

Chronic clenbuterol treatment compromises force production without directly altering skeletal muscle contractile machinery

G. Py^{1,2}, C. Ramonatxo^{1,2}, P. Sirvent³, A. M. J. Sanchez^{1,2}, A. G. Philippe^{1,2}, A. Douillard^{1,2}, O. Galbès^{1,2}, C. Lionne⁴, A. Bonniou², A. Chopard^{1,2}, O. Cazorla⁵, A. Lacampagne⁵ and R. B. Candau^{1,2}

¹Faculté des Sciences du Sport, Université Montpellier, 700 avenue du Pic Saint-Loup, F-34060 Montpellier, France

²INRA, UMR866, Université Montpellier, 2 Place Viala, F-34060 Montpellier, France

³Clermont Université, Université Blaise Pascal, EA3533, Laboratoire des Adaptations Métaboliques à l'Exercice en Conditions Physiologiques et Pathologiques (AME2P), BP 80026, F-63171 Aubière Cedex, France

⁴Centre d'Etudes d'Agents Pathogènes et Biotechnologies pour la Santé, UMR-5236 CNRS-UM1-UM1, France

⁵U1046 INSERM, UMR 9214 CNRS, Physiologie et Médecine Expérimentale du Cœur et des Muscles, University of Montpellier, CHRU Montpellier, Montpellier, France

Key points

- Clenbuterol is an adrenergic receptor agonist known to induce skeletal muscle hypertrophy and a shift towards faster muscle fibres, when administered chronically at high doses.
- However, when normalized to the muscle surface area, the increase in muscle force is no longer increased and even depressed.
- We show that muscle contraction and relaxation force kinetics were significantly reduced particularly in fast contracting muscles.
- We show that action potential-elicited Ca^{2+} transients were depressed in the fast contracting muscle.
- Our data show that chronic clenbuterol treatment reduces contractile efficiency, with altered contraction and relaxation kinetics, but without directly altering the contractile machinery. Lower Ca^{2+} release during contraction could partially explain these deleterious effects.

Abstract Clenbuterol is a β_2 -adrenergic receptor agonist known to induce skeletal muscle hypertrophy and a slow-to-fast phenotypic shift. The aim of the present study was to test the effects of chronic clenbuterol treatment on contractile efficiency and explore the underlying mechanisms, i.e. the muscle contractile machinery and calcium-handling ability. Forty-three 6-week-old male Wistar rats were randomly allocated to one of six groups that were treated with either subcutaneous equimolar doses of clenbuterol ($4 \text{ mg kg}^{-1} \text{ day}^{-1}$) or saline solution for 9, 14 or 21 days. In addition to the muscle hypertrophy, although an 89% increase in absolute maximal tetanic force (P_0) was noted, specific maximal tetanic force (sPo) was unchanged or even depressed in the slow twitch muscle of the clenbuterol-treated rats ($P < 0.05$). The fit of muscle contraction and relaxation force kinetics indicated that clenbuterol treatment significantly reduced the rate constant of force development and the slow and fast rate constants of relaxation in extensor digitorum longus muscle ($P < 0.05$), and only the fast rate constant of relaxation in soleus muscle ($P < 0.05$). Myofibrillar ATPase activity increased in both relaxed and activated conditions in soleus ($P < 0.001$), suggesting that the depressed specific tension was not due to the myosin head alteration itself. Moreover, action potential-elicited Ca^{2+} transients in flexor digitorum brevis fibres (fast twitch fibres) from clenbuterol-treated animals demonstrated decreased amplitude

G. Py and C. Ramonatxo contributed equally to the study.

after 14 days (-19% , $P < 0.01$) and 21 days (-25% , $P < 0.01$). In conclusion, we showed that chronic clenbuterol treatment reduces contractile efficiency, with altered contraction and relaxation kinetics, but without directly altering the contractile machinery. Lower Ca^{2+} release during contraction could partially explain these deleterious effects.

(Received 10 November 2014; accepted after revision 27 January 2015; first published online 27 February 2015)

Corresponding author G. Py: Faculté des Sciences du Sport, Université Montpellier, INRA, UMR866 Dynamique Musculaire et Métabolisme, 2 place Viala, F-34060 Montpellier, France. E-mail: guillaume.py@univ-montp1.fr

Abbreviations AP, action potential; CSA, cross-sectional area; EDL, extensor digitorum longus; FDB, flexor digitorum brevis; SOL, soleus.

Introduction

β_2 -Adrenergic agonists, the most effective bronchodilators used to prevent asthma in humans, act by inhibiting smooth muscle contraction and increasing epithelial mucus clearance. In addition to their anti-asthma effect, chronic administration of these agonists causes a dramatic increase in skeletal muscle growth in various mammalian species (Kim *et al.* 1992; Mersmann, 1998; Kissel *et al.* 2001) and enhances human sports performance (Collomp *et al.* 2005; Sanchez *et al.* 2012; Hostrup *et al.* 2014a,b).

Synthetic β_2 -agonists such as cimaterol, clenbuterol, fenoterol, formoterol, salbutamol and salmeterol are based on the chemical structure of adrenaline and promote muscle growth through the stimulation of β_2 -adrenoceptors and subsequent activation of downstream signalling pathways. Clenbuterol, one of the selective β_2 -adrenergic agonists, is known to induce a specific protein anabolic effect in skeletal muscle (Mersmann, 1998; Kissel *et al.* 2001; Bricout *et al.* 2004; Lynch & Ryall, 2008). Because of this effect, clenbuterol has been extensively studied, especially for its therapeutic potential in various diseases affecting muscle mass or weakness. Effects of β_2 -adrenergic agonist treatment on muscle mass have been demonstrated in the contexts of muscular dystrophy, disuse (Ricart-Firinga *et al.* 2000), ageing, denervation and amyotrophic lateral sclerosis in both animals and humans (Lynch & Ryall, 2008). In addition to the effect on muscle mass, chronic clenbuterol treatment induces a fibre-type transition from slow to fast twitch fibres in animal models (Zeman *et al.* 1988; Polla *et al.* 2001; Mounier *et al.* 2007; Douillard *et al.* 2011). The phenotypic and metabolic changes induced by clenbuterol have raised questions about the impact of this treatment on muscle contractile function and ATPase activity. Previous studies have shown greater force-producing capacity in clenbuterol-treated muscle in both fast and slow twitch muscles (Dodd *et al.* 1996). Nevertheless, when maximal tetanic contraction was corrected for muscle cross-sectional area, muscle force was unchanged and even altered in soleus muscle (McCormick *et al.* 2010). These results suggest a potentially deleterious effect of chronic clenbuterol treatment on the skeletal muscle

contractile machinery. A preliminary study suggested that chronic clenbuterol treatment induces significant changes in the calcium (Ca^{2+}) signals associated with excitation–contraction coupling in fast twitch skeletal muscles (Sirvent *et al.* 2014). However, the mechanisms responsible for the changes in clenbuterol-induced contractility are not well understood.

Ca^{2+} handling is the primary regulator of force generation by cross-bridges in striated muscle. A reduction in Ca^{2+} release from the sarcoplasmic reticulum at each contraction would explain, at least in part, the reduced contractile function. However, muscle contractile function depends on contraction and relaxation processes. Relaxation involves at least three processes: termination of Ca^{2+} release, the kinetics of calcium dissociation from troponin and cross-bridge detachment, and Ca^{2+} uptake by the sarcoplasmic reticulum and myoplasmic Ca^{2+} buffering (Poggesi *et al.* 2005). Strong evidence indicates that cross-bridge kinetics is the major determinant of the time course of striated muscle relaxation and is responsible for ‘load-dependent’ changes in relaxation kinetics. Last, the kinetics of myosin head detachment from the thin filament is related to ATP binding. Thus, beyond muscle hypertrophy, an effect of clenbuterol on contraction and relaxation kinetics is expected.

The aim of the present study was to test the effect of chronic clenbuterol treatment on muscle force generation and relaxation kinetics in slow and fast twitch muscles and on myofibrillar ATPase activity in both relaxed and activated conditions. In addition, Ca^{2+} signalling was evaluated as it is the main regulator of contraction and relaxation kinetics.

