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Effects of terbutaline on force and intracellular calcium in slow-twitch skeletal muscle fibres of the rat

[1]Tuyen N.V. Ha, [1]Giuseppe S. Posterino & *,[1]Martin W. Fryer

[1]School of Physiology and Pharmacology, University of New South Wales, Sydney, New South Wales, 2052, Australia

1 The effect of the β_2-adrenoceptor agonist, terbutaline, was investigated on simultaneously measured force and intracellular free calcium ($[Ca^{2+}]_i$) in intact rat soleus muscle fibres, and on contractile protein function and Ca^{2+} content of the sarcoplasmic reticulum (SR) in skinned fibres.

2 Terbutaline (10 μM) had no significant effect on either resting force or $[Ca^{2+}]_i$. Exposure to terbutaline increased both the integral of the indo-1 ratio transient and peak twitch force by 37%.

3 At sub-maximal (10 Hz) stimulation frequencies, terbutaline accelerated force relaxation but had highly variable effects on tetanic force amplitude. The corresponding indo-1 ratio transients were significantly larger, and faster to decay than the controls.

4 Terbutaline increased tetanic force at near maximal stimulation frequencies (50 Hz) by increasing tetanic $[Ca^{2+}]_i$. Force relaxation was accelerated at this frequency with no significant change in the indo-1 ratio transient decay rate.

5 All of terbutaline's effects on force and indo-1 ratio transients in intact fibres were completely blocked and reversed by ICI 118551 (1 μM).

6 Mechanically skinned fibres isolated from intact muscles pre-treated with terbutaline showed no significant changes in SR Ca^{2+} content, myofilament $[Ca^{2+}]_i$-sensitivity or maximum force generating capacity.

7 The results suggest that terbutaline primarily modulates force by altering the amplitude and decay rate of the $[Ca^{2+}]_i$ transient *via* phosphorylation of both the ryanodine receptor (RR) and the SR pump regulatory protein, phospholamban (PLB). The high variability of responses of slow-twitch muscles to β_2-agonists probably reflects individual differences in basal phosphorylation levels of PLB relative to that of RR.

Keywords: β_2-adrenoceptor; terbutaline; slow-twitch; phospholamban; ryanodine receptor; intracellular calcium; sarcoplasmic reticulum

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; n_{Ca}, Hill coefficient for cooperativity of Ca^{2+} binding; PKA, cyclic AMP-dependent protein kinase, PLB, phospholamban; P_o, maximum Ca^{2+}-activated force; RR, ryanodine receptor; SR, sarcoplasmic reticulum

Introduction

A long-standing question in β-adrenoceptor pharmacology has been the mechanism of action by which sympathomimetic amines exert distinct effects on the contractility of different skeletal muscle types (Bowman, 1980). Twitches and unfused tetanic contractions are potentiated and slowed by β_2-agonists in fast-twitch muscles (Bowman & Zaimis, 1958; Bowman *et al*., 1962; Tashiro, 1973; Holmberg & Waldeck, 1980; Cairns & Dulhunty, 1993a), whereas slow-twitch muscles show decreases (Bowman & Zaimis, 1958; Bowman *et al*., 1962; Holmberg & Waldeck, 1979; Tashiro, 1973), increases (Cairns & Dulhunty, 1993a) or no change (Slack *et al*., 1997) of contraction amplitude in the presence of a consistently accelerated relaxation.

In order to determine the mechanisms underlying such differences it is necessary to consider the factors influencing the amplitude and time course of skeletal muscle contraction as well as the signal transduction pathway of β_2-adrenoceptors. Given that SR Ca^{2+} handling is a key determinant of mammalian skeletal muscle contraction, and that activation of β_2-adrenoceptors leads to activation of the cyclic AMP/cyclic AMP-dependent protein kinase (PKA) pathway, then it is likely that the differences and variability in response to β_2-agonists are related to differences in the phosphorylation of key proteins involved in SR Ca^{2+} release and uptake. Two such proteins are the SR Ca^{2+} release channel/ryanodine receptor (RR), which is found in all skeletal muscles, and phospholamban (PLB), an inhibitory protein associated with the SR Ca^{2+} uptake pump in slow-twitch and cardiac muscles (Kirchberger & Tada, 1976). Phosphorylation of the RR leads to an increase in its open probability (Suko *et al*., 1993; Hain *et al*., 1994), whilst phosphorylation of PLB releases its basal inhibitory effect on the SR Ca^{2+} uptake pump (Kirchberger & Tada, 1976).

Simultaneous measurement of force and the intracellular free calcium ion concentration ($[Ca^{2+}]_i$) in single murine fast-twitch fibres (Cairns *et al*., 1993) showed that terbutaline increased tetanic tension by enhancing SR Ca^{2+} release. In contrast, the mechanism underlying β_2-agonist-induced force changes in slow-twitch skeletal muscle is not fully understood.

Almost all previous studies have investigated the effects of β_2-agonists and membrane-permeant cyclic AMP derivatives using force measurements alone. Cairns & Dulhunty (1993b) showed that terbutaline's force potentiating effects on rat soleus muscle fibres were not due to changes in the action potential, Na^+/K^+ pump activity, extracellular Ca^{2+} influx, or glycolysis, and speculated that the drug acted through a cyclic AMP-dependent increase in SR Ca^{2+} release. Slack *et al*. (1997) found no effect of isoprenaline on contraction amplitude in either wild-type (WT) or PLB-deficient mouse

* Author for correspondence; E-mail: M.Fryer@unsw.edu.au

soleus but found that PLB was essential for the speeding of relaxation by the β-agonist. Interestingly, a recent study measuring $[Ca^{2+}]_i$ in these fibres found no change in the $[Ca^{2+}]_i$ transient amplitude or kinetics when dibutyryl cyclic AMP was applied to either WT or PLB-deficient fibres (Liu *et al*., 1997).

