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RESEARCH PAPER

β_3-Adrenoceptor agonist stimulation of the Na^+,K^+-pump in rat skeletal muscle is mediated by β_2- rather than β_3-adrenoceptors

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Background and purpose: In cardiac muscle, BRL 37344, a selective β_3-adrenoceptor agonist, activates the Na^+,K^+-pump via NO signalling. This study investigated whether BRL 37344 also activates the Na^+,K^+-pump via β_3-adrenoceptors in skeletal muscle.
Experimental approach: Isolated rat soleus muscles were incubated between 1 and 60 min in buffer. Intracellular Na^+,K^+ content and Na^+,K^+-pump activity were measured using flame photometry and ouabain-suppressible $^{86}Rb^+$ uptake, respectively. Additional muscles were mounted on force transducers and stimulated (60 Hz for 2 s) every 10 min.
Key results: BRL 37344 (10^{-8}-10^{-5} M) induced a concentration- and time-dependent reduction in intracellular Na^+, and increased ouabain-suppressible $^{86}Rb^+$ uptake by up to 112%. BRL 37344-induced reductions in intracellular Na^+ were blocked by the β_1/β_2-adrenoceptor antagonist, nadolol (10^{-7} M), and the β_2-adrenoceptor antagonist, ICI 118,551 (10^{-7}-10^{-5} M), but not by β_3- or β_1-adrenoceptor antagonists, SR 59230A (10^{-7} M) and CGP 20712A (10^{-7}-10^{-5} M), respectively. Another β_3-adrenoceptor agonist, CL 316,243, did not alter intracellular Na^+. BRL 37344-induced reductions in intracellular Na^+ were not blocked by L-NAME, an NOS inhibitor, or ODQ, a guanylyl cyclase inhibitor. The NO donors, SNP and SNAP, did not alter intracellular Na^+. BRL 37344 rapidly recovered force in muscles depressed by high $[K^+]_o$, an effect that was blocked by nadolol, but not L-NAME.
Conclusions and implications: In rat soleus muscle, the β_3-adrenoceptor agonist BRL 37344 stimulated the Na^+,K^+-pump via β_2-adrenoceptors. A more selective β_3-adrenoceptor agonist did not affect Na^+, K^+ homeostasis in skeletal muscle. NO did not seem to mediate Na^+,K^+-pump stimulation in skeletal muscle.
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Keywords: β_3-adrenoceptors; Na^+,K^+-pump; nitric oxide; skeletal muscle; β_2-adrenoceptors; BRL 37344; Na^+content; β_1-adrenoceptors; ouabain-suppressible $^{86}Rb^+$ uptake; K^+ content

Abbreviations: BAT, brown adipose tissue; BRL 37344, 4-(2-[{2-hydroxy-2-(3-chlorophenyl)ethyl}-amino]propyl)-phenoxyacetic acid; CGP 20712A, 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)-propyl}amino]ethyoxy) benzamide; CL 316,243, disodium (*R,R*)-5-(2-[{2-(3-chlorophenyl)-2-hydroxyethyl}-amino]propyl)-1,3-benzodioxole-2,2-dicarboxylate; ICI 118,551, (−)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,a]quinoxilin-1-one; PKG, cGMP-dependent protein kinase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SR 59230A, 3-(2-ethylphenoxy)-1-([1*S*]-1,2,3,4-tetrahydronapth-1-ylamino)-2*S*-2-propranol oxalate; TCA, trichloroacetic acid

Introduction

In skeletal muscle, the ability to generate repeated muscle contractions depends on the maintenance of membrane excitability, which is compromised by the passive Na^+ influx and K^+ efflux associated with each action potential. Excitability depends on the membrane-bound Na^+,K^+-ATPase (Na^+,K^+-pump), which actively transports Na^+ out of the cell, and K^+ back into the cell to restore the transsarcolemmal Na^+ and K^+ gradients. Indeed, during periods of repeated action potentials, the accelerated passive ion fluxes overwhelm the capacity of the Na^+,K^+-pump, leading to a net Na^+ gain and K^+ loss, a resulting membrane depolarization and a reduction in muscle function (Sejersted and Sjøgaard, 2000).

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In skeletal muscle, the Na^+,K^+-pump is acutely stimulated by numerous factors, among which the most potent are the catecholamines and synthetic agonists acting via β_2-adrenoceptors. This leads to activation of adenylate cyclase, increasing cAMP, and resulting in protein kinase A activation (Clausen, 2003). This increases the affinity of the Na^+,K^+-pump for intracellular Na^+ (Buchanan *et al*., 2002), leading to elevated Na^+ efflux and K^+ influx, and membrane hyperpolarization (Clausen and Flatman, 1980).

The β_3-adrenoceptor, or the so-called atypical β-adrenoceptor (Arch and Kaumann, 1993), first became known as a potential anti-obesity target through its effect of increasing energy expenditure and lipolysis in brown adipose tissue (BAT), and therefore the metabolic rate of rats and mice (Arch *et al*., 1984; Yen *et al*., 1984). However, similar effects were not found in humans, due to their lack of BAT (reviewed in (Arch and Kaumann (1993)). Whereas expression of the β_3-adrenoceptor is highly abundant in BAT, expression of the β_3-adrenoceptor in skeletal muscle remains controversial. In mammalian skeletal muscle, the β_3-mRNA and protein have been detected in some studies (Evans *et al*., 1996; Chamberlain *et al*., 1999), but not in others (Granneman *et al*., 1991; Thomas and Liggett, 1993; De Matteis *et al*., 2002). Furthermore, the existence of functional β_3-adrenoceptors in skeletal muscle has been suggested by studies in which selective β_3-adrenoceptor agonists have inhibited proteolysis (Navegantes *et al*., 2006), and stimulated metabolic oxidation (Board *et al*., 2000) and glucose utilization (Liu *et al*., 1996).

Recent evidence in cardiac myocytes suggests that the β_3-adrenoceptor stimulates the Na^+,K^+-pump. In mouse cardiac myocytes, the β_3-adrenoceptor agonist, 4-(2-[{2-hydroxy-2-(3-chlorophenyl)ethyl}-amino]propyl)-phenoxyacetic acid (BRL 37344), stimulated the endothelial isoform of nitric oxide synthase (NOS), eNOS (Barouch *et al*., 2002). Stimulation of NOS activates soluble guanylyl cyclase, leading to increased cGMP and activation of cGMP-dependent protein kinase (PKG) (Kobzik *et al*., 1994). Furthermore, in rabbit cardiac myocytes, the nitric oxide (NO) donor, sodium nitroprusside (SNP), stimulated the Na^+,K^+-pump-mediated current, an effect that was abolished by inhibition of both guanylyl cyclase and PKG (William *et al*., 2005). In the same preparation, BRL 37344 stimulated the Na^+,K^+-pump-mediated current, an effect that was abolished by inhibition of NOS (Bundgaard *et al*., 2006a). Taken together, these results indicate that in cardiac myocytes, β_3-adrenoceptor agonists stimulate the Na^+,K^+-pump and that this effect is mediated via activation of NOS and signalling by NO. As this signalling is likely to be restricted to sarcolemmal microdomains with a large content of Na^+,K^+-pumps (Liu *et al*., 2003), NO-induced stimulation of the Na^+,K^+-pump may be more selective and rapid than β_2-adrenoceptor stimulation mediated by cAMP. This would be important in skeletal muscle, since a rapid stimulation of the Na^+,K^+-pump would delay the loss in membrane excitability, and hence, the decline of muscle function associated with repeated muscle contractions. This would be of further physiological interest because the β-adrenergic system in skeletal muscle would not only involve effects mediated by cAMP, but possibly also cGMP. However, it is unknown whether β_3-adrenoceptors and/or NO stimulate the Na^+,K^+-pump in skeletal muscle.