Methods

All experiments complied with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publications No. 85-23, Revised 1996). All experiments were approved by the Research Ethics Committee of Languedoc-Roussillon region [‘Ethique sur l’Expérimentation Animale’ approved the experimental protocol (# 36)].

Animals

Forty-three 6-week-old male Wistar rats (body mass: 236.3 ± 3.8 g; Charles River, Wilmington, MA, USA) were randomly allocated to one of six groups and housed in standard cages with a 12:12 h light–dark cycle and food and water available *ad libitum*. Treated rats received subcutaneous equimolar doses of clenbuterol ($4 \text{ mg kg}^{-1} \text{ day}^{-1}$) for 9 (CB-G9, $n = 7$), 14 (CB-G14, $n = 7$) or 21 days (CB-G21, $n = 8$). Body weights were measured daily and injection doses adjusted accordingly. To control for maturation effects due to the treatment duration, control rats were recruited and received a daily subcutaneous injection of an identical volume of saline solution for 9 (CTL-G9, $n = 7$), 14 (CTL-G14, $n = 7$) or 21 days (CTL-G21, $n = 7$). All measurements were made 24 h after the last treatment. While animals were anaesthetized with pentobarbital sodium, extensor digitorum longus (EDL), soleus (SOL) and flexor digitorum brevis (FDB) muscles of both left and right hindlimbs were rapidly dissected out. At the end of the surgical procedure, the animals were killed with a lethal dose of pentobarbital.

Muscle phenotype

SOL and EDL muscle phenotypes were determined by the Kaiser and Brooke myosin-ATPase coloration method (Brooke & Kaiser, 1970). All twitch fibres from each muscle were selected and identified as being type I fibres, type IIa fibres or type IIx-IIb fibres.

Kinetics of muscle force activation and relaxation kinetics

EDL and SOL muscles from the right hindlimb were surgically exposed. The excised muscles were immediately placed in a custom-built Plexiglas chamber filled with Krebs-Ringer solution [composition (in mM): NaCl 11.9, KCl 0.5, CaCl_2 0.125, MgSO_4 0.1, KH_2PO_4 0.1, glucose 1, NaHCO_3 2.5, mannitol 0.11] equilibrated with 95% $\text{O}_2/5\%$ CO_2 gas and maintained at 25°C , pH 7.4.

Contractile properties of the right EDL and SOL were assessed *in vitro* according to the methods described in detail previously (Lynch *et al.* 2001; Ryall *et al.* 2002). *In vitro* contractile measurements began with the determination of the muscle optimal length (L_0) for isometric tension development. After a 15 min equilibration in the bath, the muscle was connected to an isotonic force transducer (model 305B; Cambridge Instruments, Aurora Scientific, Inc., Ontario, Canada) and stimulated along its entire length with platinum wire electrodes. After the determination of L_0 from the maximum isometric twitch force, all subsequent contractile properties were measured

at L_0 . Optimum twitch fibre length (L_f) was determined by multiplying L_0 by previously determined ratios of fibre length to muscle length: 0.44 for EDL and 0.71 for SOL (Brooks & Faulkner, 1988; Ryall *et al.* 2002). Isometric tetanic tension was determined at different frequencies of stimulation (701B Stimulator; Aurora Scientific), ranging from 1 to 100 Hz for SOL and 150 Hz for EDL (pulse duration 0.5 ms, train duration 500 ms). A rest period of 1 min was applied between each tetanic stimulation. Maximal isometric tetanic tension (P_0) was determined from the plateau of the frequency–force curve. After all measurements, the muscle was removed from the bath, trimmed of connective tissue, blotted dry and weighed on an analytical balance. Specific force (sP_0 ; N cm^{-2}) was calculated for each muscle according to the well-accepted procedure that accounts for muscle cross-sectional area, determined after dividing muscle mass by the product of L_f and 1.06 mg mm^{-3} , according to Lynch *et al.* (2001).

Fitting of muscle mechanical kinetics

A non-linear regression was used to fit the data obtained in the CTL-G21 and CB-G21 groups only to a model that defines the kinetics of muscle force production and relaxation in EDL and SOL muscles during a single 500 ms twitch (pulse duration of 0.5 ms at 100 Hz for SOL and 150 Hz for EDL). The model used to fit the force development and relaxation kinetics for EDL and SOL and determine the parameter values is presented in Fig. 1. Data were fitted using GraphPad Prism 4 (GraphPad Prism Software, San Diego, CA, USA). During the force production phase, the rate constant of the force development (k_{ACT} , s^{-1}) was determined. The time course of full force relaxation was biphasic, starting with a slow, seemingly linear, phase (rate constant, Slow k_{REL} , s^{-1}) followed, after a ‘shoulder’, by a fast, approximately monoexponential, relaxation phase (rate constant, Fast k_{REL} , s^{-1}).

Myofibril preparation

SOL (slow twitch) muscle from CTL-G21, CB-G14 and CB-G21 groups as well as EDL (fast twitch) muscle from CTL-G21, CB-G9, CB-G14 and CB-G21 groups was used for these experiments. Briefly, muscles were immersed in ice-cooled Ringer buffer [composition (in mM): Tris-HCl 50, pH 7.0, NaCl 100, KCl 2, MgCl_2 2, EGTA 1, dithiothreitol 1, phenylmethanesulfonyl fluoride 0.2, leupeptin 0.01, pepstatin 0.005 and NaN_3 0.5] at rest length. From these latter SOL and EDL muscles, myofibrils were prepared as previously described (Candau *et al.* 2003). The total myosin head concentration in the myofibrillar suspension was measured by absorbance at 280 nm of a 1:10 dilution of the suspension in 2% SDS

(Houadjeto *et al.* 1992), based on the assumption that the molar extinction coefficient and percentage of myosin in SOL and EDL myofibrils are the same as in psoas myofibrils (Herrmann *et al.* 1994; Iorga *et al.* 2004).

Myofibrillar ATPase activity

ATPase measurements were carried out in a home-built, thermostatically controlled, Rapid Flow Quench apparatus (Barman & Travers, 1985). Experiments were performed at 4°C to slow down the unloaded shortening velocity (V_0), and thus to increase the duration of the unloaded shortening phase (t_B) and improve the time resolution. The chosen temperature of 4°C, although it slows the shortening velocity, still allows the myofibrils to behave and contract normally (Candau *et al.* 2003).

In the Rapid Flow Quench apparatus, myofibrils were mixed with [γ - 32 P]ATP (2.5 μ M myosin heads + 25 μ M [γ - 32 P]ATP in the reaction mixture at 4°C). Reaction mixtures were quenched in acid (22% trichloroacetic acid and 1 mM KH_2PO_4) at different times and the [32 P]Pi was assayed. Reaction mixtures with incubation times from 200 ms to 10 s were obtained with this apparatus. For longer incubation times, myofibrils and [γ - 32 P]ATP were mixed in a beaker at 4°C, where one sample of the reaction mixture was taken and quenched every 10–15 s for 3–10 min. The amounts of total Pi (i.e. free Pi plus myosin head-bound Pi) were determined in the quenched reaction mixtures by the filter paper method of Reimann and Umfleet (1978). Relaxing and activating buffers were used to obtain relaxed and activated conditions, respectively, i.e. 50 mM Tris-acetate, pH 7.4, 100 mM