A major limitation of most of the aforementioned studies is that they measured either force or $[Ca^{2+}]_i$, but not both. Thus, it is possible that some of the drug-induced changes in contraction were related to changes in contractile protein and/or crossbridge function rather than changes in $[Ca^{2+}]_i$. The aim of the present study was to use complementary intact and skinned fibre techniques in order to clarify the relationship between force and $[Ca^{2+}]_i$-handling during application of the specific β_2-agonist terbutaline. Results from our experiments measuring both force and $[Ca^{2+}]_i$ suggest that most, but not all, of terbutaline's actions on contractility may be explained by alterations of SR Ca^{2+} handling. Some of the preliminary results have previously been communicated in abstract form (Ha & Fryer, 1997).

Methods

Intact muscle fibre bundles

Adult (2–4 months) male Wistar rats weighing 200–300 g were killed by fluothane overdose and the soleus muscles rapidly removed. A small, tendon-to-tendon bundle of 10–40 fibres was isolated from the muscle under Krebs solution and then transferred to a perfusion chamber situated on the stage of a Nikon TMD inverted microscope. Within the chamber the preparation was suspended horizontally between a fixed hook and an isometric force transducer (BG10, Kulite, U.S.A.) and was perfused with Krebs solution (aerated with 95% O_2, 5% CO_2) at a constant rate of 2.1 ± 0.2 ml min^{-1} and a temperature of $22 \pm 1°C$. Supramaximal electrical field stimulation (70–100 V, 1 ms duration) was delivered from a Grass SD9 stimulator, the pulse sequence of which was controlled by the input from a Grass S48 stimulator. Each bundle was given an initial equilibration period of at least 1 h prior to the start of the experiment. At the end of this period the stimulation parameters and the muscle length were adjusted to produce optimum twitch force. Twitches were typically elicited at 0.033 Hz. Force-frequency curves were obtained by varying tetanic frequency for 2 s duration tetani over the 2–80 Hz range.

Fluorescence ratio measurement

$[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator indo-1. Glass micropipettes (10–20 MΩ resistance with internal 3 M KCl) were pulled from thin walled filament glass (Clark Electromedical, England) with a P87 micropipette puller (Sutter Instruments, U.S.A.). The micropipettes were then filled with 10 mM indo-1 (pentapotassium salt, Molecular Probes, OR, U.S.A.) which was dissolved in double distilled water. The micropipette was clamped into a perspex holder which was connected to a unity gain headstage (HS-2, Axon Instruments, U.S.A.). A single fibre in each muscle bundle was injected with dye by applying 150–200 kPa of pressure (Picospritzer II, General Valve Co., U.S.A.) to the mouth of the micropipette. Fifteen minutes after injection the muscle bundle was inverted so that the injected fibre lay close to the cover slip constituting the bottom of the bath. After contractile integrity was established (comparison with pre-injection controls) the fluorescence objective (Fluor 20X, N.A. 0.75, Nikon, Japan) was focused on the injected fibre under excitation (360 nm) from a Xenon UV lamp (Nikon, Japan). The final intracellular concentration of indo-1 present in the injected fibre was estimated by comparing its fluorescence to that measured for a known concentration of indo-1 dissolved in a mock intracellular solution (see Solutions below) in a glass capillary tube whose internal dimensions were similar to those of a single fibre. The average concentration of intracellular indo-1 estimated in this manner was 15 ± 3 μM ($n = 12$). Fluorescence emission was simultaneously measured at 410 and 480 nm through two separate photomultiplier tubes (Thorn, EMI, England). Photomultiplier currents were converted to voltages by a current-to-voltage amplifier with a gain of 0.1 μA V^{-1} and a filter time constant of 1 ms. Photomultiplier outputs were recorded on a chart recorder and also onto computer using a 16-bit data-acquisition card (National Instruments, Australia) supporting in-house software (Voltsamp, University of New South Wales, Australia). Muscle autofluorescence was recorded from the targeted injection site of the muscle bundle prior to indo-1 injection. The autofluorescence values for 410 and 480 nm (typically $<1\%$ of the post-injected signal) were subtracted from all recorded signals prior to calculation of the 410/480 nm fluorescence emission ratio. Photobleaching was minimized by keeping periods of excitation illumination as short as possible, and by reducing the intensity of the illuminating light 100 fold using a neutral density filter (ND2, Nikon, Japan).

Calculation of $[Ca^{2+}]_i$

The 410/480 nm fluorescence emission ratio (hereon referred to as 'ratio') is related to the $[Ca^{2+}]_i$ by the following equation (adapted from Grynkiewicz *et al*., 1985):