The primary aim of this study was therefore to investigate the effect of the β_3-adrenoceptor agonist, BRL 37344, on the Na^+,K^+-pump in isolated rat skeletal muscle. This would also yield information regarding the possible existence of the β_3-adrenoceptor in skeletal muscle. The second aim was to investigate whether Na^+,K^+-pump stimulation in skeletal muscle can be mediated by an NO signalling system.

Here, we show that the β_3-adrenoceptor agonist, BRL 37344, stimulates the Na^+,K^+-pump but that this occurs via the β_2-, rather than the β_3-adrenoceptors. Results with the more selective β_3-adrenoceptor agonist, disodium (*R,R*)-5-(2-[{2-(3-chlorophenyl)-2-hydroxyethyl}-amino]propyl)-1,3-benzodioxole-2,2-dicarboxylate (CL 316,243) (Dolan *et al*., 1994), suggest that β_3-adrenoceptors expressed in skeletal muscle are not involved in Na^+ and K^+ homeostasis. Furthermore, NO does not appear to mediate Na^+,K^+-pump stimulation in rat skeletal muscle.

Methods

Animals and preparation of muscles

Experiments were carried out using 4-week-old Wistar rats, weighing approximately 60–70 g. The animals were fed *ad libitum* and were maintained in a temperature-controlled environment (21°C) with constant day length (12 h). The animals were killed by cervical dislocation, followed by decapitation, with intact soleus muscles, a predominantly slow-twitch fibre muscle (weighing 20–30 mg), dissected out as previously described (Nielsen and Clausen, 1996). All handling and use of animals complied with Danish animal welfare regulations.

Muscles were equilibrated for 30 min at 30°C in standard Krebs–Ringer bicarbonate buffer (KR) (pH 7.4), containing the following (in mM): 122.1 NaCl, 25.1 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5.0 D-glucose, and were bubbled continuously with a mixture of 95% O_2 and 5% CO_2. In buffer with 11.0 mM K^+, an equivalent amount of Na^+ was omitted to maintain iso-osmolarity.

Incubation experiments

For each of the following interventions, muscles were placed in polyethylene baskets and following equilibration for 30 min in standard KR, were incubated at 30°C in the appropriate buffers for the indicated durations (final volume 2 ml). Control muscles were incubated for durations matching their respective experimental muscles in standard KR.

Effect of β_3*-adrenoceptor agonists*

The majority of experiments involved incubating muscles between 1 and 60 min in KR containing the β_3-adrenoceptor agonist, BRL 37344 (10^{-10}–10^{-5} M) (Langin *et al*., 1991). To further test the effect of β_3-adrenoceptor activation on Na^+ homeostasis, some experiments involved incubating muscles for 30 min in KR containing the more selective

β_3-adrenoceptor agonist, CL 316,243 (10^{-7} M, 10^{-5} M) (Dolan *et al*., 1994).

Effect of β*-adrenoceptor antagonists*
To investigate whether the effect of BRL 37344 on intracellular Na^+ content was mediated via the β_3-, β_1/β_2-, β_2- or β_1-adrenoceptors, muscles were preincubated for 15 min in KR containing the β_3-adrenoceptor antagonist, 3-(2-ethylphenoxy)-1-([1*S*]-1,2,3,4-tetrahydronapth-1-ylamino)-2*S*-2-propanol oxalate (SR 59230A); (10^{-7} M, 10^{-5} M) (Nisoli *et al*., 1996), the β_1/β_2-adrenoceptor antagonist, nadolol (10^{-7} M) (Bond and Clarke, 1988), the selective β_2-adrenoceptor antagonist, (−)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol (ICI 118,551); (10^{-7} M, 10^{-5} M) (O'Donnell and Wanstall, 1980) or the selective β_1-adrenoceptor antagonist, 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl} amino]ethyoxy) benzamide (CGP 20712A); (10^{-7} M, 10^{-5} M) (Dooley *et al*., 1986), respectively.

Selectivity of β*-adrenoceptor antagonists*
To investigate the selectivity of SR 59230A (for β_3-adrenoceptors), nadolol (for β_1/β_2- adrenoceptors), ICI 118,551 (for β_2-adrenoceptors) and CGP 20712A (for β_1-adrenoceptors), muscles were preincubated for 15 min in KR containing SR 59230A (10^{-7} M, 10^{-5} M), nadolol (10^{-7} M), ICI 118,551 (10^{-5} M) or CGP 20712A (10^{-5} M), and were then incubated for 30 min in KR containing the β_2-adrenoceptor agonist, salbutamol (10^{-7} M) (Baker, 2005) with or without SR 59230A (10^{-7} M, 10^{-5} M), nadolol (10^{-7} M), ICI 118,551 (10^{-5} M) or CGP 20712A (10^{-5} M).

Effect of NO inhibitors and donors
Inhibition of NOS was induced by preincubating muscles for 60 min in KR containing *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; 10^{-5} M, 10^{-3} M), an inhibitor of NOS (Rees *et al*., 1990). 1*H*-[1,2,4]oxadiazolo[4,3,a]quinoaxilin-1-one (ODQ) (10^{-5} M) was used to inhibit NO-sensitive guanylyl cyclase (Garthwaite *et al*., 1995).

Muscles were exposed to NO by incubating muscles for 30 min in KR containing SNP (10^{-5} M, 10^{-4} M) (Young and Leighton, 1998a) or for 60 min in KR containing *S*-nitroso-*N*-acetylpenicillamine (SNAP; 10^{-5}–10^{-3} M), a nitrosothiol derivative (Holm *et al*., 1998).

Effect of noradrenaline – mediated via the β_3-, β_2- *or* β_1-*adrenoceptors*
To investigate whether the reduction in intracellular Na^+ content induced by noradrenaline was mediated via the β_3-, β_2- or β_1-adrenoceptors, muscles were preincubated for 15 min in KR containing SR 59230A (10^{-7} M), nadolol (10^{-6} M), ICI 118,551 (10^{-7} M) or CGP 20712A (10^{-7} M), and were then incubated for 30 min in KR containing noradrenaline (10^{-5} M) with or without SR 59230A (10^{-7} M), nadolol (10^{-6} M), ICI 118,551 (10^{-7} M) or CGP 20712A (10^{-7} M).

Measurement of intracellular Na^+ *and* K^+ *contents*
Following incubation, muscles were immediately transferred to ice-cold Na^+-free Tris-sucrose buffer and underwent a 4×15 min washout to remove extracellular Na^+. Following washout, muscles were blotted, tendons cut off, muscle wet weight determined, and soaked overnight in 0.3 M trichloroacetic acid (TCA) to give complete extraction of ions from the tissue (Clausen *et al*., 1993). The Na^+ and K^+ contents in the TCA extract was measured by flame photometry (FLM3, Radiometer, Copenhagen, Denmark) with lithium as internal standard. Values for Na^+ content were then multiplied by 1.46 to correct for the loss of intracellular Na^+ during the ice-cold washout (Everts and Clausen, 1992). In contrast, the loss of K^+ during the washout was minimal (Everts and Clausen, 1992).