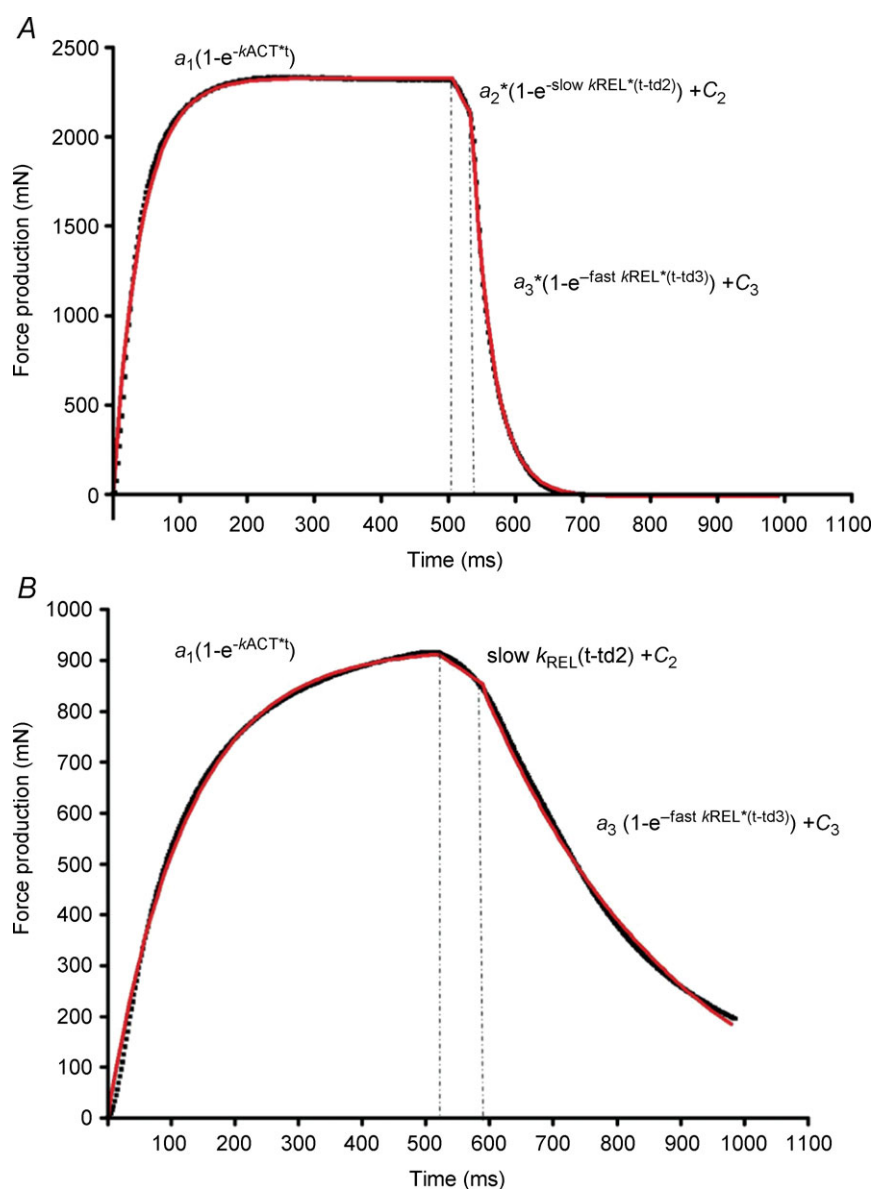


Figure 1. Fitting of force activation and relaxation in EDL (A) and SOL (B) muscles
Recording shows the fitting curve (red line) obtained from the data curve (black line) recorded at 150 and 100 Hz for EDL and SOL, respectively. The kinetics of force development was described by one exponential increase: $a_1(1-e^{-k_{ACT}t})$, where a_1 is the value of force at the maximal curve asymptote (mN); e is the exponential function; k_{ACT} (s^{-1}) is the rate constant of the force development; and x is the time (ms). The kinetics of force relaxation was described using two exponential slow and fast decays: $a_2(1-e^{-slow\ k_{REL}*(t-td2)}) + C_2$ and $a_3(1-e^{-fast\ k_{REL}*(t-td3)}) + C_3$ where C_2 and C_3 are the force levels at the beginning of each decay; $slow\ k_{REL}$ and $fast\ k_{REL}$ are the rate constants of the slow and fast phases of tension decline, respectively; a_2 and a_3 are the basal relaxation force level C_2 and C_3 , respectively; and $td2$ and $td3$ are the time delays at the beginning of each decay.

potassium acetate, 5 mM KCl and either 0.1 mM CaCl₂ and 2 mM magnesium acetate for the activating buffer (with Ca²⁺), or 2 mM EGTA and 5 mM magnesium acetate for the relaxing buffer (without Ca²⁺).

Modelling ATPase activity kinetics

Although the first phase of steady-state ATP hydrolysis was used as a measure of the ATPase activity during unloaded shortening of the calcium-activated myofibrils, the entire kinetics of ATP hydrolysis was modelled with a three-component model to determine the kinetic parameters:

$$\text{At } t < t_B, [\text{Total Pi}] / [\text{myosin heads}] = A + [k_F \cdot t] \quad (1)$$

$$\text{At } t > t_B, [\text{Total Pi}] / [\text{myosin heads}] = A + [k_F \cdot t] \\ + [A + k_F \cdot t_B + k_S \cdot (t - t_B)] \quad (2)$$

where the third component eqn (2) starts when $t > t_B$, $[\text{Total Pi}]/[\text{myosin heads}]$ is the amount of total phosphate determined per myosin head (mol of ATP hydrolysed per mol of myosin heads), A is the amplitude (mol/mol) of the Pi burst, k_F is the rate constant (s^{-1}) of the fast steady state during the unloaded shortening phase in activated conditions, k_S is the rate constant (s^{-1}) of the steady state during 'over-contraction' when the thin filaments of the two halves of the sarcomeres start to overlap each other (a non-physiological state), and t_B is the time break (s) corresponding to the unloaded shortening phase duration (Lionne *et al.* 1996). The model parameters were determined using an iterative process that minimizes the sum of the mean squares between the total phosphate measured and that predicted by the model. The ATP cost of contraction during the unloaded shortening phase was determined based on the number of ATPs consumed per myosin head ($\text{ATP} \cdot t_B$) until t_B .

The titration of the myofibril preparation ([active site]), i.e. the number of fully active ATPase sites per total sites, was estimated from the amplitude (A) of the Pi burst as proposed by Iorga *et al.* (2004) for Pi burst experiments on SOL myofibrils. Titration experiments were performed in relaxed conditions because (i) A is similar in relaxed and Ca²⁺-activated conditions, (ii) the cleavage step that controls the Pi burst amplitude occurs in the detached state whatever the condition and (iii) A is determined more accurately in relaxed than in activated conditions, due a slower reaction compared with activated conditions.

The limiting step of the cross-bridge cycle, the isomerization step that occurs just before Pi release (k_4), was evaluated from the catalytic activity (k_{cat}) and the equilibrium constant of the ATP cleavage step (K_3) (Iorga *et al.* 2004):

$$k_4 = k_{\text{cat}} (1 + K_3) / K_3 \quad (3)$$

where $k_{\text{cat}} = k_5 / [\text{active site}]$, and $K_3 > 10$.

Stopped-flow experiments

The low temperature retained for the enzyme kinetic experiments allowed measurement of the kinetics of the ATP-induced myosin head detachment from the thin filament and the ATP cleavage (K_3) (Stehle & Brenner, 2000). The experiment was carried out in a Hi-Tech Scientific stopped-flow apparatus (model SF-61 DX2; Hi-Tech Ltd, Salisbury, UK). The excitation wavelength was 295 nm and emission was >320 nm using a cut-off filter (WG320; Hi-Tech) to measure tryptophan fluorescence. For each experimental condition, a series of 12 shots was carried out and averaged. The fluorescence of the myofibrillar solution, without ATP, was set as 100% just before the start of the experiment. Fluorescence time courses were fitted with a double exponential model of rate constants $k_{\text{Trp fast}}$ and $k_{\text{Trp slow}}$. The fast phase is related to the ATP-induced detachment of the myosin head from the actin filaments. Its kinetics is hyperbolically dependent on ATP concentration, and the initial slope of the dependence gives information on the rate of detachment. The slow phase kinetics is governed by the ATP cleavage step and is independent of ATP concentration. For further details, see Stehle and Brenner (2000).

Modelling ATPase activity as a function of twitch fibre type

To identify the changes in ATPase activity due to changes in the twitch fibre type in response to clenbuterol treatment, a multilinear regression analysis including specific ATPases for each twitch fibre type was used:

$$k_i = k_{\text{ff-I}} \cdot \text{F-I} + k_{\text{ff-IIa}} \cdot \text{F-IIa} + k_{\text{ff-IIx/b}} \cdot \text{F-IIx/b} \\ + k_{\text{ssF-I}} \cdot \text{F-I} + k_{\text{ss-IIa}} \cdot \text{F-IIa} + k_{\text{ssF-IIx/b}} \cdot \text{F-IIx/b} \quad (4)$$

where F-I, F-IIa and F-IIx/b were the percentages of twitch fibre types I, IIa and IIx/b, respectively, and k_{ss} was the relaxed ATPase steady state, assumed to be equal to $0.125k_f$.

