On the other hand, treatment of the fiber bundles with clenbuterol and formoterol alone decreased Po, and their effects were not reversed by ICI. In contrast, salbutamol on its own led to an increase in Po, and pretreatment of the fiber bundles with ICI completely abolished its effects on Po, *P < 0.05 when the data indicated were compared with the control recorded before (Con) and after (WO) the experiments.
about its expression at the protein level as well as its degree of phosphorylation after β2-adrenoceptor agonist stimulation. It is also uncertain whether its phosphorylation can account for the changes in force we observed in this study. Therefore, in another experiment, we examined the expression and phosphorylation of the β2-adrenoceptor in small muscle fiber bundles isolated from the EDL (fast twitch) and soleus (slow twitch) muscles of adult mice before and after treatment with β2-adrenoceptor agonists. In all fiber bundles examined, the receptor was present irrespective of whether they were from fast- or slow-twitch muscles (Fig. 7). Furthermore, in each fiber type, the phosphorylated and nonphosphorylated forms of the receptor were reciprocal, i.e., when a fiber expressed high concentrations of one form, it expressed low levels of the other. Thus, fast-twitch fibers mainly expressed the nonphosphorylated isoform, whereas slow-twitch fibers predominantly expressed the phosphorylated form, of the receptor (Fig. 7, A and B). Moreover, treatment of the bundles with any of the β2-adrenoceptor agonists increased the phosphorylation of the receptor in slow-twitch muscle fiber bundles (see Fig. 7C).

Effects of treatment of muscle fiber bundles with β2-adrenoceptor agonists on the phosphorylation of PLB. It is generally believed that the lusitrophic effect of β-adrenoceptor stimulation in cardiac muscles arises from the phosphorylation of PLB, a small sarcoplasmic reticulum Ca2+-ATPase pump-associated protein, by PKA (18, 22). However, whether similar changes in the phosphorylation of PLB occur in skeletal muscle fibers after β2-adrenoceptor agonist treatment is uncertain. It is also uncertain whether the changes in its phosphorylation contributed to the changes in force reported elsewhere.

Fig. 6. The PKA inhibitor (PKAi) 14–22 amide does not abolish the effects of clenbuterol and formoterol on P0 in isolated intact mouse skeletal muscle fiber bundles. A: P0 traces showing the effects of treatment of a fast-twitch muscle fiber bundle with standard Ringer solution at the start (Con) and end of the experiment (WO), standard Ringer solution plus the PKAi 14–22 amide, clenbuterol alone, and clenbuterol plus the PKAi 14–22 amide on P0. B: maximum isometric contractions recorded from another fast-twitch muscle fiber bundle treated with standard Ringer’s solution at the start (Con) and end of the experiment (WO), standard Ringer solution plus salbutamol, and salbutamol plus the PKAi 14–22 amide. C: summary data showing the effects of treatment of 5 muscle fiber bundles per experiment with standard Ringer solution at the start (Con) and end of the experiment (WO), standard Ringer solution plus clenbuterol, formoterol, or salbutamol, and the combination of each agonist and the PKAi 14–22 amide on P0. Note that treatment of the muscle fiber bundles with PKAi alone did not significantly affect P0. On the other hand, clenbuterol and formoterol decreased P0, and their effects were not significantly altered by treatment of the fiber bundles with the agonist plus 14–22 amide. In contrast, salbutamol increased P0, and treatment of the fiber bundles with a combination of salbutamol plus 14–22 amide increased P0 further. *P < 0.05 when the indicated data were compared with the control recorded before (Con) and after (WO) the experiments; †P < 0.001 between the two values.
in this report. Therefore, in another experiment, we examined the effects of treatment of small muscle fiber bundles with clenbuterol, formoterol, and salbutamol on the phosphorylation of PLB. Treatment of the fiber bundles with clenbuterol increased the phosphorylation of PLB in fast-twitch fiber bundles but decreased it in fast-twitch fiber bundles (Fig. 8, A and B). Similar results were obtained when fiber bundles were treated with formoterol (results not shown). In contrast, treatment of the fiber bundles with salbutamol had no effect on the phosphorylation of PLB in slow-twitch fiber bundles. However, it completely abolished the phosphorylation of PLB in fast-twitch muscle fiber bundles (Fig. 8, C and D).