Measurement of $^{86}Rb^+$ *uptake rate and* Na^+,K^+*-pump activity*
$^{86}Rb^+$ has previously been shown to be a reliable tracer for determination of Na^+,K^+-pump-mediated K^+ transport (Clausen *et al*., 1987). To investigate the time course of the effects of BRL 37344 on total $^{86}Rb^+$ uptake, following equilibration, muscles were incubated between 1 and 20 min in buffer containing $^{86}Rb^+$ (0.1 μCi ml^{-1}) without or with 10^{-5} M BRL 37344. In the next series of experiments, we investigated the effect of BRL 37344 on Na^+,K^+-pump activity, by measuring ouabain-sensitive $^{86}Rb^+$ uptake. Following equilibration, muscles were preincubated for 15 min without or with 10^{-3} M ouabain, followed by a further 5 min incubation in KR containing $^{86}Rb^+$ (0.1 μCi ml^{-1}) without or with 10^{-3} M ouabain and/or 10^{-5} M BRL 37344. All muscles then underwent a 4×15 min washout in ice-cold Na^+-free Tris-sucrose buffer to remove extracellular $^{86}Rb^+$ and Na^+. Following washout, muscles were blotted, tendons cut off, muscle wet weight determined, and soaked overnight in 2 ml 0.3 M TCA in 4 ml counting vials. Muscles were then taken for counting of $^{86}Rb^+$ activity by Cerenkov radiation in a β-counter. The amount of $^{86}Rb^+$ activity retained after the washout was calculated and expressed as the relative uptake of the $^{86}Rb^+$ activity from the incubation medium by the muscle. The K^+ uptake was then calculated by converting the relative uptake of $^{86}Rb^+$ to K^+ using the concentration of K^+ in the incubation medium (for details see (Buchanan *et al*., 2002)). Previous studies have reported that the loss of $^{86}Rb^+$ during the washout was minimal (Buchanan *et al*., 2002), and therefore no correction was made for $^{86}Rb^+$ uptake values.

Measurement of $^{22}Na^+$ *influx*
The effect of BRL 37344 on the Na^+ influx was determined by measuring the initial rate of $^{22}Na^+$ influx, as described elsewhere (Clausen and Kohn, 1977). Following equilibration, muscles were preincubated for 15 min in KR without or with 10^{-3} M ouabain. Muscles were then incubated for 2 min in KR containing $^{22}Na^+$ (0.5 μCi ml^{-1}) without or with 10^{-3} M ouabain and without or with 10^{-5} M BRL 37344. All muscles then underwent a 4×15 min washout in ice-cold Na^+-free Tris-sucrose buffer to remove all extracellular $^{22}Na^+$. Following washout, muscles were blotted, tendons

cut off, muscle wet weight determined, and the activity of the $^{22}Na^+$ retained in the muscles was determined by γ-counting. After correction for the loss of intracellular $^{22}Na^+$ during the washout, the Na^+ influx was calculated from the specific activity of $^{22}Na^+$ in the incubation buffer (Clausen and Kohn, 1977).

Measurement of force

Intact muscles were mounted at optimal force generating length on electrodes for isometric contractions and equilibrated for 30 min in KR at 30°C. Muscles were then exposed to field stimulation across the central region through platinum electrodes, using 2 s trains of 0.2 ms 12 V pulses at 60 Hz every 10 min. Force was measured using force displacement transducers and recorded with a chart recorder and/or digitally on a computer. The mean absolute force produced under control conditions was 0.37 ± 0.02 N ($n = 24$), with results expressed as a percentage of the control force produced in standard KR.

Chemicals and isotopes

All chemicals were of analytical grade. BRL 37344, ouabain, nadolol, SR 59230A, salbutamol, CL 316,243, CGP 20712A, ICI 118,551, L-NAME, ODQ , SNP and SNAP were purchased from Sigma Chemicals (St Louis, MO, USA). $^{86}Rb^+$ (0.1 μCi ml^{-1} buffer) and $^{22}Na^+$ (0.5 μCi ml^{-1} buffer) was from Amersham International (Aylesbury, Buckinghamshire, UK).

Statistical analysis

All data are presented as either mean ± s.e.m. or for the sake of clarity as mean with s.e.m. The statistical differences between two groups was analysed using a nonpaired-samples Student's *t*-test. The statistical difference between three or more groups was analysed using a one-way ANOVA. Differences were located with a Student–Newman–Keuls *post hoc* test. Significance was accepted at $P < 0.05$.

Results

Concentration- and time-dependent effects of BRL 37344

The effect of six different concentrations of BRL 37344 (10^{-10}–10^{-5} M, all 60 min) on the intracellular Na^+ and K^+ contents was initially investigated (Figure 1). There was no effect of BRL 37344 on intracellular Na^+ content at concentrations of 10^{-10} M and 10^{-9} M (Figure 1a). However, intracellular Na^+ content was significantly reduced with concentrations of BRL 37344 exceeding 10^{-9} M, with the largest reduction (56%) occurring with 10^{-5} M BRL 37344 (Figure 1a). In contrast, the only significant effect of BRL 37344 on intracellular K^+ content occurred at a concentration of 10^{-6} M, where intracellular K^+ content was 5% higher than in control muscles ($P < 0.01$, Figure 1b). In the presence of ouabain at a concentration (10^{-3} M) sufficient to block the Na^+,K^+-pumps, BRL 37344 (10^{-5} M) produced no significant change in Na^+ (+ouabain, 29.5 ± 1.4; ouabain + BRL 37344, 27.9 ± 0.8 μmol(g wet wt)$^{-1}$, $n = 4$) or

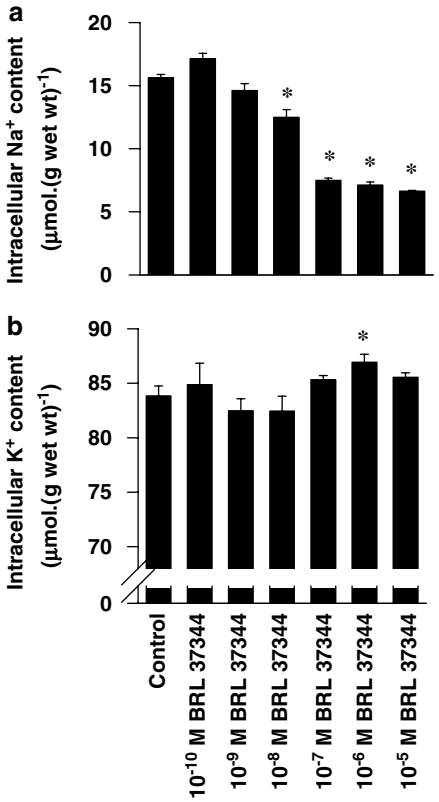

Figure 1 Concentration-dependent effect of BRL 37344 on (**a**) intracellular Na^+ and (**b**) K^+ contents in rat soleus muscle. Muscles were placed in polyethylene baskets, equilibrated for 30 min in standard KR and then incubated for 60 min without or with 10^{-10}–10^{-5} M BRL 37344. Muscles were then washed for 4 × 15 min in ice-cold Na^+-free Tris-sucrose buffer, blotted, tendons removed, weighed and taken for flame photometric analysis of Na^+ and K^+ content. Data are means with s.e.m.; $n = 4$–11. *$P < 0.01$ vs control.

K^+ contents (+ouabain, 70.5 ± 0.3; ouabain + BRL 37344, 69.8 ± 1.6 μmol(g wet wt)$^{-1}$, $n = 4$). Moreover, in comparison to the control values given in Figure 1, ouabain alone produced a large increase in intracellular Na^+ content and a large reduction in intracellular K^+ content.

Figure 2 shows the time course of the effect of BRL 37344 (10^{-5} M) on intracellular Na^+ and K^+ content. The reduction in intracellular Na^+ content was significant as early as 2 min after the onset of incubation with BRL 37344 (11%) and the decline continued until after 20–60 min when the intracellular Na^+ content approached a plateau around 50% lower than controls (Figure 2a). The elevation in intracellular K^+ content with BRL 37344 was significantly different from controls at 5 (6%), 10 (5%) and 20 min after the onset of incubation (5%, Figure 2b).

BRL 37344 (10^{-5} M) also increased total $^{86}Rb^+$ uptake, by 42, 57, 11 and 20% at 2, 5, 10 and 20 min following the onset of incubation, respectively (Figure 3). This indicates an early stimulation of the Na^+,K^+-pump.