$$[Ca^{2+}] = (R - R_{min}/R_{max} - R) \cdot K_D \cdot \beta$$

where R is the experimentally recorded 410/480 nm fluorescence emission ratio, R_{min} is the minimum ratio at very low $[Ca^{2+}]$ (pCa ~ 9; pCa = −log $[Ca^{2+}]$), R_{max} is the maximum ratio attained at saturating $[Ca^{2+}]$ (pCa ~ 4), K_D is the estimated dissociation constant between Ca^{2+} and indo-1, and β is a factor describing the 480 nm fluorescence emission at saturating $[Ca^{2+}]$ divided by its value at minimum $[Ca^{2+}]$. These parameters were estimated in both microcuvettes and in intact muscle fibres. The latter were used to convert R values into $[Ca^{2+}]_i$. In cuvettes, R was measured in a series of solutions containing 20 μM indo-1 and varying $[Ca^{2+}]$ in the range pCa 4–9. Three separate droplets of each Ca^{2+} solution were sandwiched between two coverslips and the readings performed in triplicate. This technique estimated R_{min} as 0.02, R_{max} as 0.4, K_D as 200 nM, and β as 3.5. R_{min} in intact muscle fibres (0.035) was estimated by measuring R after (1) co-injecting 0.25 M EGTA with the 10 mM indo-1 and measuring R ($n = 4$), or (2) permeabilizing indo-1 injected fibres with 50 μM β-escin in a calcium-free, K^+-based Krebs solution containing EGTA ($n = 2$). R_{max} in intact fibres was obtained in two experiments where indo-1 injected fibres were permeabilized with 50 μM β-escin in a K^+-based Krebs solution. Intermediate $[Ca^{2+}]$ were obtained in the fibre by co-injecting various heavily buffered CaEGTA/EGTA solutions with the indo-1. All calibration parameters obtained for indo-1 within the muscle fibre were similar to those found in microcuvettes, except for R_{min} which was found to be significantly larger (0.035 vs 0.02).

Force and fluorescence ratio analysis

Post-experimental analysis of force and ratio data was performed using commercial software (Graphpad Prism v. 2.01, San Diego, U.S.A.) in conjunction with an in-house software program (Ratio, University of New South Wales, Australia). The peak amplitude and relaxation rate were determined for force and ratio recordings acquired during twitches. The relaxation rate was calculated by fitting a single exponential function to the lower 70% of each response. Similar analyses were performed for tetanic responses except that the lower 50% of the response was chosen as the exponential fitting region. The tetanic force response also displays a brief linear phase of relaxation (sometimes referred to as the 'shoulder') which occurs shortly after the last stimulus pulse. The slope of this phase of relaxation was determined by linear regression.

The absolute values of the decay rates of the indo-1 ratio transients in rat slow-twitch muscles were highly variable (fastest rate constant was 14 s^{-1} for twitches, most tetanic decays were $\sim$1 s^{-1}) but were much slower than the estimated Ca^{2+} dissociation rate constant for indo-1 previously estimated in intact fast-twitch mouse skeletal muscle fibres (at least 98 s^{-1}, Westerblad & Allen, 1994b). Thus, indo-1 kinetics did not limit temporal resolution of the decay rates. All indo-1 ratio traces were analysed before digital filtering. The digital filtering settings of 100 Hz for twitches and 10 Hz for tetani were used for clarity of Figures 1–3 only.

Force-pCa curves were established in intact fibres by measuring the mean force and $[Ca^{2+}]_i$ achieved during the last 500 ms of a 2 s tetanus during tetanic stimulation frequencies varying from 2–80 Hz.

Mechanically-skinned fibre technique

The mechanically-skinned fibre preparation was used as previously described (Lamb *et al*., 1994). Fibres were mechanically-skinned and then mounted onto a transducer (KG3, Scientific Instruments, Germany) to monitor isometric force. The length and diameter of each fibre was measured under paraffin oil prior to the start of each experiment. Next, Ca^{2+}-sensitivity of the contractile proteins was determined by exposing the skinned fibre to a series of solutions (heavily buffered with Ca-EGTA/EGTA) containing progressively increasing free $[Ca^{2+}]$ (Stephenson & Williams, 1981). Maximum Ca^{2+}-activated force (P_o) was determined in each fibre and expressed in terms of fibre cross sectional area ($N.cm^{-2}$). Sub-maximal force responses were normalized to P_o. Complete activation curves were performed at least twice for any experimental condition in each fibre. Force-pCa curves were fitted to the averaged data by regression analysis using a modified form of the Hill equation. The pCa eliciting 10% (pCa_{10}) and 50% (pCa_{50}) of P_o were estimated from these curves as well as the Hill coefficient (n_{Ca}) describing the curve steepness.

Effects of terbutaline on contractile protein function and SR Ca^{2+} content

Two different skinned fibre preparation procedures were used to determine the effect of terbutaline on contractile protein sensitivity. The first method assessed the direct effect of 10 μM 'myoplasmic' terbutaline on the force-pCa curve. Skinned fibres were prepared from soleus muscles that had been rapidly removed from the animal, lightly blotted on filter paper, and then placed into paraffin oil. Next, force-pCa curves were performed before, during, and after exposure to 10 μM terbutaline in the myoplasmic bathing solution (total of six curves).

The second method assessed whether terbutaline caused any persistent changes in contractile protein sensitivity following its application to intact muscle bundles. In these experiments paired soleus bundles from the same muscle were set up in parallel and stimulated in Krebs solution at 0.033 Hz. The test bundle was perfused with 10 μM terbutaline until twitch potentiation was at its maximum (usually $\sim$30 min). Both bundles were then blotted on filter paper and placed into paraffin oil for skinned fibre preparation. Fibres were dissected alternately from the test and control bundle during the course of the day.

Terbutaline effects on the SR Ca^{2+} content were assessed in skinned fibres prepared using the second method above. The endogenous SR Ca^{2+} content was determined using a previously described method (Fryer & Stephenson, 1996).