Ca²⁺-transient measurement

Due to its relatively small size and the technical constraints associated with Ca²⁺-transient measurement, the FDB muscle (fast twitch) was selected, dissected and enzymatically dissociated at 37°C for 1 h with 3 mg ml⁻¹ collagenase (type 1; Sigma, St Louis, MO, USA) dissolved in an external medium (in mM: NaCl 145, KCl 4, CaCl₂ 1.8, MgSO₄ 1, Hepes 10 and glucose 10, pH 7.4). Bundles of fibres were then transferred to an external medium without collagenase, and the fibres were mechanically separated by gentle trituration. Intact single fibres were

Table 1. Time course of clenbuterol-induced phenotype shift in EDL and SOL muscles

	EDL				SOL				
	CTL-G21	CB-G9	CB-G14	CB-G21	CTL-G21	CB-G9	CB-G14	CB-G21	
Fibre types (%)	I	7.5 ± 2.2	7.3 ± 2.6	7.6 ± 2.8	5.4 ± 1.4	80.0 ± 7.4	72.0 ± 8.3	69.4 ± 9.5*	60.7 ± 8.5***
	Ila	25.6 ± 1.9	14.7 ± 4.3*	9.9 ± 2.4**	11.0 ± 3.7**	14.8 ± 8.5	16.9 ± 8.4	20.4 ± 9.7	29.1 ± 10**
	IIX-IIb	66.9 ± 2.7	78.0 ± 4.3*	82.4 ± 3.1**	83.6 ± 4.7**	5.3 ± 3.6	11.1 ± 1.8*	10.2 ± 2.8*	10.2 ± 6.8*

Values are mean percentages (\pm SD, $n = 8$) of the different fibre types in EDL and SOL. *Different from CTL-G21; **different from CTL-G21 and CB-G9; ***different from CTL-G21, CB-G9 and CB-G14. $P < 0.05$. Because non-statistically differences exist between CTL-G9, CTL-G14 and CTL-G21 groups for both fibre type distribution and CSA, results of the CTL-G21 group only are given.

plated on extracellular matrix (Sigma)-coated coverslips attached across a 12 mm hole in the bottom of 35 mm Petri dishes. FDB fibres bathed in an external medium were loaded for 30 min at room temperature with Fluo-3 AM (5 μ M; TefLabs, Austin, TX, USA). Action potential (AP)-induced Ca^{2+} transients were triggered by field stimulation with a single 2 ms pulse just above the stimulation threshold. Fluorescence images were acquired in line-scan mode (spatial [x] vs. temporal [t], 1.5 ms per line) with a confocal system (Zeiss LSM 510 Meta, 63 \times objective, NA = 1.2, H₂O immersion; Oberkochen, Germany). Fluo-3 was excited with an argon/krypton laser at 488 nm, and the emitted fluorescence was recorded at 525 nm. Single-fibre Ca^{2+} transients were identified on raw fluorescence images. Image strips of the manually identified area of a single fibre were extracted. After subtracting the photomultiplier offset current, strip images were converted to $\Delta F/F$, where F is the mean resting fluorescence of the fibre calculated from the image area preceding the AP stimulation and subsequent Ca^{2+} transient. The temporal fluorescence time courses of each fibre were generated by spatial compression of the $\Delta F/F$ images, resulting in a mean spatial F value for each temporal coordinate.

Statistics

Variables were compared between groups using ANOVA and the Newman-Keuls *post hoc* multiple comparison procedure when significance was detected. The significance of the model parameters retained in the kinetic analysis was tested by an F-test. Significance was set at $P < 0.05$. All values are expressed as mean \pm SEM.

Results

Morphometric properties, shift in muscle phenotype

Clenbuterol treatment for 21 days resulted in significant increases in muscle mass, more pronounced in EDL (+22%; 220.9 \pm 5.4 mg CB-G21 vs. 176.3 \pm 8.3 CTL-G21; $P < 0.001$) than SOL (+9%; 202.0 \pm 5.8 mg CB-G21

vs. 176.7 \pm 6.1 mg CTL-G21; $P < 0.01$). The muscle mass/body mass ratio was not different compared with control groups. In the clenbuterol-treated rats, the ratio was unchanged in SOL (0.53 \pm 0.01 CB-G21 vs. 0.54 \pm 0.01 CTL-G21) but significantly increased in EDL (0.59 \pm 0.01 CB-G21 vs. 0.52 \pm 0.02 CTL-G21; $P < 0.001$).

Non-statistically significant differences were found between CTL-G9, CTL-G14 and CTL-G21 groups for both fibre type distribution or cross-sectional area (CSA) (data not shown for CTL-G9 and CTL-G14 groups) and thus we decided to present results of the CTL-G21 group only. EDL muscle underwent a phenotypic shift toward faster muscle twitch fibres, as determined by the myosin ATP (Table 1). This shift was noticeable from day 9 onward. The increased percentage of type IIX-IIb twitch fibres at day 9 was accompanied by a decreased percentage of type Ila twitch fibres. At day 21, a 17% ($P < 0.05$) increase and a 15% ($P < 0.05$) decrease in IIX-IIb and Ila twitch fibre types, respectively, were observed compared with CTL-G21. Similar results in the SOL muscle were found (Table 1). At day 14, the proportion of type I muscle fibres was 11% ($P < 0.05$) lower in the treated group compared with CTL-G14 group. The percentage of type IIX-IIb twitch fibres was significantly higher in the treated group compared with CTL-G9 at day 9 (+6%, $P < 0.01$). Later (i.e. between day 9 and day 21), no further increase in the percentage of type IIX-IIb twitch fibres was observed. After 21 days of treatment, the percentage of type Ila fibres was significantly increased from 14 to 29% (Table 1).

Kinetics of muscle force activation and relaxation

Muscle force development. In EDL muscles, maximal isometric tension (P_o) exhibited an 89% increase in CB-G21 compared with CTL-G21 (2232 \pm 192 vs. 1177 \pm 206 mN, respectively; $P < 0.05$, Fig. 2A). However, when P_o was corrected to account for muscle CSA, sP_o was not different in EDL among groups (12.3 \pm 1.0 vs. 12.0 \pm 1.8 N cm⁻² in CB-G21 and CTL-G21, respectively, Fig. 2A).

In SOL muscle, no significant difference in P_o was observed between CB-G21 and CTL-G21 (1648 ± 133 vs. 1365 ± 50 mN, respectively) (Fig. 2B). However, clenbuterol treatment significantly reduced SOL sP_o in CB-G21 compared with CTL-G21 (11.9 ± 0.7 vs. 14.0 ± 0.6 N cm⁻²; $P < 0.05$, Fig. 2B).

Kinetics of force development and relaxation. Fitting of the kinetics of force activation and relaxation for EDL

and SOL gave high determination coefficients, with a mean r^2 value greater than 0.997 and 95% confidence intervals inferior to 1% for EDL and SOL. Figure 2C and D illustrates representative recordings from fitting curves obtained from G21-CB and CTL-G21 for both EDL and SOL. The results obtained from fitting the muscle mechanical kinetics showing the clenbuterol effect after 21 days of treatment are summarized in Table 2. In EDL, the main clenbuterol effect was observed for k_{ACT} with a significant decrease compared with the untreated