Effects of β2-adrenoceptor agonists on cAMP concentrations in fiber bundles. In most tissues, β2-adrenoceptor stimulation activates adenylate cyclase, leading to an increase in intracellular cAMP levels (10, 17, 36, 42), and treatment of rat skeletal muscle fibers with dibutryl-cAMP has similar effects on force as terbutaline (6, 12). Therefore, in another experiment, we investigated the effects of treatment of muscle fiber bundles with either 50 μM clenbuterol or 500 nM salbutamol on cAMP concentrations. Both agonists led to a two- to threefold increase in the concentration of cAMP in all bundles examined irrespective of whether they were from fast- or slow-twitch muscles (Fig. 9). Furthermore, pretreatment of the fiber bundles with 10 μM ICI-118551 completely reversed the increase in cAMP induced by the agonists in both fiber types (Fig. 9, A and B). For example, treatment of the fast-twitch fiber bundles with clenbuterol increased the cAMP levels from 556 ± 10 to 1,371 ± 57 pmol/g muscle (n = 3 fibers), and pretreatment of the bundles with ICI-118551 reversed this to 157 ± 12 pmol/g muscle.

DISCUSSION

Although the acute effects of terbutaline (5, 12) and salbutamol (43) on force production in mammalian skeletal muscle fiber bundles have previously been investigated, this is the first time those of a long-acting β2-adrenoceptor agonist have been examined. The results we present here show that the effects of treatment of small, intact mammalian skeletal muscle fiber bundles with β2-adrenoceptor agonists on force production depend on the class of the agonist and not on the fiber type composition of the muscle, as previously suggested (2, 4, 11, 15, 38, 41). Thus, treatment of the fiber bundles with the short-acting β2-adrenoceptor agonist salbutamol increased force production in both fast- and slow-twitch fiber bundles, whereas treatment with the long-acting β2-adrenoceptor agonists clenbuterol and formoterol had the opposite effect on force (see Figs. 2 and 3).

It is also noteworthy that the effects of salbutamol reported in the present study and those presented elsewhere (5, 12, 43) are similar (for example, compare the results shown in Figs. 2 and 3 of the present study with those shown in Figs. 3 and 4 of Ref. 5). Thus, treatment of the fiber bundles with either salbutamol or terbutaline led to a 14–20% increase in both twitch and tetanic force. Also, the increase in force occurred in both fiber types irrespective of whether the bundles were from mice (present study and Ref. 4) or rats (Refs. 5 and 12). Therefore, it is likely that an increase in force is a general property of all short-acting β2-adrenoceptor agonists in adult mammalian skeletal muscles irrespective of their fiber type composition, and the differences in force observed in some previous studies (2, 3, 15) probably reflect the preparations used or the stimulation techniques used.
One of the adverse effects of β2-adrenoceptor agonists, particularly after oral administration or inhalation at high doses, is skeletal muscle tremors (21, 23). Although the molecular mechanisms underlying the tremors are still poorly understood, some researchers believe that they arise from the action of the agonists on peripheral β2-adrenoceptors (29). However, as the results reported in this and previous studies (5, 6, 12, 43) clearly demonstrate, treatment of small skeletal muscle fiber bundles with various concentrations of clenbuterol (5–100 μM), formoterol (5–150 μM), salbutamol (0.001–1.0 μM) (present study and Ref. 43) and terbutaline (0.1–10 μM) (Refs. 5 and 6) did not induce contractures in any of the preparations. Therefore, we do not think that the tremors arise from the direct actions of these compounds on β2-adrenoceptors in mammalian skeletal muscles. Instead, we speculate that they arise either from the action of these compounds on the autonomic nervous system or from the hypokalemia they induce in vivo (9).

It is generally believed that both short- and long-acting β2-adrenoceptor agonists exert their positive inotropic effects in mammalian skeletal muscles through the β2-adrenoceptor (5, 12). Therefore, their effects should be easily reversed by either propranolol (a general β-blocker) or ICI-118551 (a β2-adrenoceptor-specific blocker). However, as the results reported in the present study show, this is not always the case (see Figs. 4 and 5). Thus, both inhibitors completely abolished the increase in force induced by salbutamol, and ICI-118551 also reversed the increase in cAMP induced by both clenbuterol and salbutamol (see Fig. 9). In contrast, both inhibitors had no effect on the decline in force induced by both clen-
buterol and formoterol. Our interpretation of these observations is that in mammalian skeletal muscle fiber bundles, short-acting β2-adrenoceptor agonists modulate force mainly through the β2-adrenoceptor, whereas long-acting β2-adrenoceptor agonists do not. This suggestion is further supported by the observations that ICI-118551 (12) and propranolol (5) also abolished the effects of terbutaline on force production in isolated rat skeletal muscle fiber bundles, whereas ICI-118551 and knockout of the β2-adrenoceptor had no effect on the increase in glucose uptake induced by clenbuterol in mouse skeletal muscle fiber bundles (32). Similarly, mechanically removing the sarcolemma in rat fast- and slow-twitch skeletal muscle fibers did not abolish the effects of clenbuterol on sarcoplasmic reticulum Ca2+ loading (1). Taken together, all these observations suggest that clenbuterol has effects on force production and glucose uptake in mammalian skeletal muscle fibers that are not mediated via the β2-adrenoceptor.