Effect of BRL 37344 on Na^+,K^+-pump activity

Ouabain (10^{-3} M) decreased $^{86}Rb^+$ uptake from 394 ± 23 to 177 ± 7 nmol(g wet wt)$^{-1}$ min^{-1} ($n = 4$). The difference,

216 ± 26 nmol(g wet wt)$^{-1}$, is a measure of Na^+,K^+-pump activity. Incubation with 10^{-5} M BRL 37344 (5 min) increased this ouabain-sensitive $^{86}Rb^+$ uptake to 458 ± 12 nmol (g wet wt)$^{-1}$ min^{-1} ($n=4$). In the presence of ouabain, there was no effect of BRL 37344 on $^{86}Rb^+$ uptake (+ouabain, 177 ± 7; ouabain + BRL 37344, 165 ± 9 nmol(g wet wt)$^{-1}$ min^{-1}, $n=4$). These results indicate that the increase in total $^{86}Rb^+$ uptake with BRL 37344 (Figure 3) was due to increased Na^+,K^+-pump activity.

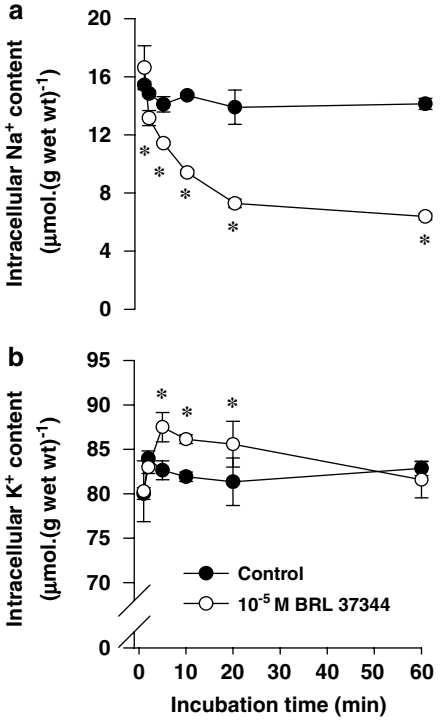

Figure 2 Time course of the effects of 10^{-5} M BRL 37344 on (**a**) intracellular Na^+ and (**b**) K^+ contents in rat soleus muscle. Muscles were incubated for 1, 2, 5, 10, 20 or 60 min without or with 10^{-5} M BRL 37344. Experimental conditions as described in Figure 1. Data are means $\pm$ s.e.m.; $n=4$–8. *$P<0.05$ vs control.

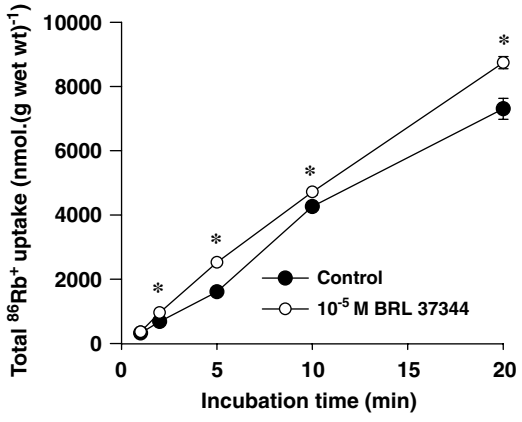

Figure 3 Time course of the effects of 10^{-5} M BRL 37344 on total $^{86}Rb^+$ uptake in rat soleus muscle. Muscles were transferred into KR containing $^{86}Rb^+$ (0.1 μCi ml^{-1}) and incubated for 1, 2, 5, 10 or 20 min without or with 10^{-5} M BRL 37344. Experimental conditions as described in Figure 1. Data are means $\pm$ s.e.m.; $n=4$. *$P<0.02$ vs control.

Effect of BRL 37344 on Na^+ influx

Incubation with 10^{-5} M BRL 37344 did not significantly attenuate $^{22}Na^+$ influx measured over a 2 min period neither in the absence (control, 598 ± 22; + BRL 37344, 564 ± 18 nmol g^{-1} min^{-1}, $n=4$–5) nor in the presence of 10^{-3} M ouabain (+ouabain, 784 ± 42; ouabain + BRL 37344, 705 ± 8 nmol g^{-1} min^{-1}, $n=5$).

Effect of β-adrenoceptor antagonists on the BRL 37344-induced reduction in intracellular Na^+ content

The β_3-adrenoceptor antagonist, SR 59230A. At a concentration of 10^{-7} M, the β_3-adrenoceptor antagonist, SR 59230A, had no effect on the reduction in intracellular Na^+ content induced by 10^{-7} M BRL 37344 (Figure 4). However at a concentration of 10^{-5} M, SR 59230A attenuated the reduction in intracellular Na^+ content induced by 10^{-7} M BRL 37344, by 13% (Figure 4).

The β_1/β_2-adrenoceptor antagonist, nadolol and the β_2-adrenoceptor antagonist, ICI 118,551. Preincubation with the β_1/β_2-adrenoceptor antagonist, nadolol (10^{-7} M) and with the selective β_2-adrenoceptor antagonist, ICI 118,551 (10^{-5} M), completely suppressed the reduction in intracellular Na^+ content induced by 10^{-7} M BRL 37344 (Figure 5). At the lower concentration of 10^{-7} M, preincubation with ICI 118,551 partially suppressed the reduction in intracellular Na^+ content induced by BRL 37344 (control, 15.7 ± 0.5;

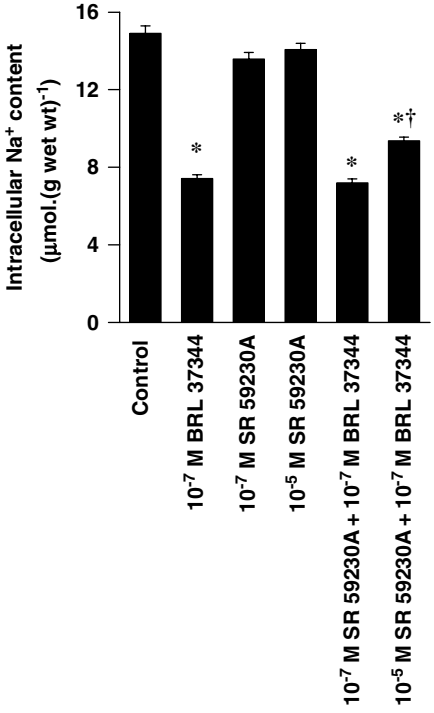

Figure 4 Effect of β_3-adrenoceptor antagonist, SR 59230A, on BRL 37344-induced change in intracellular Na^+ content in rat soleus muscle. Muscles were preincubated for 15 min without or with 10^{-7} M or 10^{-5} M SR 59230A, incubated for 30 min without or with the indicated additions, and were then treated as described in Figure 1. Data are means with s.e.m.; $n=4$. *$P<0.01$ vs control, $^{\dagger}P<0.001$ vs 10^{-7} M BRL 37344.

+BRL 37344, 9.2±0.3; ICI 118,551+BRL 37344, 12.4±0.5 μmol(g wet wt)$^{-1}$, $n=4$–12).

The β_1-adrenoceptor antagonist, CGP 20712A. Preincubation with the selective β_1-adrenoceptor antagonist, CGP 20712A (10^{-5} M), had no effect on the reduction in intracellular Na^+ content induced by 10^{-7} M BRL 37344 (Figure 5). Similar effects were found with 10^{-7} M CGP 20712A (control, 15.7±0.5; +BRL 37344, 9.2±0.3; CGP 20712A+BRL 37344, 10.0±0.7 μmol(g wet wt)$^{-1}$, $n=4$–12).

Selectivity of SR 59230A, nadolol, ICI 118,551 and CGP 20712A
The effects of preincubation with SR 59230A (10^{-7} M, 10^{-5} M), nadolol (10^{-7} M), ICI 118,551 (10^{-5} M) and CGP 20712A (10^{-5} M) on the reduction in intracellular Na^+ content induced by the β_2-adrenoceptor agonist, salbutamol (10^{-7} M), was used to assess the selectivity of these antagonists (Table 1).