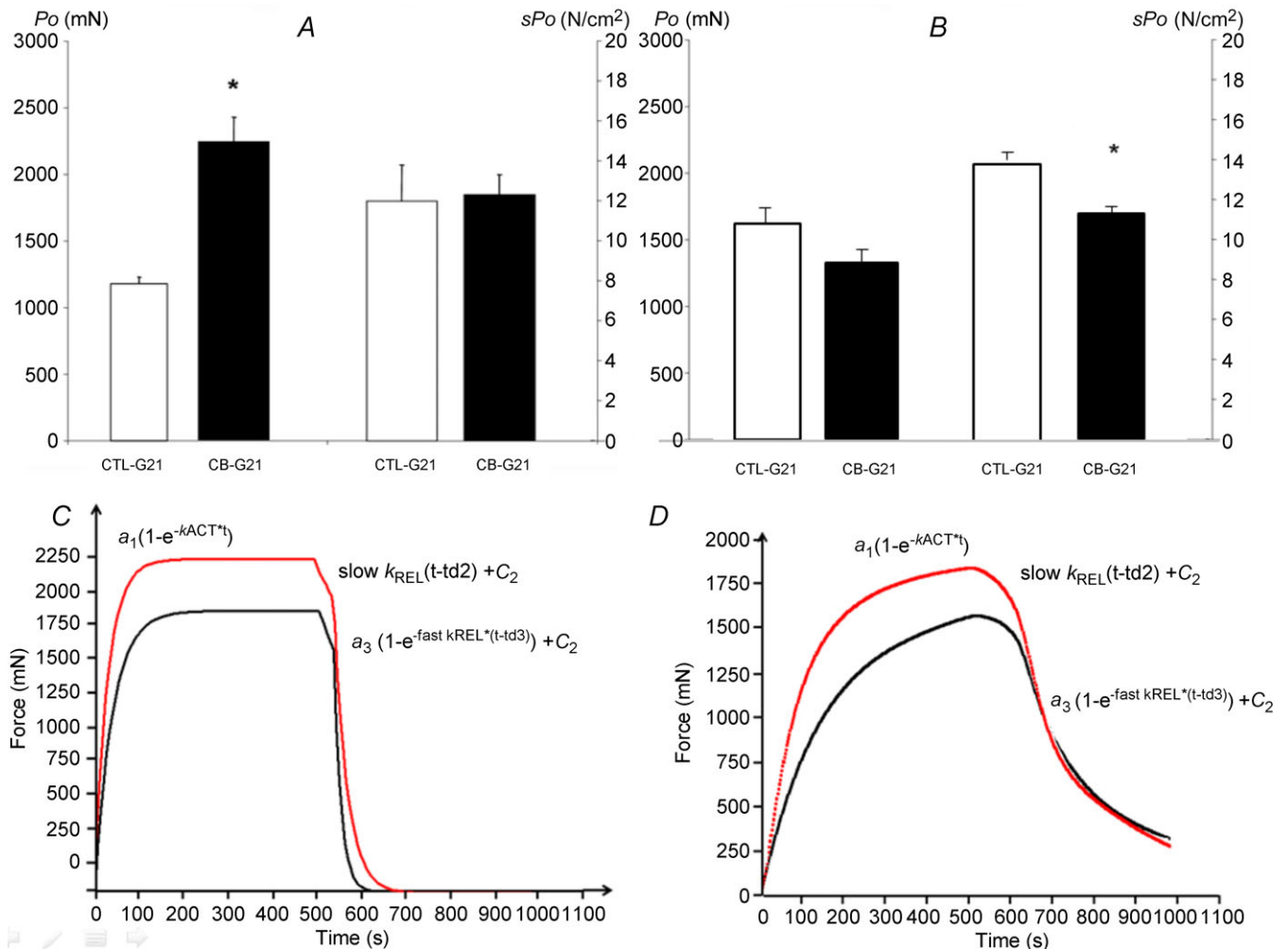


Figure 2. Effect of clenbuterol treatment on maximal isometric tetanic power (P_o), specific isometric tetanic power (sP_o), and force development and force relaxation kinetics in EDL (A, C) and SOL (B, D) muscle

Mean (\pm SEM) EDL (A) and SOL (B) maximal isometric tetanic power (P_o , left vertical axis) and specific maximal isometric tetanic power (sP_o , right vertical axis) recorded from CTL-G21 (white bar) and CB-G21 animals (dark bar). C and D, recording shows representative fitting curves in a CB-treated rat (red line) and a CTL-treated rat (black line) in EDL muscle (C) and SOL muscle (D). The kinetics of force development was described by one exponential increase: $a_1(1-e^{-k_{ACT}t})$, where a_1 is the value of force at the maximal curve asymptote (mN); e is the exponential function; k_{ACT} (s^{-1}) is the rate constant of the force development; and x is the time (ms). The kinetics of force relaxation was described using two exponential slow and fast decays: $a_2(1-e^{-slow\ k_{REL}(t-td2)} + C_2)$ and $a_3(1-e^{-fast\ k_{REL}(t-td3)} + C_3)$ where C_2 and C_3 are the force levels at the beginning of each decay; $slow\ k_{REL}$ and $fast\ k_{REL}$ are the rate constants of slow and fast phases of tension decline, respectively; a_2 and a_3 are the basal relaxation forces level C_2 and C_3 , respectively; and $td2$ and $td3$ are the time delay at the beginning of each decay. * $P < 0.05$ CTL-G21 vs. CB-G21.

Table 2. Clenbuterol alters contraction and relaxation kinetics in fast muscle

	EDL		SOL	
	CTL-G21 (<i>n</i> = 7)	CB-G21 (<i>n</i> = 8)	CTL-G21 (<i>n</i> = 7)	CB-G21 (<i>n</i> = 8)
k_{ACT} (s^{-1})	23.1 ± 0.8	19.3 ± 1.2**	8.5 ± 0.8	7.6 ± 0.0
Slow k_{REL} (s^{-1})	3.4 ± 0.1	1.8 ± 0.0**	1.8 ± 0.2	1.4 ± 0.1
Fast k_{REL} (s^{-1})	38.7 ± 2.2	28.7 ± 1.6***	10.4 ± 1.7	6.3 ± 0.5*

Kinetic parameters for force development and relaxation during maximal tetanic electrostimulation following clenbuterol treatment in EDL and SOL muscles. Values are expressed as means ± SEM. k_{ACT} , rate constant of force development; Slow k_{REL} , rate constant of the slow relaxation phase; Fast k_{REL} , rate constant of the fast relaxation phase; CTL-G21, untreated control group; CB-G21, clenbuterol-treated group. * P < 0.05; ** P < 0.01; *** P < 0.001.

condition (Table 2; P < 0.01). In addition, clenbuterol treatment significantly decreased Slow k_{REL} and Fast k_{REL} (Table 2; P < 0.01 and P < 0.001, respectively). In SOL muscle, a significant decrease in Fast k_{REL} was observed in CB-21 compared with CTL-G21 (Table 2; P < 0.05).

Chemical kinetics

Slow-type muscle. Relaxed condition. A significant increase in myosin ATPase activity was observed during clenbuterol treatment compared with CTL (Figs 3A and 4A, P < 0.001) in myofibrils prepared from SOL muscle.

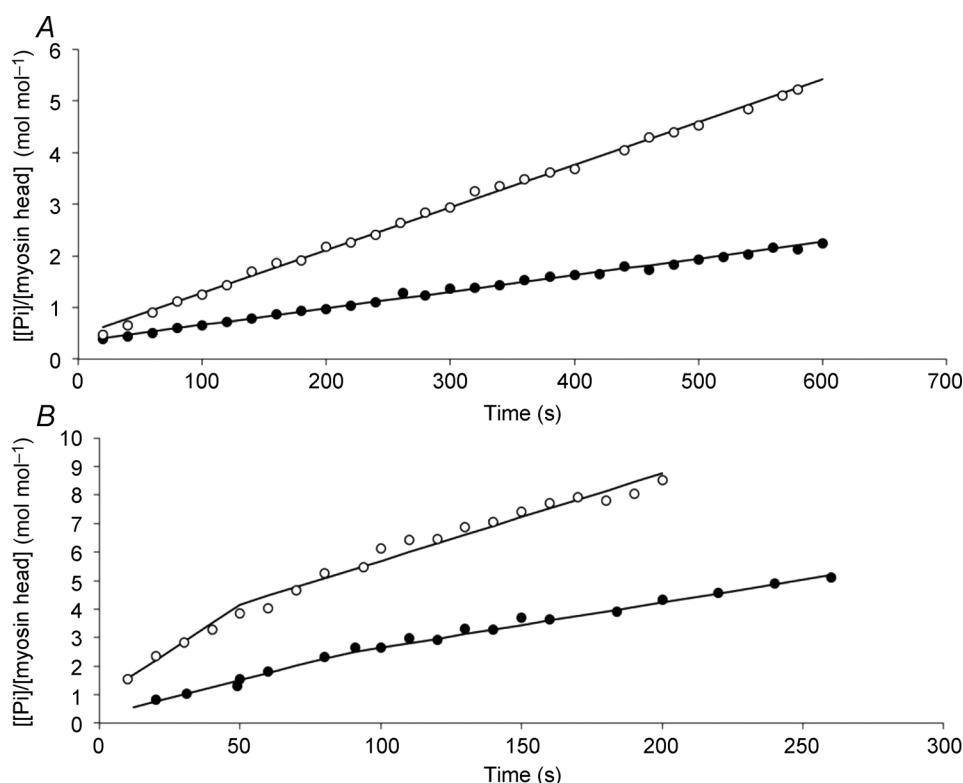


Figure 3. Time courses of SOL myosin ATPase in relaxed (A) or Ca^{2+} -activated (B) myofibrils in control group (filled circles), and after 21 days of clenbuterol treatment (open circles)

