Reducing chloride conductance prevents hyperkalaemia-induced loss of twitch force in rat slow-twitch muscle

Maarten Geert van Emst[1], Sjoerd Klarenbeek[1], Arend Schot[1], Jaap Jan Plomp[2], Arie Doornenbal[1] and Maria Elisabeth Everts[1]

[1]*Department of Pathobiology, Division of Anatomy and Physiology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.158, 3508 TD Utrecht, The Netherlands*
[2]*Departments of Neurology and Neurophysiology, Leiden University Medical Centre, P.O. Box 9604, 2300 RC Leiden, The Netherlands*

Exercise-induced loss of skeletal muscle K^+ can seriously impede muscle performance through membrane depolarization. Thus far, it has been assumed that the negative equilibrium potential and large membrane conductance of Cl^- attenuate the loss of force during hyperkalaemia. We questioned this idea because there is some evidence that Cl^- itself can exert a depolarizing influence on membrane potential (V_m). With this study we tried to identify the possible roles played by Cl^- during hyperkalaemia. Isolated rat soleus muscles were kept at 25 °C and twitch contractions were evoked by current pulses. Reducing $[Cl^-]_o$ to 5 mM, prior to introducing 12.5 mM K_o^+, prevented the otherwise occurring loss of force. Reversing the order of introducing these two solutions revealed an additional effect, i.e. the ongoing hyperkalaemia-related loss of force was sped up tenfold after reducing $[Cl^-]_o$. However, hereafter twitch force recovered completely. The recovery of force was absent at $[K^+]_o$ exceeding 14 mM. In addition, reducing $[Cl^-]_o$ increased membrane excitability by 24%, as shown by a shift in the relationship between force and current level. Measurements of V_m indicated that the antagonistic effect of reducing $[Cl^-]_o$ on hyperkalaemia-induced loss of force was due to low-Cl^--induced membrane hyperpolarization. The involvement of specific Cl^- conductance was established with 9-anthracene carboxylic acid (9-AC). At 100 μM, 9-AC reduced the loss of force due to hyperkalaemia, while at 200 μM, 9-AC completely prevented loss of force. To study the role of the $Na^+-K^+-2Cl^-$ cotransporter (NKCC1) in this matter, we added 400 μM of the NKCC inhibitor bumetanide to the incubation medium. This did not affect the hyperkalaemia-induced loss of force. We conclude that Cl^- exerts a permanent depolarizing influence on V_m. This influence of Cl^- on V_m, in combination with a large membrane conductance, can apparently have two different effects on hyperkalaemia-induced loss of force. It might exert a stabilizing influence on force production during short periods of hyperkalaemia, but it can add to the loss of force during prolonged periods of hyperkalaemia.

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Corresponding author M. G. van Emst: Department of Pathobiology, Division of Anatomy and Physiology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.158, 3508 TD Utrecht, The Netherlands. Email: m.vanemst@vet.uu.nl

The substantial Cl^- conductance of mammalian skeletal muscle has a stabilizing influence on membrane potential (V_m). Blocking the ClC-1 channel or replacing extracellular Cl^- with foreign anions results in myotonia (Landau, 1952; Bryant & Morales-Aguilera, 1971; Bretag, 1987). As such, Cl^- is likely to affect muscular fatigue by influencing the K^+-related force depression.

Hyperkalaemia can occur during brief periods of intense exercise. The interstitial $[K^+]$ in human muscle can reach values as high as 10 mM during fatiguing exercise (Juel *et al.* 2000). A K^+-induced depolarization of V_m can render the muscle fibre inexcitable and cause loss of force if V_m depolarizes to above −60 mV (Juel, 1986; Clausen & Everts, 1991; Cairns *et al.* 1995, 1997; Overgaard *et al.* 1999; Clausen & Overgaard, 2000; Pedersen *et al.* 2003). *In vitro* experiments established that stimulating mouse soleus muscle until it fatigues, raises interstitial $[K^+]$ from 5 to 10 mM

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and concomitantly lowers $[K^+]_i$ from 168 to 136 mM (Juel, 1986). The change in $[K^+]_o/[K^+]_i$ depolarized V_m to −58 mV at 37 °C. Therefore, the reported changes in K^+ concentration gradients during exercise appear large enough to contribute to the development of muscular fatigue. However, several mechanisms have been identified, which might act concertedly *in vivo* to diminish both the amount of K^+ released during exercise and the effect of a rise in $[K^+]_o$ on V_m and membrane excitability: increased muscle temperature, circulating catecholamines and intracellular acidification (Clausen *et al.* 1993; Nielsen *et al.* 2001; Overgaard & Nielsen, 2001; Yensen *et al.* 2002; Pedersen *et al.* 2003). In addition, it has been repeatedly suggested that the large Cl^- conductance of mammalian muscle fibres may prevent excessive depolarization during repeated stimulation (Dulhunty, 1979; Coonan & Lamb, 1998; Wallinga *et al.* 1999; Sejersted & Sjøgaard, 2000).