Salbutamol induced a 47% reduction in intracellular Na^+ content, that was not blocked by preincubation with 10^{-7} M SR 59230A, but was attenuated to a 38% reduction compared to controls by preincubation with 10^{-5} M SR 59230A (Table 1). The reduction in intracellular Na^+ content induced by salbutamol was blocked by preincubation with nadolol and ICI 118,551 but not CGP 20712A (Table 1).

Effect of the β_3-adrenoceptor agonist, CL 316,243, on intracellular Na^+ content
To further investigate whether β_3-adrenoceptor agonists were involved in Na^+ homeostasis in skeletal muscle, the effects of the more selective β_3-adrenoceptor agonist, CL 316,243, on intracellular Na^+ content was examined. There was no effect of CL 316,243 on intracellular Na^+ content at any of the concentrations tested (control, 14.8±0.6; +10^{-7} M CL 316,243, 14.1±0.3; +10^{-5} M CL 316,243, 14.2±0.5 μmol(g wet wt)$^{-1}$, all $n=6$–7).

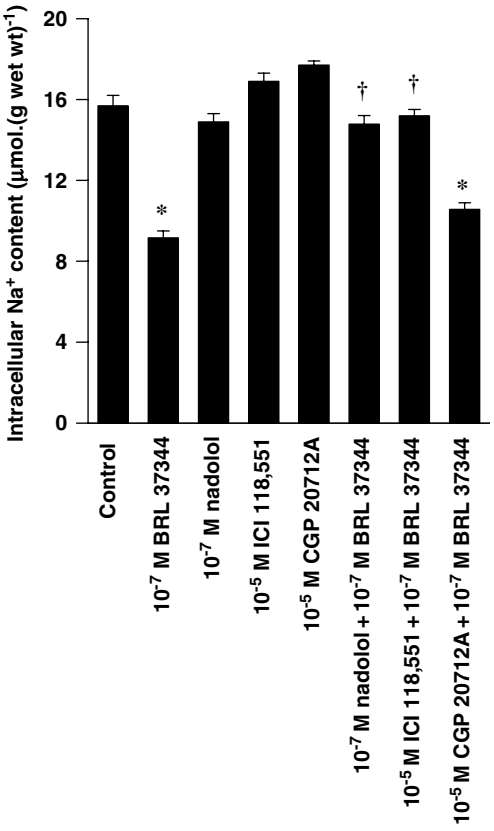

Figure 5 Effect of the β_1/β_2-adrenoceptor antagonist, nadolol, β_2-adrenoceptor antagonist, ICI 118,551, and β_1-adrenoceptor antagonist, CGP 20712A, on BRL 37344-induced change in intracellular Na^+ content in rat soleus muscle. Muscles were preincubated for 15 min without or with 10^{-7} M nadolol, 10^{-5} ICI 118,551 or 10^{-5} M CGP 20712A, incubated for 30 min without or with the indicated additions and were then treated as described in Figure 1. Data are means with s.e.m.; $n=4$–12. *$P<0.001$ vs control, $^{\dagger}P<0.001$ vs 10^{-7} M BRL 37344.

Table 1 Effects of SR 59230A, nadolol, ICI 118,551 and CGP 20712A on the salbutamol-induced change in intracellular Na^+ content in rat soleus muscle

Preincubation (15 min)	*Incubation (30 min)*	*Intracellular Na^+ content (μmol(g wet wt)$^{-1}$)*
No additions	No additions	15.2±0.7
No additions	10^{-7} M Salbutamol	8.0±0.3*
10^{-7} M SR 59230A	10^{-7} M SR 59230A	14.8±0.8
10^{-5} M SR 59230A	10^{-5} M SR 59230A	14.1±0.6
10^{-7} M Nadolol	10^{-7} M Nadolol	15.4±1.0
10^{-5} M ICI 118,551	10^{-5} M ICI 118,551	16.8±0.3
10^{-5} M CGP 20712A	10^{-5} M CGP 20712A	16.8±0.4
10^{-7} M SR 59230A	10^{-7} M SR 59230A+10^{-7} M salbutamol	7.7±0.4*
10^{-5} M SR 59230A	10^{-5} M SR 59230A+10^{-7} M salbutamol	9.4±0.4*,†
10^{-7} M Nadolol	10^{-7} M Nadolol+10^{-7} M salbutamol	13.2±0.6†
10^{-5} M ICI 118,551	10^{-5} M ICI 118,551+10^{-7} M salbutamol	17.0±0.3†
10^{-5} M CGP 20712A	10^{-5} M CGP 20712A+10^{-7} M salbutamol	8.8±0.2*

Abbreviations: CGP 20712A, 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl}amino]ethyoxy) benzamide; ICI 118,551, (−)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol; KR, Krebs–Ringer bicarbonate buffer; SR 59230A, 3-(2-ethylphenoxy)-1-([1*S*]-1,2,3,4-tetrahydronapth-1-ylamino)-2*S*-2-propranol oxalate.
Muscles were placed in polyethylene baskets, equilibrated for 30 min in standard KR and preincubated for 15 min without or with 10^{-7} M SR 59230A, 10^{-5} M SR 59230A, 10^{-7} M nadolol, 10^{-5} M ICI 118,551 or 10^{-5} M CGP 20712A. Muscles were then incubated for 30 min without or with the indicated additions, washed for 4×15 min in ice-cold Na^+-free Tris-sucrose buffer, blotted, tendons removed, weighed and taken for analysis of Na^+ content. Data are means±s.e.m., $n=4$–10. *$P<0.001$ vs control, $^{\dagger}P<0.05$ vs 10^{-7} M salbutamol.

Table 2 Effect of the NOS inhibitors, L-NAME and ODQ, on the BRL 37344-induced change in intracellular Na^+ content in rat soleus muscle

Preincubation (60 min)	*Incubation (5 min)*	*Intracellular Na^+ content (μmol(g wet wt)$^{-1}$)*
No additions	No additions	14.8±0.3
No additions	10^{-7} M BRL 37344	12.9±0.3*
No additions	10^{-5} M BRL 37344	11.4±0.1*
10^{-5} M L-NAME	10^{-5} M L-NAME	14.4±0.3
10^{-3} M L-NAME	10^{-3} M L-NAME	14.8±0.6
10^{-5} M ODQ	10^{-5} M ODQ	14.5±0.3
10^{-5} M L-NAME	10^{-5} M L-NAME + 10^{-7} M BRL 37344	12.8±0.2*
10^{-5} M L-NAME	10^{-5} M L-NAME + 10^{-5} M BRL 37344	10.6±0.4*
10^{-3} M L-NAME	10^{-3} M L-NAME + 10^{-5} M BRL 37344	11.3±0.2*
10^{-5} M ODQ	10^{-5} M ODQ + 10^{-7} M BRL 37344	13.0±0.4*

Abbreviations: BRL 37344, 4-(2-[{2-hydroxy-2-(3-chlorophenyl)ethyl}-amino]propyl)-phenoxyacetic acid; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,a]quinoxilin-1-one.
Muscles were preincubated for 60 min without or with 10^{-5} M or 10^{-3} M L-NAME, or 10^{-5} M ODQ, were incubated for 5 min without or with the indicated additions, and then treated as described in Table 1. Data are means±s.e.m., $n=4$–8.
*$P<0.01$ vs control.

Table 3 Effect of NO donors, SNP and SNAP, on intracellular Na^+ content in rat soleus muscle

Incubation (30 or 60 min)	*Intracellular Na^+ content (μmol(g wet wt)$^{-1}$)*
Control	15.9±0.5
10^{-5} M SNP	14.6±0.4
10^{-4} M SNP	14.7±0.1
10^{-5} M SNAP	17.0±0.5
10^{-4} M SNAP	15.3±0.3
10^{-3} M SNAP	16.1±0.5

Abbreviations: NO, nitric oxide; NS, nonsignificant; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine.
Muscles were incubated for 30 min without or with 10^{-5} M or 10^{-4} M SNP or for 60 min without or with 10^{-5} M, 10^{-4} M or 10^{-3} M SNAP. Muscles were then treated as described in Table 1. Data are means±s.e.m., $n=3$–4. NS.