Reaction mixtures were 3 μM myofibrils (as myosin heads) plus 30 μM [γ - ^{32}P]ATP at 4°C. The reaction mixtures were quenched at the times indicated and the total [^{32}P]Pi concentrations were determined. In the relaxed condition, the amplitude of Pi burst and ATPase activity were 0.34 ± 0.05 vs. 0.45 ± 0.07 mol mol $^{-1}$, and 0.0083 ± 0.0011 s $^{-1}$ for the control and 21-day clenbuterol-treated groups, respectively. In the Ca^{2+} -activated condition, the slope of the fast linear phase (ATPase activity in unloaded shortening), slope of the slow linear phase, duration of the unloaded shortening phase (dashed lines) and the ATP cost of shortening were 0.025 ± 0.004 vs. 0.065 ± 0.01 s $^{-1}$, 0.016 ± 0.003 vs. 0.031 ± 0.006 s $^{-1}$, 87 ± 9 vs. 50 ± 5 s and 2.4 ± 0.2 vs. 4.2 ± 0.4 mol mol $^{-1}$ for the control (CTL-G21) and 21-day clenbuterol-treated groups (CB-G21), respectively.

This increase was explained by the twitch fibre shift from slow to fast type ($P < 0.001$). The lack of significant change ($P > 0.05$) in the titration index suggests that the number of myosin heads fully competent in the myofibril suspension, from an enzymatic point of view, was

not affected by the clenbuterol treatment. The fast rate constant of the tryptophan fluorescence signal increased and the steeper initial slope of the dependence with ATP concentration ($P < 0.01$; Fig. 4B) indicated an increase in the rate constant of ATP binding-induced myosin

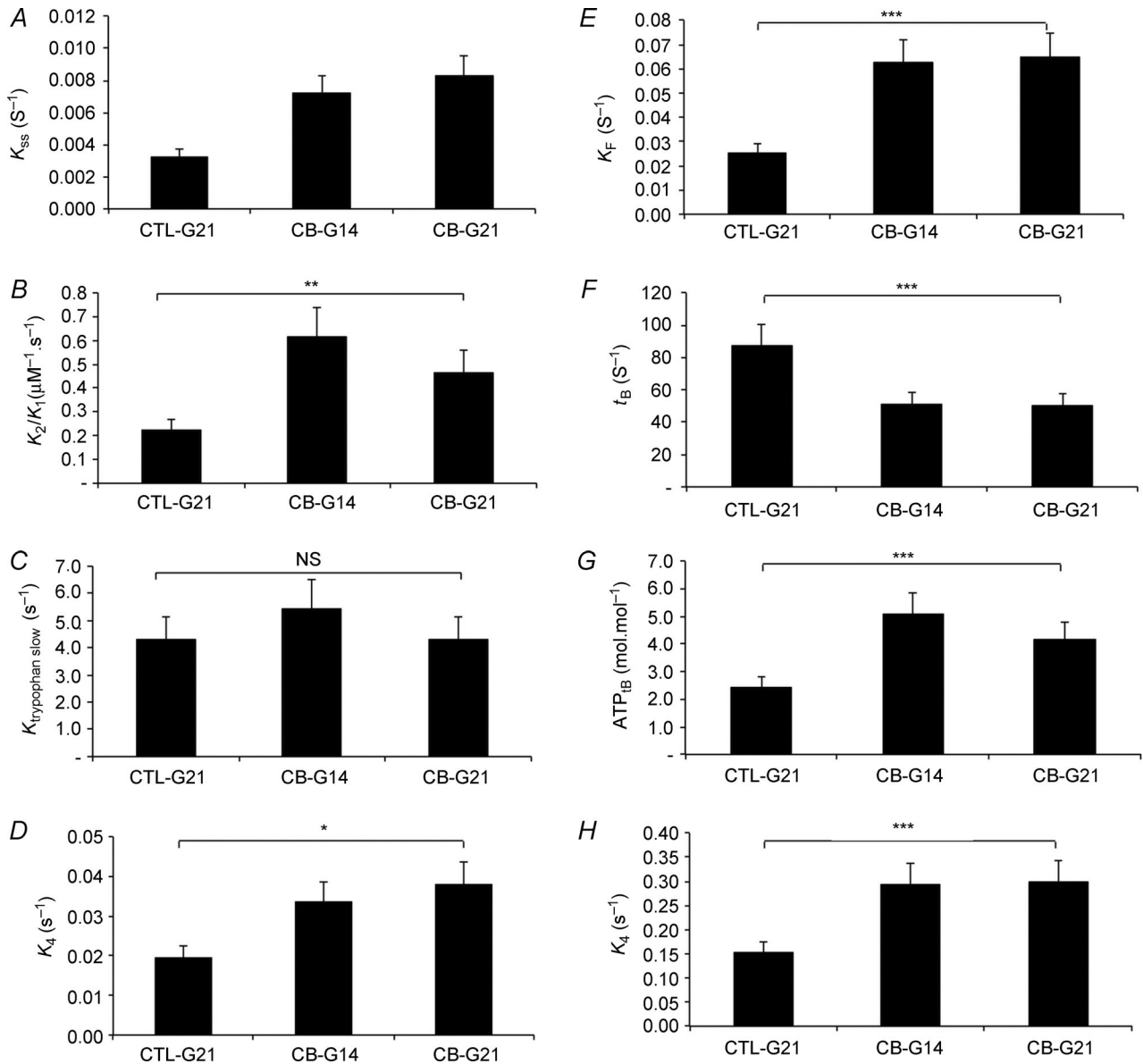


Figure 4. Effect of clenbuterol on SOL myofibrillar ATPase activity in relaxed (A–E) and Ca^{2+} -activated (F–H) conditions at 4°C

Reaction mixture concentrations were 3 and 30 μM for myosin heads and [γ - ^{32}P]ATP concentrations in Rapid Flow Quench experiments, respectively. In Stopped-Flow experiments (B and C), myosin head concentration was 1 μM reaction mixture, and ATP concentrations ranged from 30 μM to 1 mM. CTL-G21, control group; CB-G14, 14-day clenbuterol-treated group; CB-G21, 21-day clenbuterol-treated group; k_{ss} , rate constant of the steady-state phase in relaxed condition; k_2/k_1 , ATP binding-induced myosin detachment obtained from the initial slope of the regression of $k_{tryptophan\ fast}$ as a function ATP concentration; $k_{tryptophan\ slow}$, rate constant observed for the slow phase of tryptophan increase when mixing myofibrils with ATP; k_4 , rate constant of Pi release (the isomerization step preceding diffusion of Pi); k_F , rate constant of the fast steady-state phase in activated condition; t_B , duration of the unloaded shortening phase, an index of unloaded shortening velocity; ATP_{t_B} , ATP consumed during the unloaded shortening phase. Because no significant difference was detected between CTL-G9, CTL-G14 and CTL-G21 groups, results of the CTL-G21 group are presented. NS, not significant. ** $P < 0.01$; *** $P < 0.001$.

detachment with clenbuterol treatment compared with the control group. In contrast, the rate constant of the tryptophan fluorescence slow phase was not altered by clenbuterol treatment ($P > 0.05$; Fig. 4C). As this slow phase was attributed to the ATP cleavage step (Stehle & Brenner, 2000), the present result suggests a lack of clenbuterol effect on this ATP cleavage step. The increase in the rate constant of Pi release under clenbuterol treatment ($P < 0.001$; Fig. 4D) suggests that the enhancement in relaxed ATPase activity was due to an increase in the Pi release step, a rate-limiting step in the steady state of ATPase activity.