During hyperkalaemia Cl^- ions flow into the muscle fibre because their chemical gradient is no longer balanced by V_m. In turn, the influx of these anions slows down membrane depolarization. Therefore, Cl_o^- lowers the rate of the K^+-induced membrane depolarization (Hodgkin & Horowicz, 1959; Dulhunty, 1978; McCaig & Leader, 1984). In addition, Dulhunty (1978, 1979) demonstrated that Cl^- is also capable of reducing the extent of the K^+-induced membrane depolarization. This effect of Cl^- on V_m is most likely to be related to the transport of Cl^- into the muscle fibre by the bumetanide-sensitive $Na^+-K^+-2Cl^-$ (NKCC1) transporter, as shown in rats and mice (Betz *et al.* 1984; Harris & Betz, 1987; Aickin *et al.* 1989; Geukes Foppen *et al.* 2002). As with Na^+ and K^+, any ion which is actively transported across the sarcolemma has an equilibrium potential that differs from V_m (Dulhunty, 1978; Aickin *et al.* 1989), i.e. an actively transported ion tends to pull the V_m towards its own equilibrium potential. A strong influence of Cl^- on V_m causes the membrane to behave like a weak K^+ electrode. In fact, the sensitivity of V_m in extensor digitorum longus (EDL) or soleus muscle to changes in $[K^+]_o$ ranges from 55 down to 35 mV per decade $[K^+]_o$ (Dulhunty, 1980; Mølgaard *et al.* 1980; Chua & Dulhunty, 1988; Siegenbeek van Heukelom, 1991; Cairns *et al.* 1997; Yensen *et al.* 2002). So, Cl^- is capable of reducing both the rate and the extent of K^+-induced membrane depolarization. This implies that a reduction in Cl^- conductance should lead to a more rapid and more pronounced loss of force during hyperkalaemia. Recently, Cairns *et al.* (2004) showed that tetanus depression in mouse soleus muscle, due to raised $[K^+]_o$, occurred more rapidly and to a greater extent at low $[Cl^-]_o$.

However, one final aspect of the influence of Cl^- on V_m has not yet been considered. The intracellular accumulation of Cl^-, combined with a large specific conductance (Dulhunty, 1979; Coonan & Lamb, 1998), could lead to substantial membrane depolarization because the equilibrium potential of Cl^- has become less negative than V_m (Aickin *et al.* 1989). Hyperpolarizing responses up to 20 mV have been measured in soleus, EDL, sternomastoid and lumbrical muscle from rat or mouse, after eliminating Cl^- conductance or Cl^- accumulation (Dulhunty, 1978; Betz *et al.* 1984; Aickin *et al.* 1989). In contrast, several other studies found no difference between V_m and the equilibrium potential for Cl^- (E_{Cl}) under resting conditions (Blum & Westphal, 1981; DeCoursey *et al.* 1981; Donaldson & Leader, 1984; McCaig & Leader, 1984). If Cl^- exerts a substantial depolarizing influence on V_m, and the V_m at which twitch force decreases is fixed at around −60 mV (Cairns *et al.* 1997), then this could make the fibres more prone to hyperkalaemia-induced loss of force. It has been found that fatigue during tetanic contractions was attenuated after reducing Cl^- conductance in EDL muscle of developing rats (De Luca *et al.* 1990). Most of the results from Cairns *et al.* (2004) illustrate that low $[Cl^-]_o$ principally accelerates the fatigue with continuous stimulation, but one graph illustrates that the final loss of force can be less when $[Cl^-]_o$ is low. Whether an increase in $[K^+]_o$ caused the tetanus depression was not examined.

The aim of this investigation was to identify the roles played by Cl_o^- and specific Cl^- conductance during hyperkalaemia-induced loss of twitch force in isolated rat soleus muscle. We tested the hypothesis that Cl^- has an antagonistic effect on the developmental course of hyperkalaemia-induced loss of force, but adds to the final loss of force during prolonged hyperkalaemia. To this end, twitch force was studied under various conditions: in the presence and near absence of extracellular Cl^-, with and without the addition of the ClC-1 blocker 9-anthracene-carboxylic-acid (9-AC), and finally, with and without the addition of the loop-diuretic bumetanide. We expected a more rapid loss of force during hyperkalaemia after reducing $[Cl^-]_o$ or adding 9-AC, because the stabilizing effect of a large Cl^- conductance on V_m is lost. In addition, we expected that less force would be lost after reducing Cl^- conductance or blocking the NKCC1 transporter because of the associated hyperpolarization.