Effect of NOS inhibitors and NO donors on the BRL 37344-induced reduction in intracellular Na^+ content

Muscles were preincubated with the NOS inhibitor, L-NAME (10^{-5} M, 10^{-3} M) or with the guanylyl cyclase inhibitor, ODQ (10^{-5} M), to investigate whether the Na^+,K^+-pump stimulation induced by BRL 37344, leading to a reduction in intracellular Na^+ content, was mediated via increased NO signalling. Neither L-NAME nor ODQ caused any significant change in intracellular Na^+ content or in the reduction in intracellular Na^+ content induced by 5 min incubation with BRL 37344 (10^{-7} M, 10^{-5} M, Table 2). There was also no effect of L-NAME (10^{-3} M) on the reduction in intracellular Na^+ content induced by 5 min incubation with 10^{-5} M salbutamol (control, 15.5±0.2; +salbutamol, 10.3±0.1; +L-NAME, 14.9±0.3; L-NAME + salbutamol, 10.2±0.2 μmol(g wet wt)$^{-1}$, $n=4$).

If increased NO were involved in Na^+,K^+-pump stimulation in skeletal muscle, the NO donors SNP and SNAP would be expected to reduce intracellular Na^+ content. However, there was no significant effect of either SNP (30 min) or SNAP (60 min) on intracellular Na^+ content at any of the concentrations tested (SNP, 10^{-5}–10^{-4} M; SNAP, 10^{-5}–10^{-3} M, Table 3).

Effect of nadolol or L-NAME on the BRL 37344-induced force recovery in muscles depressed by high $[K^+]_o$

In human skeletal muscle, intense exercise has been shown to increase the interstitial $[K^+]$ to as high as 11–12 mM (Nordsborg *et al*., 2003). Under these conditions, Na^+,K^+-pump stimulation is essential to restore the *trans*-sarcolemmal K^+ gradients to protect membrane excitability. It was therefore investigated whether BRL 37344 could induce force recovery in muscles depressed by high extracellular $[K^+]$ ($[K^+]_o$) (11 mM), and whether any force recovery could be blocked by preincubation with nadolol or L-NAME (Figure 6).

Incubation of muscles at 11 mM K^+ reduced force to 16±5% of initial force (Figure 6a). In these muscles, BRL 37344 produced a rapid force recovery, with force returning to 70±12 and 92±1% of initial force, at only 10 min following the addition of 10^{-7} M and 10^{-5} M BRL 37344, respectively (Figure 6a). Very similar force recoveries were also seen with 10^{-7} M and 10^{-5} M salbutamol (data not shown). The force recovery observed with 10^{-5} M BRL 37344 was completely blocked by preincubation with 10^{-5} M nadolol (Figure 6a), but was not affected by preincubation with 10^{-5} M L-NAME (Figure 6b).

Effect of SR 59230A, nadolol, ICI 118,551 and CGP 20712A on the noradrenaline-induced reduction in intracellular Na^+ content

As shown in Table 4, 10^{-5} M noradrenaline induced a 52% reduction in intracellular Na^+ content, that was not altered by preincubation with either 10^{-7} M SR 59230A or 10^{-7} M CGP 20712A, but was blocked by preincubation with 10^{-6} M nadolol and 10^{-7} M ICI 118,551.

Discussion

This study investigated whether the β_3-adrenoceptor agonist, BRL 37344, modulated Na^+,K^+-pump activity in isolated rat soleus muscle, as well as the mechanisms of its actions. The first main finding was that BRL 37344 stimulates the Na^+,K^+-pump, and that this occurred via the β_2-adrenoceptors, rather than the β_3-adrenoceptors. The second main finding using the more selective β_3-adrenoceptor agonist, CL 316,243 (Dolan *et al*., 1994), was that any β_3-adrenoceptors expressed in skeletal muscle

are not involved in Na^+ and K^+ homeostasis. Finally, NO did not appear to mediate Na^+,K^+-pump stimulation in rat skeletal muscle.

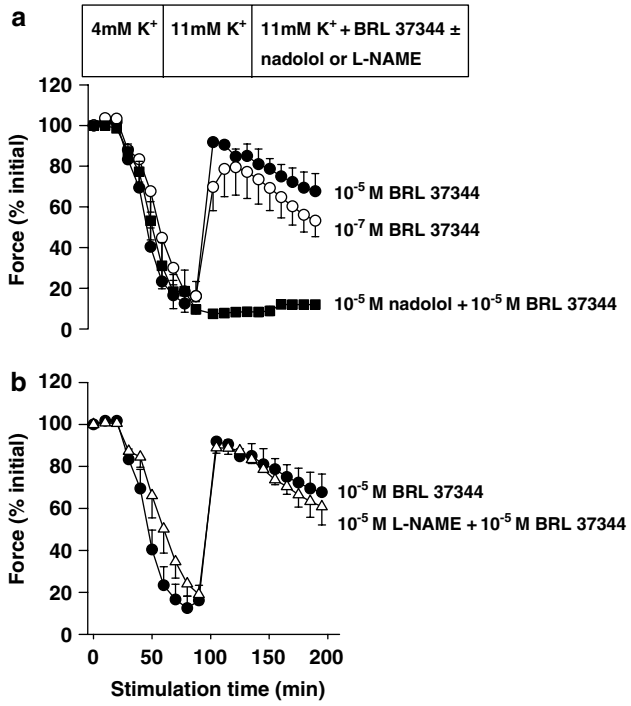

Figure 6 Effect of the β_1/β_2-antagonist, nadolol, and the NOS inhibitor, L-NAME, on the BRL 37344-induced force recovery in high $[K^+]_o$-depressed rat soleus muscles. Muscles were mounted on force transducers and stimulated at 60 Hz (0.2 ms, 12 V) for 2.0 s every 10 min for 20 min, were then incubated in KR containing high $[K^+]_o$ (11 mM) and stimulated for a further 170 min. At 90 min of stimulation, the following additions were made; (**a**) 10^{-5} M BRL 37344; 10^{-7} M BRL 37344 or both 10^{-5} M nadolol and 10^{-5} M BRL 37344, or (**b**) 10^{-5} M BRL 37344; or both 10^{-5} M L-NAME and 10^{-5} M BRL 37344. (**a**) Nadolol or (**b**) L-NAME were added 15 min before the addition of BRL 37344. Data are means ± s.e.m. (10^{-5} M nadolol + 10^{-5} M BRL 37344), or are mean + s.e.m. (10^{-5} M BRL 37344) or mean – s.e.m. (10^{-7} M BRL 37344, 10^{-5} M L-NAME-10^{-5} M BRL 37344) for clarity; $n = 4$–8.