Ca²⁺-activated condition. In this condition and over the present time scale, the time curve was biphasic (Fig. 3B). The fast steady state corresponds to the unloaded shortening phase (Lionne *et al.* 1996). During this phase, myofibrils were shortened at maximal velocity in the unloaded condition. The slow steady state corresponds to a pseudo-isometric phase (non-physiological phase). The time corresponding to the transition between these two phases is the duration of the unloaded shortening (t_B). A significant increase in the slope of the fast steady state (i.e. myosin ATPase activity) was observed under clenbuterol treatment in activated SOL myofibrils compared with controls ($P < 0.001$; Fig. 4E). In association with this increase, a shorter duration of the unloaded phase was noted ($P < 0.001$; Fig. 4F), suggesting an increase in unloaded shortening velocity. Interestingly,

the amount of ATP hydrolysed during the unloaded shortening phase increased under clenbuterol treatment (Fig. 4G; $P < 0.001$). Similarly to the relaxed condition, an increase ($P < 0.001$) in Pi release rate was also noted in the Ca²⁺-activated condition, suggesting that the increased myofibrillar ATPase was due to an increase in the rate constant of Pi release. The multilinear regression analysis revealed that the increases in the Pi release rate constant and myofibrillar ATPase activity in SOL were mainly explained ($P < 0.001$) by the phenotype shift induced by clenbuterol.

Fast-type muscle. No significant change was seen in myofibrillar ATPase activity (Fig. 5) in myofibrils prepared from EDL muscle. The rate constant of the fast and slow steady-state phases (Fig. 5A and B, respectively), the duration of the unloaded shortening phase (Fig. 5C) and the amount of ATP consumed during this shortening (Fig. 5D) were not modified by clenbuterol treatment. The multilinear regression analysis revealed that the increase in the Pi release rate constant and myofibrillar ATPase activity in EDL were not significantly explained by the phenotype shift induced by clenbuterol.

Ca²⁺ transients

The action potential-elicited Ca²⁺ transients in field-stimulated intact FDB twitch fibres from the clenbuterol-treated animals showed a progressively

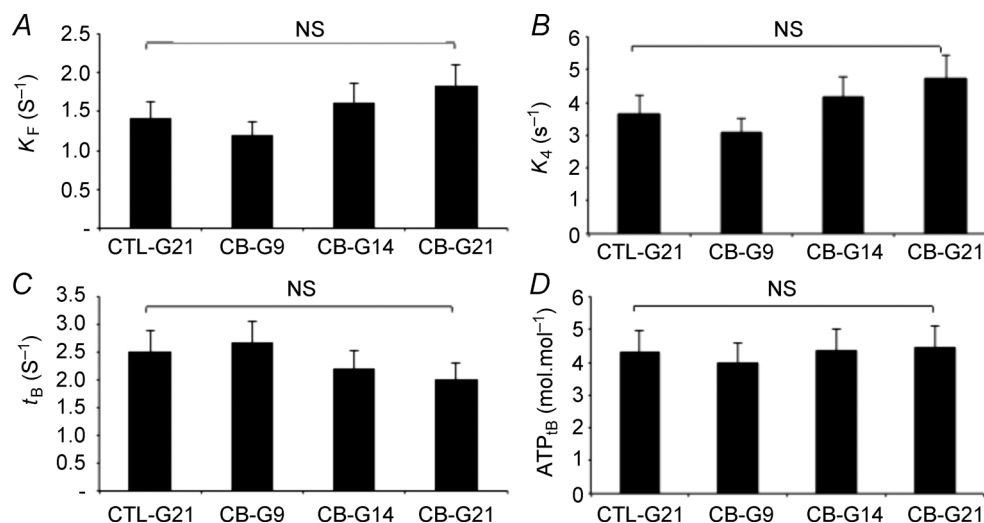


Figure 5. Effect of clenbuterol on EDL myofibrillar ATPase activity in Ca²⁺-activated conditions at 4°C. Reaction mixture concentrations were 3 and 30 μM for myosin heads and [γ -³²P]ATP concentrations in Rapid Flow Quench experiments, respectively. CTL-G21, control group; CB-G9, 9-day clenbuterol-treated group; CB-G14, 14-day clenbuterol-treated group; CB-G21, 21-day clenbuterol-treated group; k_4 , rate constant of Pi release (the isomerization step preceding diffusion of Pi); k_F , rate constant of the fast steady-state phase in activated condition; t_B , duration of the unloaded shortening phase, an index of unloaded shortening velocity; ATP_{t_B}, ATP consumed during the unloaded shortening phase. Because no significant difference was detected between CTL-G9, CTL-G14 and CTL-G21 groups, results of the CTL-G21 group are presented. NS, not significant.

decreased amplitude after 14 days (-19% , $P < 0.01$) and 21 days (-25% , $P < 0.01$), but not after 9 days, when compared with the control groups (Fig. 6).

Discussion

In this study, we showed that chronic clenbuterol administration induced a strong side effect, in contrast to the positive functional effect associated with muscle hypertrophy and absolute maximal tetanic force (P_0). In agreement with the study of Head & Ha (2011) showing that clenbuterol treatment induces a loss in force production, we demonstrated that this loss is linked to (i) a diminution of the contraction and relaxation kinetics during muscle force generation, especially in fast-type muscle, and (ii) calcium handling.

Chronic clenbuterol treatment leads to hypertrophy but reduced specific force in slow twitch muscle

We confirmed previous studies showing that chronic clenbuterol administration increases both muscle mass and absolute isometric force, especially in fast-type muscle (Mounier *et al.* 2007). In the present study, the 89% increase in EDL absolute P_0 in clenbuterol-treated rats ($4 \text{ mg kg}^{-1} \text{ day}^{-1}$) is greater than the 31% increase found by Zeman *et al.* (1988) for the same duration of treatment with rats receiving about $1.6 \text{ mg kg}^{-1} \text{ day}^{-1}$ or the 20% increase in maximal isometric force in the study of Ryall *et al.* (2002) with the commonly used dose of $2 \text{ mg kg}^{-1} \text{ day}^{-1}$. Thus, it seems that a dose-response effect exists between clenbuterol and the related increase in maximal tetanic force for treatment lasting at least 2–4 weeks (Zeman *et al.* 1988; Ryall *et al.* 2002). Moreover at the dose of $1.6 \text{ mg kg}^{-1} \text{ day}^{-1}$ it seems that no further increase in type II fibre CSA or P_0 occurs in EDL muscle between 2 and 12 weeks of treatment (Zeman

et al. 1988). Nevertheless, when normalized to CSA, the maximal tetanic force was unchanged and even depressed in slow twitch muscle. Moreover, it is well known that high dose clenbuterol administration, as in our study, causes inflammation and apoptonecrosis in muscle early on in the treatment and that these effects seem to be greater in slow twitch fibres (Burniston *et al.* 2005; Douillard *et al.* 2011). This could be due to higher β_2 -adrenoceptor and protein G α s subunit density in slow-type as opposed to fast-type muscles (Martin *et al.* 1989). The main consequences of the apoptonecrotic period could be delayed muscle growth and an increased number of infiltrated cells and damaged fibres. Thus, these transient deleterious effects might explain the subsequent decrease in the maximal tetanic contraction obtained at 9, 14 and 21 days in SOL and, to a lesser extent, the decrease in the maximal tetanic contraction obtained at 14 days in EDL.

Clenbuterol treatment did not increase specific myofibrillar ATPase activity

Our results show that the myofibrillar ATPase activities and the content of type II twitch fibres increased in the same proportion in slow twitch muscle during clenbuterol treatment, indicating that the ATPase activity associated with each twitch fibre type was not altered. This suggests that the depressed specific tension was not due to the contractile machinery itself. Two myofilament proteins are possibly phosphorylated in response to β -adrenoceptor activation, i.e. troponin-I, which reduces the affinity for Ca^{2+} binding to troponin-C, and myosin binding protein-C (C-protein), which increases the maximal myosin ATPase rate, although neither protein seems phosphorylated in mammalian skeletal muscle under β -adrenergic agonist treatment (Manning & Stull, 1982; Gruen *et al.* 1999). The amino acid residues that are phosphorylated by protein kinase A in cardiac troponin-I or C-protein are absent from the skeletal muscle isoforms of these proteins (Shaffer & Gillis, 2010). Another protein, myosin light-chain kinase, is phosphorylated under β -agonist treatment in smooth muscle, but again this does not occur in skeletal muscle (Manning & Stull, 1982). β -Agonists such as clenbuterol seem to have no notable ergogenic effect on myosin head function in skeletal muscle, except for the well-known change in twitch fibre type.