Methods

Animal handling and muscle preparation

Male Wistar rats were obtained from laboratory stock (GDL, Utrecht University, the Netherlands). All twitch force experiments were performed using rats weighing 190–200 g. For measurements of V_m we used rats weighing 140 g. These smaller animals were chosen because of the size and limitations of the set-up. Animals were fed *ad libitum* and kept under a 12 h light (during the daytime)/12 h dark regime, at 21 °C. Rats were

killed by cervical dislocation. Next, intact soleus muscles weighing between 95 and 115 mg (wet weight) were dissected out in approximately 15 min. During dissection muscles were kept moist with a modified Krebs–Ringer bicarbonate buffer solution. All procedures were approved by the Utrecht University Committee for experiments on animals and were in accordance with the Dutch law on experimental animals.

After dissection, soleus muscles were mounted vertically in thermostatically controlled Perspex measuring chambers (40 ml). A small part of the proximal fibula was used to fix the muscle at the bottom of the chamber. At the other end of the muscle, a size 10 fishing hook was pierced trough the tendon and connected to the force tranducer using Ethicon EH781P 5-0 thread (Johnson & Johnson Medical, Amersfoort, the Netherlands). Next, a tension of 50 mN was applied to the soleus by adjusting the position of the force transducer. In preliminary experiments we had established that this pre-tension resulted in optimal twitch force.

All muscles were allowed to acclimatize for 45 min at 25 °C in modified Krebs–Ringer bicarbonate buffer solution. A temperature of 25 °C was chosen for the following two reasons. First, to avoid myotonia; reducing Cl^- conductance triggered myotonia at 35 °C, but not at 25 °C, although an occasional myotonic contraction could still be observed. Thus, we were compelled to perform our experiments at 25 °C. Second, a bath temperature of 25 °C reduces metabolic requirements and thus ensures sufficient oxygenation of the central muscle fibres. It has been shown (Segal & Faulkner, 1985) that as incubation temperature exceeds 25 °C, the critical radius for oxygen diffusion becomes less than the average radius of 70–90 mg muscles. Although the weight of our soleus muscles was around 100 mg (without tendons), we did not find any indication of a progressively larger anoxic core with time. Stability of the muscle preparation was established during preliminary experiments lasting 250 min. During these experiments, both peak isometric twitch force and membrane excitability, as determined by the isometric twitch-force–current-strength relationship, were measured repeatedly and remained stable.

Signal conditioning and stimulation protocol

Muscles were stimulated directly through two platinum/iridium (90/10%) wire electrodes of 0.2 mm diameter (Drijfhout, Amsterdam, the Netherlands). These were inserted horizontally through the muscle, 1 cm apart. Electrodes had been mechanically sharpened.

Single 0.2 ms TTL voltage pulses were obtained once each minute from a programmable pulse-generator (Master-8, A.M.P.I., Israel) and converted into constant unipolar current pulses with a two-channel stimulus isolator (BSI-2, BAK Electronics Inc., Germany). The performance and accuracy of the stimulus isolator at different current levels was routinely checked at 1 and 10 kΩ with a Tektronix TDS 3012 digital oscilloscope (Beaverton, OR, USA). During stimulation, the muscles were no longer submerged in conductive fluid. The fluid was pumped out of the chamber 5 s in advance of the stimulus, only to be pumped back in 1 s after stimulation. Timing was controlled by the programmable pulse generator. The advantages of stimulating muscle suspended in air are that much less current is needed to evoke the contractions and, more importantly, the results become much more reproducible (Van der Heijden *et al.* 1998).

Membrane excitability of each muscle was repeatedly established during an experiment. This was done by increasing the current level from 0 to 0.5 mA in 0.05 mA steps, and from 0.5 to 1.5 mA in 0.1 mA steps, and recording twitch force at each stimulus level. These twitch-force–stimulus-level relationships are referred to as twitch curves. All further stimulation was performed at a stimulus level of 1.5 mA, which was at least 1.5 times the current needed to evoke maximum twitch force.

Isometric force development was measured using FT03 force displacement transducers (GRASS FT03, W. Warwick, RI, USA). The force transducers were connected with a signal conditioner (Cyber Amp 380, Axon Instruments). The output signal from the transducer was amplified 2000 times and low-pass filtered at 100 Hz before A/D conversion took place. The signal was sampled (250 Hz) using LabVIEW 3.1 (National Instruments, Austin, TX, USA).

Before each experiment transducers were calibrated using weights of 5, 10, 20 and 50 g. The relation between weight and voltage output was highly linear. The inverse slope of this relation was used for off-line analyses of muscle twitch force (1 g = 9.8 mN).