BRL 37344 stimulated the Na^+,K^+-pump via β_2-adrenoceptors

The β_3-adrenoceptor agonist, BRL 37344, induced a concentration- and time-dependent reduction in intracellular Na^+ content that was significant after only 2 min of incubation. As this effect was blocked by ouabain, it was mediated via Na^+,K^+-pump stimulation, as also demonstrated by the BRL 37344-induced increase in ouabain-sensitive $^{86}Rb^+$ uptake. The decrease in intracellular Na^+ content could not be attributed to inhibition of Na^+ influx, the initial rate of $^{22}Na^+$ influx being unaffected by BRL 37344. However, the reduction in intracellular Na^+ content induced by BRL 37344 was completely blocked by preincubation with the β_1/β_2-adrenoceptor antagonist, nadolol, but not by preincubation with the β_3-adrenoceptor antagonist, SR 59230A (10^{-7} M). At this concentration, we demonstrated that SR 59230A does not act on the β_2-adrenoceptors since there was no effect of preincubation with this antagonist, on the reduction in intracellular Na^+ content induced by the β_2-adrenoceptor agonist, salbutamol (10^{-7} M). At the concentrations of 10^{-6} M and 3×10^{-6} M, SR 59203A has previously been shown to act as a selective β_3-adrenoceptor antagonist; having no effect on the increase in cAMP induced with salbutamol in rat cerebellum (Nisoli *et al.*, 1996) and completely blocking the relaxant effects of several selective β_3-adrenoceptor agonists in rat colon (Kaumann and Molenaar, 1996), respectively. However, at the higher concentration of 10^{-5} M, SR 59230A appeared to act also on the β_2-adrenoceptors in the present study since the reductions in intracellular Na^+ content induced by BRL 37344 and salbutamol were both slightly attenuated (~10–13%, $P<0.05$) by this preincubation. The lack of selectivity of SR 59230A at a concentration of 10^{-5} M is likely to reflect the considerable heterogeneity that exists between the structure of the β_1-, β_2- and β_3-adrenoceptors, their structure between species, and also between the affinities of these β-adrenoceptors for antagonists and agonists (Arch, 2000). We also demonstrated that nadolol prevents the reduction in intracellular Na^+ content induced by salbutamol. Importantly, salbutamol has previously been shown to be highly selective for the β_2-adrenoceptors (Baker, 2005).

Table 4 Effect of SR 59230A, nadolol, ICI 118,551, CGP 20712A on the noradrenaline-induced change in intracellular Na^+ content in rat soleus muscle

Preincubation (15 min)	*Incubation (30 min)*	*Intracellular Na^+ content (μmol(g wet wt)$^{-1}$)*
No additions	No additions	15.9 ± 0.5
No additions	10^{-5} M Noradrenaline	7.6 ± 0.3*
10^{-7} M SR 59230A	10^{-7} M SR 59230A	14.1 ± 0.3
10^{-6} M Nadolol	10^{-6} M Nadolol	14.7 ± 0.3
10^{-7} M ICI 118,551	10^{-7} M ICI 118,551	16.9 ± 0.4
10^{-7} M CGP 20712A	10^{-7} M CGP 20712A	16.0 ± 1.4
10^{-7} M SR 59230A	10^{-7} M SR 59230A + 10^{-5} M noradrenaline	6.9 ± 0.2*
10^{-6} M Nadolol	10^{-6} M Nadolol + 10^{-5} M noradrenaline	14.7 ± 0.3†
10^{-7} M ICI 118,551	10^{-7} M ICI 118,551 + 10^{-5} M noradrenaline	15.7 ± 0.1†
10^{-7} M CGP 20712A	10^{-7} M CGP 20712A + 10^{-5} M noradrenaline	7.7 ± 0.8*

Abbreviations: CGP 20712A, 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl}amino]ethyoxy) benzamide; ICI 118,551, (−)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol; SR 59230A, 3-(2-ethylphenoxy)-1-([1*S*]-1,2,3,4-tetrahydronapth-1-ylamino)-2*S*-2-propranol oxalate.

Muscles were preincubated for 15 min without or with 10^{-7} M SR 59230A, 10^{-6} M nadolol, 10^{-7} M ICI 118,551 or 10^{-7} M CGP 20712A, were incubated for 30 min without or with the indicated additions and then treated as described in Table 1. Data are means ± s.e.m., $n = 4$–13.

*$P<0.001$ vs control, †$P<0.001$ vs 10^{-5} M noradrenaline.

Furthermore, the force recovery induced by BRL 37344 in high $[K^+]_o$-depressed muscles was completely blocked by preincubation with nadolol. Although nadolol acts as a β_1/β_2-adrenoceptor antagonist, the effects shown here with nadolol are mediated via the β_2-adrenoceptors since the reduction in intracellular Na^+ content induced with 10^{-7} M BRL 37344 was blocked by preincubation with the selective β_2-antagonist, ICI 118,551 (10^{-7} M, 10^{-5} M) (O'Donnell and Wanstall, 1980), but not with the selective β_1-adrenoceptor antagonist, CGP 20712A (10^{-7} M, 10^{-5} M) (Dooley *et al*., 1986). The selectivity of these antagonists was demonstrated by the suppression of the reduction in intracellular Na^+ content induced with the β_2-adrenoceptor agonist salbutamol (10^{-7} M) by preincubation with ICI 118,551 (10^{-5} M) but not CGP 20712A (10^{-5} M). Furthermore, in rat soleus muscle, salbutamol was at least 100 times more potent than the β_1-adrenoceptor agonist H133/22 in stimulating the Na^+,K^+-pump (Clausen and Flatman, 1980). Taken together, these results demonstrate that at concentrations exceeding 10^{-9} M, BRL 37344 rapidly and potently stimulated the Na^+,K^+-pump in skeletal muscle, but that this effect was mediated by the β_2-, rather than the β_3- or the β_1-adrenoceptors. Previous studies in rat and mouse skeletal muscle using selective β_2-adrenoceptor antagonists have demonstrated that at low concentrations ($<10^{-9}$ M), BRL 37344 acts as a selective β_3-adrenoceptor agonist, whereas at higher concentrations ($>10^{-9}$ M), BRL 37344 also activates the β_2-adrenoceptors (Liu *et al*., 1996; Board *et al*., 2000). The present results support these findings since no reduction in intracellular Na^+ content was observed with either 10^{-10} M or 10^{-9} M BRL 37344.

No effect of selective β_3-adrenoceptor agonist on Na^+,K^+-pump in rat soleus muscle

There was no effect of the more selective β_3-adrenoceptor agonist, CL 316,243, on the intracellular Na^+ content in skeletal muscle. This contrasts the reduction in intracellular Na^+ content induced by BRL 37344 ($>10^{-9}$ M) that was mediated via β_2-adrenoceptors, providing evidence that CL 316,243 does not act via the β_2-adrenoceptors. Indeed, it has previously been shown in isolated rat soleus and EDL muscles that the proteolysis induced by CL 316,243 was completely abolished by SR 59230A but not ICI 118,551 (Navegantes *et al*., 2006).

These results with CL 316,243 are in keeping with our observation of a lack of reduction in intracellular Na^+ content with low concentrations of BRL 37344 (10^{-10} M, 10^{-9} M), which appear to reflect activation independent of the β_2-adrenoceptors (Liu *et al*., 1996; Board *et al*., 2000). Together, these findings suggest (i) that if β_3-adrenoceptors are present in skeletal muscle, they are not involved in Na^+ and K^+ homeostasis in skeletal muscle, (ii) that CL 316,243 and low concentrations of BRL 37344 ($<10^{-9}$ M) do not affect any β_3-adrenoceptors present in rat soleus muscle, or (iii) that β_3-adrenoceptors are expressed in very low amounts, or not at all, in rat soleus muscle. Despite the above-mentioned study finding evidence for a functional β_3-adrenoceptor in the regulation of proteolysis (Navegantes *et al*., 2006), it remains unclear whether β_3-adrenoceptors are indeed expressed in skeletal muscle. Whereas several studies have found evidence for expression of the β_3-adrenoceptor agonist mRNA (Evans *et al*., 1996) and protein (Sillence *et al*., 1993) in rat skeletal muscle, others have not (Granneman *et al*., 1991; McNeel and Mersmann, 1999). In humans, β_3-mRNA was not detected in several types of skeletal muscle, including the soleus, intercostal, posterior tibialis and gastrocnemius muscles (Thomas and Liggett, 1993). However, these results need to be interpreted with caution, as the muscles were taken post mortem from a 43-year-old man who was brain-dead from a massive cerebral vascular haemorrhage. Indeed, the β_3-adrenoceptor protein was identified in two out of three samples of human gastrocnemius muscle using immunohistochemistry and a high-affinity monoclonal antibody for β_3 (Mab72c) (Chamberlain *et al*., 1999). However, in a later study using the same antibody, the β_3-adrenoceptor protein was not detected in any of three samples of human pectoralis muscle (De Matteis *et al*., 2002). Further work with additional monoclonal antibodies specific for β_3 is therefore required to confirm the presence or absence of β_3-adrenoceptors in skeletal muscle. It is also possible that CL 316,243 and low concentrations of BRL 37344 may act via an as yet, uncharacterized β-adrenoceptor in skeletal muscle (Board *et al*., 2000). As discussed by Board *et al*. (2000), the existence of such a receptor would support the discrepancy in results pertaining to β_3-adrenoceptor expression in skeletal muscle.