Calcium handling is altered under chronic clenbuterol treatment

Another explanation is that the loss of force production induced by clenbuterol reflects different aspects of problems in calcium handling.

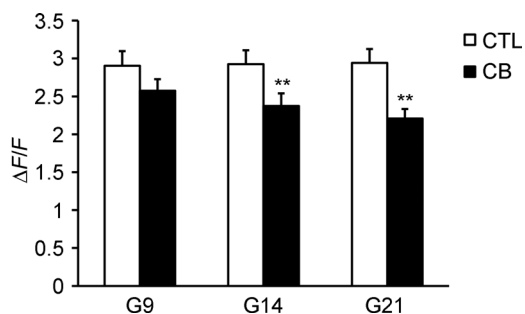


Figure 6. Effect of clenbuterol treatment on FDB Ca^{2+} transient amplitude

Bar graphs comparing amplitude for single-fibre AP-elicited Ca^{2+} transients in FDB fibres of control (CTL; $n = 7$) or clenbuterol-treated (CB; $n = 8$) rats after 9, 14 and 21 days of treatment. Amplitude was calculated from individual exponential fits and averaged. $**P < 0.01$.

We found an alteration in the calcium handling ability in fast-type muscle with a reduction in Ca^{2+} -transient amplitude that may explain, at least in part, the reduced kinetics of muscle contraction. Sarcolemma Ca^{2+} current and sarcoplasmic reticulum Ca^{2+} handling are altered differently during acute β_2 -agonist administration compared with chronic administration. The earlier demonstration that acute β_2 -agonists influence intracellular Ca^{2+} handling in intact skeletal muscle was achieved by administering intubutaline to isolated fast twitch FDB fibres from mice (Cairns & Dulhunty, 1993). These authors found a potentiation of myoplasmic $[\text{Ca}^{2+}]_i$ response ($[\text{Ca}^{2+}]_i$) during tetanic stimulation (20–100 Hz), especially at high stimulation frequencies. A series of subsequent studies further highlighted the acute β_2 -agonist-induced augmentation in peak twitch or tetanic $[\text{Ca}^{2+}]_i$ on skeletal muscles for doses ranging from 1 to 50 μM (Bruton *et al.* 1996; Ha *et al.* 1999; Prakash *et al.* 1999). Overall, β_2 -agonists do not change the resting $[\text{Ca}^{2+}]_i$ in rodent muscles (Prakash *et al.* 1999). A recent study by Rudolf *et al.* (2006) showed that isoprenaline (10 μM) facilitated emptying of Ca^{2+} from the lumen of the sarcoplasmic reticulum of mouse tibialis anterior muscle *in vivo* and that this effect was greater with tetanic stimulation. This strongly suggests that the increased amplitude of Ca^{2+} transients involves greater Ca^{2+} efflux from the sarcoplasmic reticulum. However, data on the effect of chronic β_2 -agonist treatment on skeletal muscle calcium homeostasis are sparse (Sirvent *et al.* 2014). In this study, high-dose chronic clenbuterol treatment had a deleterious effect given that Ca^{2+} -transient amplitudes were decreased. Although we do not understand the precise mechanisms that would explain why chronic clenbuterol treatment has the opposite effect on Ca^{2+} handling compared with that usually reported for acute treatment, the observations by Burniston *et al.* (2005) and Douillard *et al.* (2011) suggest some directions for investigation. First, chronic high doses (4 mg kg⁻¹ day⁻¹) of clenbuterol induce transient apoptosis and necrosis in fast skeletal muscle. In addition, Lavoie *et al.* (2002) used the same dose and observed a decrease of 45 and 40% in β_2 -adrenoreceptor density in SOL and gastrocnemius muscle, respectively.

Chronic clenbuterol treatment alters contraction and relaxation kinetics

Beyond the specific maximal force, the original results of our study were related to the changes in muscle contraction and relaxation kinetics induced by clenbuterol. Indeed, our data demonstrated altered contraction and relaxation kinetics during muscle force generation, especially in EDL, with significant decreases in k_{ACT} , Slow k_{REL} and Fast k_{REL} . Regarding contraction development, k_{ACT} depends

on both the cross-bridge isomerization rate constant (dependent on Pi liberation) and the Ca^{2+} release velocity (i.e. Ca^{2+} transient). In EDL, our results do not provide evidence for the first mechanism because myosin k_4 and ATPase activity did not change under clenbuterol treatment. However, we showed a reduction in the muscle Ca^{2+} transient of FDB muscle that approximates the EDL muscle fast phenotype (Fig. 6). This could contribute to the reduced kinetics of muscle contraction and strongly suggests that clenbuterol acts on some of the processes involved in excitation–contraction coupling, thereby modulating force and contractility characteristics, probably via an alteration in calcium handling. It has been well documented that muscle contraction efficiency and resistance to fatigue are altered by clenbuterol treatment (Chen & Alway, 2001; Polla *et al.* 2001). Interestingly, based on the fitting kinetics, we found altered muscle relaxation after treatment. Slow k_{REL} was dependent on the myosin head detachment rate constant and the velocity of calcium uptake, confirming the role of calcium handling ability in the alteration of the relaxation properties of skeletal muscle. In our study, the mechanism responsible for the decreased relaxation rate in EDL induced by any alteration of the myosin head detachment kinetics can be ruled out because of the lack of significant change in the ATP binding rate constant. The slowed time course of skeletal muscle contraction and relaxation that clenbuterol induced during force generation counterbalanced the positive effects associated with skeletal muscle hypertrophy and may have functional relevance for physical activities. As the rate constants of both force development and relaxation control the maximal shortening velocity, such treatment could be considered as having a negative impact on the activities of daily living. On the one hand, slowing muscle relaxation might be associated with altered agonist–antagonist coordination but, on the other, a reduced myosin head detachment rate would be associated with reduced maximal shortening velocity. Another potentially negative effect for slow twitch muscle lies in the increased ATP cost of shortening, suggesting an altered efficiency in mechanochemical transduction, which would explain, at least in part, the lower resistance to fatigue observed with high doses (He *et al.* 2000). These last negative effects are of particular importance in sports where performance depends on endurance and muscle oxidative capacity. However, even with altered contraction and relaxation kinetics, there remains the increase in absolute tetanic force that could confer to athletes the expected ergogenic aid.

In conclusion, our study shows that chronic clenbuterol treatment decreases skeletal muscle contraction and relaxation kinetics, especially in fast-type muscle, and an increase in the ATP cost for contraction was noted, as well. Because we found a decreased Ca^{2+} -transient amplitude, it seems that the reduced specific force

production or the increased fatigue was dependent on depressed calcium homeostasis induced by clenbuterol. The beneficial anabolic effects of high-dose β_2 -agonists for treatment of sarcopenia, muscle weakness and muscular dystrophy seem counterbalanced, at least in part, by deleterious effects on muscle contractility, relaxation kinetics and Ca^{2+} handling.

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Additional information

Competing interests

The authors declare that they have no conflict of interest.

Author contributions

Conception and design of the experiments: G.P. and R.C.; collection, analysis and interpretation of data: G.P., C.R., R.C., P.S., A.M.J.S., A.G.P., A.D., O.G., C.L., A.B., A.C., O.C. and A.L.; drafting the article or critically revising it for substantial intellectual content: C.R., R.C., G.P., A.M.J.S., A.G.P. and A.C.

Funding

This work was supported by the World Anti-Doping Agency, the Centre National d'Etudes Spaciales, the French Ministry of Youth and Sports, and the Fondation Française des Jeux, and it was funded in part by the University of Montpellier and the Institut National de la Recherche Agronomique (INRA). A.M.J.S., A.D. and A.G.P. received PhD scholarships from the Ministère de l'Enseignement la Recherche et de la Technologie (MENRT).