Solutions and drugs

The Krebs-bicarbonate solution contained (mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 0.6; $NaHCO_3$, 25; glucose, 11; $CaCl_2$, 2.5; *d*-tubocurarine 0.015. Foetal calf serum (0.1% v v^{-1}) was added to the Krebs solution in order to maximize fibre survival during microinjection. Di-sodium ethylenediaminetetraacetic acid (EDTA; 50 μM) was routinely added to all solutions as an anti-oxidant. Caffeine, (±)-propranolol HCl and terbutaline were each dissolved into the Krebs-bicarbonate solution and the pH adjusted to 7.4. All drugs were obtained from Sigma Australia.

Two types of skinned fibre solution were used. The force-pCa relationship in skinned fibres was determined using heavily buffered Ca-EGTA/EGTA solutions as previously described (Stephenson & Williams, 1981). The Ca^{2+} content of single skinned fibres was determined using solutions heavily buffered with Ca-BAPTA/BAPTA to ensure a more rapid chelation of Ca^{2+} and smaller pH-induced Ca^{2+} affinity changes during fibre lysis. (Fryer & Stephenson, 1996). All solutions contained ATP, creatine phosphate and had a free $[Mg^{2+}]$ of 1 mM. The pH of all solutions was adjusted to 7.10±0.01 and the osmolality was 295±5 mosmol kg^{-1}.

Calcium calibration solutions for indo-1 in cuvettes used a Ca-EGTA/EGTA buffering system (11 mM total EGTA) and also contained (mM): KCl, 111; HEPES, 11; indo-1, 0.02; pH 7.2.

Data analysis

The force per cross sectional area was calculated by multiplying the force (in N) by the muscle length (in cm) and the density of muscle tissue (1.06 g ml^{-1}) and dividing this by the bundle wet weight (in g). The effects of terbutaline on various parameters in intact bundles was expressed as the percentage change of the parameter relative to the value obtained from a control response acquired immediately prior to terbutaline addition. Values presented in the text are expressed as the mean±s.e.mean from '*n*' preparations. Statistical significance between raw data means was assessed using Student's *t*-test for unpaired and paired data where appropriate. *P* values less than 0.05 were considered significant.

Results

Effects of terbutaline on resting force and $[Ca^{2+}]_i$

No significant changes in resting force or $[Ca^{2+}]_i$ (control: 107 ± 14 nM; terbutaline: 118 ± 17 nM) were observed during 30 min perfusion with 10 μM terbutaline in 12 experiments.

Effects of terbutaline on force and $[Ca^{2+}]_i$ during twitches

Representative traces of the effect of terbutaline on the simultaneously measured twitch force and indo-1 ratio transients are shown in Figure 1. The data from 11–12 experiments of this kind are summarized in Table 1. Thirty minutes exposure to 10 μM terbutaline produced a consistent and significant increase in both peak twitch force and the rate of twitch relaxation. Indo-1 ratio transient measurements under the same conditions were less consistent but revealed a significant enhancement of amplitude in the presence of highly variable effects on the decay rate (Table 1). Whilst not significant, terbutaline tended to slow the decay of the twitch indo-1 ratio transient. Previous studies analysing $[Ca^{2+}]_i$ transients in frog skeletal muscle have shown that twitch force potentiation is often associated with a slowing of the $[Ca^{2+}]_i$ transient decay rate in the presence of little $[Ca^{2+}]_i$ transient amplitude change (Sun *et al*., 1996).

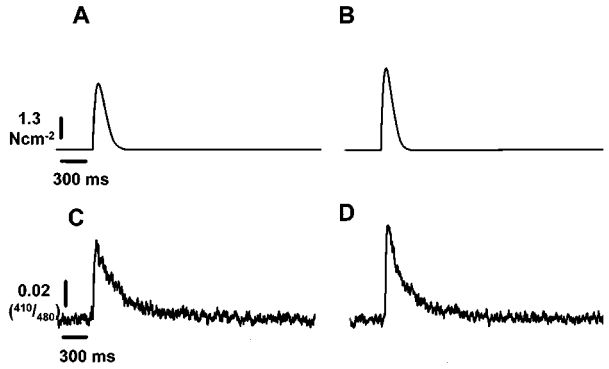

Figure 1 Effect of 10 μM terbutaline on force (upper traces) and indo-1 ratio transients (lower traces) recorded during twitches. (A and C) controls. (B and D) 30 min after terbutaline (10 μM) application. Traces are averages of 20 successive twitches elicited at 0.1 Hz (ratio traces filtered digitally at 100 Hz).

Table 1 Effects of terbutaline (10 μM) on force and indo-1 ratio transients after 30 min perfusion

	Parameter	*Twitch* (% change)	*10 Hz tetanus* (% change)	*50 Hz tetanus* (% change)
Force	amplitude	37 ± 13*	19 ± 11	29 ± 7*
	relaxation rate (linear phase)	N.D.	41 ± 12*	33 ± 10*
	relaxation rate (exponential phase)	12 ± 4*	18 ± 3*	17 ± 5*
Ratio	amplitude	19 ± 9*	36 ± 14*	30 ± 17
	relaxation rate	-26 ± 16	26 ± 8*	5 ± 9
	integral	37 ± 19	N.D.	N.D.
	n	12	12	11

*Significant difference from control ($P < 0.05$, paried *t*-test on raw data). N.D., not determined.

Given the likely influence of both the $[Ca^{2+}]_i$ transient amplitude and decay rate on twitch force, the integral of the twitch indo-1 ratio transient (area under the response) was also determined. The mean increase in the indo-1 ratio transient integral ($37 \pm 19\%$) was similar in magnitude to the mean increase in peak twitch force ($37 \pm 13\%$) but was not statistically significant (Table 1).