In rat skeletal muscle fiber bundles, the increase in force induced by short-acting β2-adrenoceptor agonists is accompanied by an increase in cAMP (43) and intracellular Ca2+ concentration (4, 6, 12). It can also be mimicked by dibutyryl-cAMP (5, 12). Therefore, it has been suggested that the increase in force arises from the enhanced release of Ca2+ from the sarcoplasmic reticulum, and, for this to occur, the phosphorylation of ryanodine/dihydropyridine receptors by PKA is necessary (6, 12). Although in the present study both clenbuterol and salbutamol increased the levels of cAMP in all fiber bundles investigated, and although this was completely reversed by pretreatment of the fiber bundles with ICI-118551, pretreatment of the fiber bundles with 14–22 amide, a PKA-specific inhibitor, had no effect on the decrease in force induced by clenbuterol and formoterol. Also, pretreatment of the fiber bundles with 14–22 amide did not abolish the increase in force induced by salbutamol. Instead, it further potentiated the effects of salbutamol on force. Some of these observations give further credence to the notion that long-acting β2-adrenoceptor agonists have direct effects on force. They also suggest that, in mammalian skeletal muscle fibers, the steps downstream of PKA may have negative effects on force production. Indeed, in cardiac muscles, the activation of PKA leads to the phosphorylation of troponin I and myosin-binding protein C, and this has been shown to decrease myofilibrillar Ca2+ sensitivity. It also enhances the cross-bridge cycling rate, and the net effect of all these actions is a reduction in twitch tension (15, 19, 30). Furthermore, as the effects of clenbuterol and formoterol were insensitive to 14–22 amide, we suggest that they are not mediated via PKA either.

Another consequence of PKA activation in striated skeletal muscles is the phosphorylation of PLB. PLB is a 22-kDa oligomeric protein that acts as a modulator of sarco(endoplas-mic reticulum Ca2+-ATPase (SERCA) in both cardiac and slow-twitch skeletal muscle fibers (18). Normally, it inhibits Ca2+ reuptake by SERCA, but once it is phosphorylated at Ser16 and Thr17 by PKA, its inhibitory effects are abolished, and this enhances the activity of SERCA, resulting in an increase in the rate of Ca2+ reuptake and, hence, tension relaxation (40). In addition to its effects on force, terbutaline also increases the rate of tension relaxation in rat slow-twitch muscle fibers (12) but not in mouse fast-twitch muscle fiber bundles (4). Therefore, these observations have been taken as an indication that terbutaline increases the phosphorylation of PLB in mammalian skeletal muscle fibers (6, 12). However, as the results shown in Fig. 8 demonstrate, salbutamol did not increase the phosphorylation of PLB in any of the fiber bundles, suggesting that short-acting β2-adrenoceptor agonists may have no effect on the phosphorylation of PLB. Indeed, dibutyryl-cAMP has been shown to have no effect on the decay of Ca2+ transients recorded from the soleus muscles of both transgenic and wild-type mice (24). Taken together, these findings suggest that an increase in the phosphorylation of PLB may not be a constant feature of treatment of mammalian skeletal muscle fiber bundles with short-acting β2-adrenoceptor agonists.

In contrast, treatment of both fast- and slow-twitch muscle fiber bundles with clenbuterol and formoterol always led to a decrease in force in both fiber types (Figs. 2 and 3) and an increase in the phosphorylation of PLB in slow-twitch fibers only (Fig. 8). Furthermore, in these fibers, the changes in force were always accompanied by a marked increase in the rate of tension relaxation. Indeed, all fiber bundles treated with >50 μM clenbuterol and 100 μM formoterol were unable to maintain tetanus, and their tetanic contractions in the presence of the drugs always looked like twitch contractions. From these observations, we suggest that the phosphorylation of PLB by both clenbuterol and formoterol may play an important role in the decline in force induced by these compounds. However, as PLB is mainly found in slow-twitch fibers, this cannot be the only mechanism underlying the reduction in force induced by these compounds in mammalian skeletal muscle fibers. Therefore, another possibility is that these compounds act directly to uncouple cycling cross-bridges. Indeed, it is well known that β-adrenoceptor stimulation can enhance cross-bridge cycling in ventricular myocytes (14), and this may explain why the actions of clenbuterol and formoterol were insensitive to propranolol and ICI-118551. However, further studies to confirm this are warranted.

In summary, the results we report here show that salbutamol, like terbutaline, increases force production in isolated intact mammalian skeletal muscle fibers, whereas clenbuterol and formoterol have the opposite effects on force. They also show that the effects of salbutamol are mediated via the β2-adrenoceptor, whereas those of clenbuterol and formoterol are not. From these results, we suggest that effects of clenbuterol and formoterol on force production in isolated, intact mammalian skeletal muscle fiber bundles may arise from their direct actions on cross-bridges.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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