If the present findings represent a lack of β_3-adrenoceptor stimulation of the Na^+,K^+-pump in skeletal muscle, this contrasts the stimulatory effect found in rabbit cardiac myocytes (Bundgaard *et al*., 2006a). This difference may reflect a tissue- or species-specific effect. Indeed, a species-specific effect has been shown for the negative inotropic effect induced by BRL 37344 (Gauthier *et al*., 1999).

The physiological catecholamines, adrenaline and noradrenaline, activate the Na^+,K^+-pump in skeletal muscle, leading to increased Na^+ efflux and K^+ influx and membrane hyperpolarization (Clausen and Flatman, 1977, 1980). As the β_3-adrenoceptor demonstrates a much higher (<30-fold) affinity for noradrenaline than adrenaline, and the opposite is true for the β_2-adrenoceptor in CHO cells (Hoffmann *et al*., 2004), the noradrenaline-induced Na^+,K^+-pump activation in skeletal muscle may be mediated via the β_3-adrenoceptors. Indeed, in rabbit cardiac myocytes, the noradrenaline-induced increase in Na^+,K^+-pump-mediated current was resistant to nadolol (Bundgaard *et al*., 2006a). However, we found that the reduction in intracellular Na^+ content induced by noradrenaline (10^{-5} M) was blocked by preincubation with nadolol (10^{-6} M) and ICI 118,551 (10^{-7} M), but not by SR 59230A8 (10^{-7} M) or CGP 20712A (10^{-7} M). Thus, in isolated rat soleus muscle, noradrenaline-induced Na^+,K^+-pump stimulation occurred via the β_2-adrenoceptors, rather than the β_3- or β_1-adrenoceptors.

No evidence for NO-induced Na^+,K^+-pump stimulation

In skeletal muscle, NO is continuously produced via the enzymatic action of NOS. At rest, NO production is low. However, during periods of repeated action potentials it may increase by up to 100% (Kobzik *et al*., 1994). Endogenous NO

depresses contractile function, as evidenced by an elevation in submaximal force with the addition of several exogenous NOS inhibitors in rat diaphragm muscle (Kobzik *et al*., 1994; Reid *et al*., 1998). Moreover, this effect was reversed with the addition of the exogenous NO donors, *S*-nitroso-*N*-acetylcysteine and SNP (Kobzik *et al*., 1994). In cardiac myocytes, NO signalling is generally restricted to sarcolemmal microdomains (Barouch *et al*., 2002) that contain significant amounts of Na^+,K^+-pumps (Liu *et al*., 2003). Owing to this colocalization, any NO-induced stimulation of the Na^+,K^+-pump would occur very selectively and rapidly, and therefore, represent a potentially important localized activator of the Na^+,K^+-pump. However, in the present study, there was no effect of L-NAME or ODQ, which inhibit NO production and NO-activated guanylyl cyclase, respectively, on the reduction in intracellular Na^+ content induced by BRL 37344. There was also no effect of preincubation with L-NAME on the reduction in intracellular Na^+ content induced by salbutamol or on the BRL 37344-induced force recovery in high $[K^+]_o$-depressed muscles. Furthermore, neither of the NO donors, SNP nor SNAP, reduced intracellular Na^+ content. These results therefore indicate that the NO signalling system is not causing stimulation of the Na^+,K^+-pump in skeletal muscle. This lack of effect of NO on the Na^+,K^+-pump cannot be explained by an absence of NO signalling in skeletal muscle, since a pathway involving NOS, soluble guanylyl cyclase, cGMP and PKG was shown in isolated rat soleus muscle (Young and Leighton, 1998a, b). Conversely, this lack of effect may reflect that NO-induced stimulation of the Na^+,K^+-pump is tissue-dependent. In rabbit cardiac myocytes, L-NAME completely abolished the increase in Na^+,K^+-pump-mediated current induced by BRL 37344 (Bundgaard *et al*., 2006a), and Na^+,K^+-pump activity was also stimulated by SNP (William *et al*., 2005). Furthermore, in mouse cardiac vesicles, knockout of the NOS isoforms, nNOS and eNOS, significantly depressed Na^+,K^+-pump activity (Zhou *et al*., 2002). In contrast, both SNP and SNAP reduced Na^+,K^+-pump activity in the renal medulla of rats (McKee *et al*., 1994; Beltowski *et al*., 2003), and SNAP reduced Na^+,K^+-pump activity in rat liver cells (Muriel and Sandoval, 2000).

BRL 37344-induced rapid force recovery in muscles depressed by high $[K^+]_o$

In humans, intense exercise has been shown to increase interstitial $[K^+]$ to as high as 11–12 mM (Nordsborg *et al*., 2003). Such a large increase reduces the *trans*-sarcolemmal $[K^+]$ gradient, leading to membrane depolarization and inactivation of the voltage-gated Na^+ channels (Ruff, 1996). This results in loss of muscle excitability, and subsequently, a reduction in force production. Indeed, in the present study, increasing the $[K^+]_o$ from 4 to 11 mM reduced force to $\sim$16% of initial force. However, BRL 37344 (10^{-5} M) recovered force production to $\sim$92% of the initial level at only 10 min after the onset of exposure. This BRL 37344-induced force recovery is likely to reflect Na^+,K^+-pump activation, leading to membrane hyperpolarization and hence, increased force production. This is closely similar to the rapid force recovery induced by β_2-adrenoceptor agonists in high $[K^+]$-depressed muscles as found in this study and earlier (Clausen *et al*., 1993). In keeping with this, the effect was suppressed by preincubation with nadolol.

Implications

Several clinical uses of β_3-adrenoceptor agonists have been proposed, including for the treatment of diabetes and obesity (Arch and Wilson, 1996), urinary bladder dysfunction (Badawi *et al*., 2005), bronchoconstriction (Broadley, 2006) and heart failure (Bundgaard *et al*., 2006b). Results from the present study suggest that the use of BRL 37344 in these clinical settings could be jeopardized by some of the well-known adverse effects seen clinically when using β_2-adrenoceptor agonists (Broadley, 2006). As BRL 37344 at concentrations exceeding 10^{-9} M appears to act as a β_2-adrenoceptor agonist in skeletal muscle, administration of this compound may induce hypokalemia via Na^+,K^+-pump stimulation. Indeed, in conscious rabbits, intravenous administration of BRL 37344 led to a significant reduction in plasma K^+ levels, that was even greater than that induced by the administration of salbutamol (Reverte *et al*., 1993). Thus, clinical use of β_3-adrenoceptor stimulation might require the use of more selective agonists, such as CL 316, 243.

In conclusion, the β_3-adrenoceptor agonist BRL 37344 stimulated the Na^+,K^+-pump to reduce intracellular Na^+ content, an effect that was mediated via the β_2-, rather than the β_3-adrenoceptors. In addition, results with the more selective β_3-adrenoceptor agonist CL 316,243 indicated that any β_3-adrenoceptors expressed in rat soleus muscle are unlikely to be involved in Na^+ and K^+ homeostasis in this tissue. Finally, NO did not seem to mediate Na^+,K^+-pump stimulation in skeletal muscle.

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Conflict of interest

The authors state no conflict of interest.

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