Effects of terbutaline on force and $[Ca^{2+}]_i$ during tetani

The effects of terbutaline on force and $[Ca^{2+}]_i$ during sub-maximal (10 Hz) and near maximal (50 Hz) tetani were investigated. Like the twitch data, the indo-1 ratio transient data for tetani was much less consistent than the force (Table 1). Figures 2 and 3 illustrate some of the larger ratio amplitude changes seen in terbutaline. Overall, terbutaline tended to increase the peak force attained during 10 and 50 Hz stimulation and increased the rate of relaxation during both the linear and exponential phases of force relaxation (Table 1). At 10 Hz stimulation, terbutaline significantly potentiated both the amplitude and relaxation rate of the indo-1 ratio transient (Figure 2, Table 1). The drug also tended to increase the peak ratio amplitude at 50 Hz (Figure 3, Table 1) but appeared to have little effect on the rate of ratio decline at this frequency (Table 1).

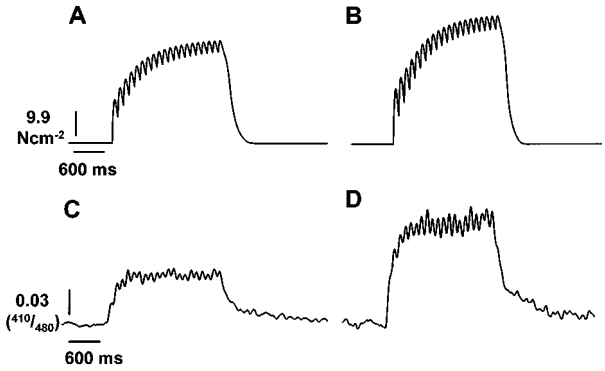

Figure 2 Effect of 10 μM terbutaline on force (upper traces) and indo-1 ratio transients (lower traces) recorded during sub-maximal (10 Hz) tetanic stimulation. (A and C) controls. (B and D) 20–40 min after terbutaline application. Traces are averages of four successive 10 Hz tetani given at 5 min intervals (ratio traces filtered digitally at 10 Hz).

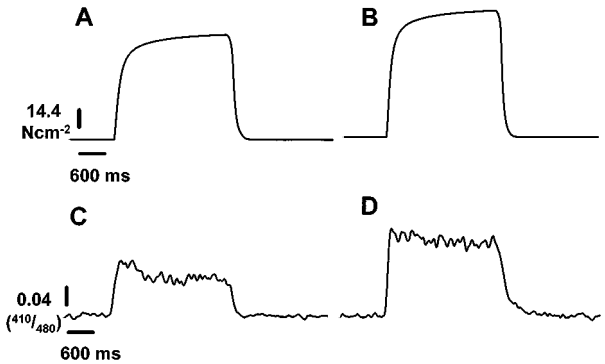

Figure 3 Effect of 10 μM terbutaline on force (upper traces) and indo-1 ratio transients (lower traces) recorded during near maximal (50 Hz) tetanic stimulation. (A and C) controls. (B and D) 20–40 min after terbutaline (10 μM) application. Traces are averages of four successive 50 Hz tetani given at 5 min intervals (ratio traces filtered digitally at 10 Hz).

Reversibility and antagonism of terbutaline effects

The effects of 30 min treatment with 10 μM terbutaline were only slightly reversed by 30 min perfusion with drug-free Krebs (19±4% reversal of twitch potentiation, $n=9$). In contrast, the application of the specific β_2-adrenoceptor antagonist ICI 118551 (1 μM) to terbutaline-treated preparations led to full reversal within 19±3 min ($n=3$). Pretreatment with the same concentration of ICI 118551 completely blocked the effects of 10 μM terbutaline on force and indo-1 ratio transients ($n=3$).

Effects of terbutaline on the contractile proteins in skinned and intact fibres

The possibility that part of terbutaline's force potentiating effect was related to an action on the contractile proteins was investigated by assessing the effect of terbutaline on submaximal (pCa_{10}, pCa_{50}) and maximal (P_o) parameters of the force-pCa relationship. This was determined by three different methods: (1) applying terbutaline to intact, indo-1 injected fibres in which the force and $[Ca^{2+}]_i$ were graded by changing the tetanic stimulation frequency (Figures 4A and 5); (2) testing the force-pCa relation in mechanically-skinned fibres which had been previously exposed to terbutaline whilst intact (Figure 4B, Table 2); and (3) directly testing the effects of 'myoplasmic' terbutaline on the force-pCa relation in skinned fibres (Table 2).

The normalized force-pCa relationship obtained from an indo-1 injected bundle using method 1 is shown in Figure 4A. In this particular preparation terbutaline caused a small rightward shift in pCa_{50} (~0.06 pCa), an effect which was partially reversed by 30 min washout of terbutaline. Even though the data from this preparation suggested that terbutaline had a small inhibitory effect on Ca^{2+}-sensitivity at low to intermediate $[Ca^{2+}]$, the mean effect was not significant at either the pCa_{10} or the pCa_{50} level over seven experiments.

Assessment of terbutaline's effects on the Ca^{2+}-sensitivity of the contractile proteins using methods 2 and 3 circumvented any problems that might have been associated with indo-1 calibration or dye binding to intracellular proteins using method 1. In these experiments fibres were treated with the non-ionic detergent (Triton X-100, 5 min) in order to remove all remaining membranes but leaving contractile protein function intact. It can be seen that the force-pCa relation obtained using method 2 (Figure 4B) was very similar to that obtained using method 1 (Figure 4A). In fact, the pCa_{50} value obtained for the control curve in Figure 5 (6.09±0.02) compares favourably with the pCa_{50} values shown for both

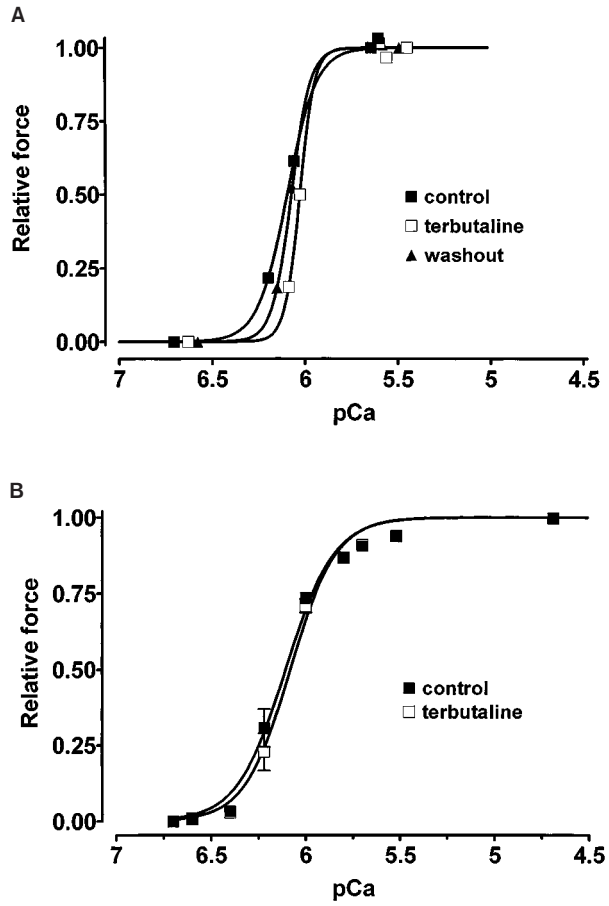

Figure 4 Comparison of the effects of 10 μM terbutaline on Ca^{2+}-sensitivity of the contractile proteins in intact (A) and mechanically skinned (B) soleus muscle fibres. Results in (A) are normalized to the same maximum force (1.00). Calculated pCa_{50} values (±95% confidence limits) were: control, 6.09±0.02; terbutaline, 6.03±0.01; wash, 6.07±0.005. (B) shows mean results ($n=4-7$) from mechanically-skinned fibres that were exposed to terbutaline whilst still intact. Curves represent the least squares computer-generated fit to a modified version of the Hill equation. Calculated values (±s.e.mean) for pCa_{50}: control, 6.11±0.02; terbutaline, 6.09±0.02.

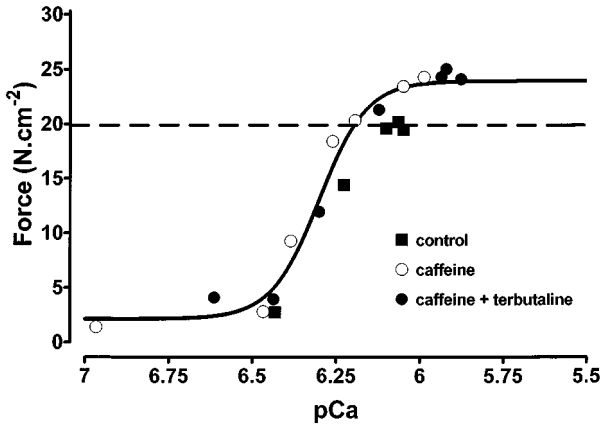

Figure 5 Terbutaline, like caffeine, enhances the apparent maximum tetanic force by potentiating steady-state tetanic $[Ca^{2+}]_i$. Points comprising the force-pCa relation were obtained from an indo-1 injected soleus muscle bundle in the following sequence: control, 15 min after 1 mM caffeine, 20 min after 1 mM caffeine plus 10 μM terbutaline. Note that the dotted line (indicating the apparent maximum Ca^{2+}-activated force (P_o) under control conditions, ~20 N.cm^{-2}) is below the value attainable in either caffeine alone or caffeine plus terbutaline. A single curve was fitted to the combination of the three data sets yielding the following parameters (±95% confidence level): pCa_{50}, 6.30±0.05, P_o, 23.9±2.4 N.cm^{-2}.

Table 2 Effects of terbutaline (10 μM) on the contractile characteristics of mechanically-skinned soleus muscle fibres

	Control	*Exposed to terbutaline whilst intact*	*Control*	*Exposed to terbutaline after skinning*
pCa_{10}	6.39±0.03	6.35±0.03	6.56±0.05	6.45±0.05
pCa_{50}	6.11±0.02	6.09±0.02	6.24±0.04	6.21±0.04
n_{Ca}	3.6±0.2	3.8±0.3	2.9±0.1	3.0±0.1
P_0 (N.cm^{-2})	42±3	43±4	34±1	33±1

Results represent the mean±s.e.mean from 5–7 fibres.

methods 2 and 3 in Table 2 (6.11 ± 0.02 and 6.24 ± 0.04 respectively). The summarized results from skinned fibre experiments using methods 2 or 3 (Table 2) showed that terbutaline had no significant effect on the Ca^{2+}-sensitivity of the contractile proteins (pCa_{10}, pCa_{50}), the steepness of the force-pCa relation (n_{Ca}), or P_o. The lack of effect of terbutaline on P_o in skinned fibres contrasts with the $29 \pm 7\%$ increase in tetanic force seen during 50 Hz stimulation in intact bundles (Table 1). One possible explanation for this discrepancy is that the contractile proteins are not saturated by $[Ca^{2+}]_i$ during high frequency tetanic stimulation under control conditions. This contention is supported by the previous demonstration (in rat soleus fibres) that caffeine (1 mM) could potentiate the apparent P_o by $\sim 20\%$ (Fryer & Neering, 1989). For this reason, the force-pCa relation was established under control conditions, after treatment with 1 mM caffeine, and after treatment with both 1 mM caffeine and 10 μM terbutaline (Figure 5).

The results in Figure 5 show that the apparent P_o under control conditions (indicated by the horizontal dashed line) was enhanced $\sim 20-25\%$ by 1 mM caffeine. Subsequent addition of terbutaline to the caffeine-treated preparation caused no further increase in P_o. The data points obtained under the three different conditions were well fitted by a single sigmoidal curve suggesting that neither intervention had much effect on the Ca^{2+}-sensitivity of the contractile proteins.

Effects of terbutaline on the SR Ca^{2+} content

It has previously been suggested that β-adrenoceptor agonists increase the Ca^{2+} transient amplitude in cardiac muscle primarily by increasing the SR Ca^{2+} content (Hussain & Orchard, 1997). To test this possibility in slow-twitch skeletal muscle we quantitatively estimated the SR Ca^{2+} content of single mechanically-skinned fibres isolated from intact muscle bundles that had been previously exposed to either terbutaline (10 μM) or the control Krebs solution for 30 min. The mean SR Ca^{2+} content from seven control fibres (two rats) was 1.25 ± 0.04 mM per fibre volume. This value compares well with earlier measurements made by Fryer & Stephenson (1996) in soleus fibres (~ 1.35 mM). The mean SR Ca^{2+} content of the seven terbutaline-treated fibres was not significantly different from control fibres (1.26 ± 0.02 mM per fibre volume).

Discussion

This study represents the first characterization of the effects of terbutaline, a selective β_2-receptor agonist, on simultaneously recorded force and $[Ca^{2+}]_i$ in slow-twitch mammalian skeletal muscle fibres. Such experiments have allowed us to discern which aspects of force modulation by terbutaline are linked with changes in $[Ca^{2+}]_i$. The results suggest that the force enhancement by terbutaline is related to an increased $[Ca^{2+}]_i$ transient, and is not a result of any change in the Ca^{2+}-sensitivity or maximum force generating capacity of the contractile proteins. In contrast, the classical speeding of twitch and tetanic force relaxation by terbutaline was often, but not always, associated with a faster indo-1 ratio transient decay rate, suggesting that both $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent mechanisms may underlie this pharmacological effect.

Terbutaline enhances force and indo-1 ratio transients

The degree of potentiation of twitch and tetanic force by 10 μM terbutaline in rat soleus bundles in the present study was quite variable, a feature previously noted by Cairns & Dulhunty (1993a) using the same preparation. The increase in twitch tension by terbutaline was consistently associated with an increase in the peak and the integral of the indo-1 ratio transient, suggesting that force potentiation resulted from enhanced SR Ca^{2+} release and/or slowed SR Ca^{2+} uptake. The latter mechanism is considered unlikely as inhibition of the SR Ca^{2+} pump would tend to increase resting $[Ca^{2+}]_i$ over time and dramatically slow the decay of the $[Ca^{2+}]_i$ transient following tetanic stimulation (Westerblad & Allen, 1994a). Terbutaline had no significant effect on resting $[Ca^{2+}]_i$ and actually accelerated the rate of $[Ca^{2+}]_i$ decay following tetanic stimulation at near physiological frequencies (10 Hz; Table 1). Whilst Figure 2 shows an example of force potentiation by terbutaline at 10 Hz, the results at this frequency were extremely variable and were not significantly different from the controls. Such variability appears to be typical of sympathomimetic drug effects on slow-twitch muscles at unfused tetanic stimulation frequencies, where both decreases (Tashiro, 1973; Holmberg & Waldeck, 1979) and increases (Cairns & Dulhunty, 1993a) of force have been reported. The present data at 10 Hz suggest that terbutaline is both enhancing SR Ca^{2+} release (which tends to increase peak force) and speeding SR Ca^{2+} uptake (which tends to suppress the peak $[Ca^{2+}]_i$ and peak force level attained). As both of these processes have the potential to be modulated by protein phosphorylation (see detailed discussion below), the variability of results might be explained by differences in the basal level of phosphorylation or sensitivity to phosphorylation at Ca^{2+} release and uptake sites. In this scheme, the final effect of terbutaline in a muscle would depend on the relative balance between the stimulation of SR Ca^{2+} release and the stimulation of SR Ca^{2+} uptake.

Mechanisms underlying terbutaline effects on $[Ca^{2+}]_i$ handling

β-adrenergic agents, through their stimulation of the cyclic AMP-dependent protein kinase (PKA) pathway, are known to promote the phosphorylation of many intracellular target proteins in skeletal muscle including the dihydropyridine (DHP)-sensitive Ca^{2+} channels in the transverse tubules (Arreola *et al*., 1987), the SR pump regulatory protein phosphlamban (Kirchberger & Tada, 1976), and the SR Ca^{2+} release channel (Suko *et al*., 1993; Hain *et al*., 1994). The increased indo-1 ratio transient in the presence of terbutaline is unlikely to be mediated by enhanced Ca^{2+} influx through DHP-sensitive Ca^{2+} channels because the effects of terbutaline remain after pre-treatment with 10 μM nifedipine (Cairns & Dulhunty, 1993b). In cardiac myocytes, potentiation of $[Ca^{2+}]_i$ transients by isoprenaline arises from an increased SR Ca^{2+} content (Hussain & Orchard, 1997). Terbutaline had no effect on the SR Ca^{2+} content in the present study, suggesting that modified gating of the SR Ca^{2+} release channel is the most likely mechanism of action. It is suggested that the increase in indo-1 ratio transient amplitude in the presence of terbutaline is a consequence of SR Ca^{2+} release channel phosphorylation by PKA, an event which has been shown to increase the channel's open probability (Suko *et al*., 1993; Hain *et al*., 1994). This mechanism has previously been put forward to explain the increase in tetanic $[Ca^{2+}]_i$ transient amplitude caused by terbutaline in fast-twitch mammalian skeletal muscle fibres (Cairns *et al*., 1993), as well as the recovery of $[Ca^{2+}]_i$ transient amplitude by dibutyryl cyclic AMP in mouse soleus fibres pre-treated with the PKA inhibitor, H-89 (Liu *et al*., 1997).

With regard to the decay rate of the indo-1 ratio transient, our results at 10 Hz stimulation show a clear acceleration in the presence of β-adrenoceptor stimulation. This contrasts with the lack of such an effect in mouse fast-twitch fibres (Cairns *et al*., 1993), a result which may be attributed to the absence of PLB in this fibre type. The importance of PLB phosphorylation in the β-adrenoceptor/PKA-mediated speeding of SR Ca^{2+} uptake in slow-twitch skeletal muscle has recently been clarified using soleus muscles from wild-type and PLB-knockout mice (Liu *et al*., 1997). This study showed that inhibition of PKA (using H-89) slowed the $[Ca^{2+}]_i$ transient decay rate in wild-type (PLB-containing) but not PLB-knockout soleus fibres, an effect which could be reversed using dibutyryl cyclic AMP. In contrast to the present results, Liu *et al*. (1997) failed to see any potentiation of $[Ca^{2+}]_i$ transient amplitude or any speeding of $[Ca^{2+}]_i$ transient decay in wild-type mouse soleus fibres exposed to a PKA-promoting intervention (dibutyryl cyclic AMP). As their study demonstrated a high basal level of PKA-induced phosphorylation, the apparent discrepancy in results can be satisfactorily explained by the presence of a lower basal phosphorylation level of both the SR Ca^{2+} release channel and PLB in fibres from our intact fibre bundles.

Effects of terbutaline on contractile protein and crossbridge function

The present study clearly demonstrated that terbutaline had no significant effect on the $[Ca^{2+}]_i$-sensitivity of the contractile proteins in rat soleus muscle fibres, suggesting that PKA-mediated phosphorylation events do not modify contractile protein sensitivity to $[Ca^{2+}]_i$ in this preparation. These results are similar to those previously found in intact (Cairns *et al*., 1993) and skinned (Fabiato & Fabiato, 1978) fast-twitch skeletal muscle fibres, but stand in contrast to the decreased $[Ca^{2+}]_i$-sensitivity due to troponin-I phosphorylation in cardiac muscle (Kurihara & Konishi, 1987).

Terbutaline had no effect on the P_o of skinned fibres (Table 2) but appeared to increase the P_o of intact fibres (Table 1). This apparent discrepancy was resolved by showing that the effect in intact fibres was simply a consequence of potentiated tetanic $[Ca^{2+}]_i$ such that the 'true' P_o could be achieved (Figure 5). An important conclusion to be drawn from this result is that the steady-state $[Ca^{2+}]_i$ level attained in 'maximum' tetanic contractions under control conditions is not saturating for force generation by the contractile proteins, a result which must be kept in mind for future experiments when drug-induced changes in P_o are being interpreted.

An effect of terbutaline on crossbridge function was suggested by the observation that twitch and tetanic force relaxation was often accelerated by terbutaline in the presence of an unchanged or even slower indo-1 ratio transient decay rate (Table 1). Such a result might arise artifactually because the indo-1 ratio transients recorded from a single fibre might not be representative of the rest of the fibres in the bundle from which the force is being measured. Soleus muscles contain 10–15% type IIa fibres (Close, 1972), so we might expect discrepancies to occur approximately 10–20% of the time. However, the occurrence of mismatches was much greater than this level, suggesting that terbutaline accelerates force relaxation by an additional $[Ca^{2+}]_i$-independent mechanism. One possibility is that terbutaline speeds the rate of crossbridge detachment, an effect which has been previously shown for adrenaline in rat papillary muscle using pseudo-random binary noise-modulated perturbation analysis (Hoh *et al*., 1988).

In conclusion, the results show that terbutaline's effects on force production in rat soleus fibres may be largely explained by changes in the amount of SR Ca^{2+} released and the speed of SR Ca^{2+} re-uptake, and are not related to changes in the $[Ca^{2+}]_i$-sensitivity or maximum-force generating capacity of the contractile proteins. Our results are consistent with a mechanism whereby terbutaline activates β_2-adrenoceptors leading to the PKA-dependent phosphorylation of both the RR and PLB. Phosphorylation of the RR leads to the increased $[Ca^{2+}]_i$ transient seen in both fast-twitch (Cairns *et al*., 1993) and slow-twitch (present study) muscles. Phosphorylation of PLB can only occur in cardiac and slow-twitch skeletal muscles, leading to faster relaxation in these muscle types. Variations in the degree of basal phosphorylation of the RR relative to PLB in different slow-twitch skeletal muscle preparations probably account for the diversity of force responses to β_2-agonists reported in the literature.

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