Solutions and chemicals

In the first set of experiments, four different solutions were used: modified Krebs–Ringer solution, high K_o^+, low Cl_o^-, and high K_o^+/low Cl_o^-. Modified Krebs–Ringer solution contained 5 mM $KHCO_3^-$ instead of 5 mM KCl. The low Cl_o^- solutions were made by replacing NaCl with sodium isethionate (isethionic acid, sodium salt; Aldrich). We chose isethionate (sodium salt) to replace Cl_o^- because it is known as one of the monovalent anions that can be used to substitute for Cl^- without affecting divalent cation activity, osmolality or Na^+ activity. Moreover, it does not seem to interact with Cl^- channels (Bretag, 1987). In an additional control experiment we used methanesulphonate ($CH_3SO_3^-$, methanesulphonic acid, sodium salt; Aldrich) to replace Cl_o^-. The salt and ionic composition of the solutions is given in Table 1.

Table 1. Composition of the extracellular solutions

	Modified Krebs–Ringer solution	High K_o^+	Low Cl_o^-	High K_o^+/ Low Cl_o^-
NaCl	125	125	0	0
Sodium isethionate	0	0	125	125
$KHCO_3$	5	12.5	5	12.5
$NaHCO_3$	20	12.5	20	12.5
$NaH_2PO_4{\cdot}H_2O$	1.2	1.2	1.2	1.2
$MgSO_4$	1.2	1.2	1.2	1.2
$CaCl_2{\cdot}2H_2O$	2.6	2.6	2.6	2.6
D-Glucose	5	5	5	5
K^+	5	12.5	5	12.5
Na^+	146.2	138.7	146.2	138.7
Cl^-	130.2	130.2	5.2	5.2

Concentrations are given in millimoles per litre. The $[K^+]_o$ was increased by equimolar replacement of $NaHCO_3$ with $KHCO_3$. The $[Cl^-]_o$ was reduced by replacement of NaCl by sodium isethionate (isethionic acid, sodium salt). The three rows at the bottom provide the concentrations of the three ions being involved in setting membrane excitability. All fluids were continuously aerated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) through glass filter plates (16–40 μm pores, duran, Schott Glas, Mainz, Germany).

In the second and third set of experiments, the modified Krebs–Ringer solution and high K_o^+ solutions, as mentioned previously, were used. To these two control solutions we added 9-AC to a final concentration of 100 μM (20 mM stock, solvent ethanol) or bumetanide to a final concentration of 100 or 400 μM (100 mM stock, solvent methanol). In the experiments with 9-AC and bumetanide, the control solutions contained an equimolar amount of the solvent, i.e. 0.5% v/v ethanol and 0.1–0.4% v/v methanol, respectively. Because (m)ethanol is a permeant solute it should only have a transient effect on cell volume. In an additional set of experiments 9-AC was tested at a concentration of 200 μM. In this case the stock solution contained 9-AC (400 mM) dissolved in DMSO.

Prior to each experiment, the Na^+, K^+ and Cl^- concentrations of the solutions were measured with ion-sensitive electrodes (AVL 983-S). The osmotic value of the solutions was verified with a freezing-point osmometer (Osmomat 030/Gonotec, Berlin, Germany). The osmolality of the solutions in Table 1 varied between 302 and 309 mosmol kg^{-1}. The osmolality of the solutions containing 0.1 or 0.4% v/v methanol ranged from 315 to 325 and from 389 to 399 mosmol kg^{-1}, respectively. The osmolality of the solutions containing 0.5% v/v ethanol ranged from 376 to 390 mosmol kg^{-1}.

All chemicals were of analytical grade. Bumetanide and 9-AC were from Sigma-Aldrich (Steinheim, Germany), TTX was from Alomone Laboratories (Jerusalem, Israel), methanol was from Sigma Diagnostics (St Louis, MO, USA) and ethanol was from Riedel-de Haën (Sigma-Aldrich, Seelze, Germany).

Intracellular measurement of resting membrane potential

The steady-state membrane potential of soleus muscle fibres was measured during incubations with media containing a range of K_o^+ concentrations (5, 8, 11, 12.5, 14 and 17 mM), at either a low (5 mM) or standard (130 mM) Cl_o^- concentration. All media contained 1 μm TTX to prevent action potentials and contractions. Soleus muscles were dissected and pinned down on the silicone-rubber-lined bottom of a small plastic Petri dish (~3 ml bath volume), containing either the modified Krebs–Ringer solution (5 mM K^+ and 130.2 mM Cl^-) or the low-Cl^- medium (5 mM K^+ and 5.2 mM Cl^-). So, each rat contributed one muscle to the high Cl_o^- group and one to the low Cl_o^- group. The bath temperature was kept at 25 °C using a Peltier device. Muscles were allowed to acclimatize for 45 min. Intracellular recordings were made using a glass capillary microelectrode filled with 3 M KCl (~15 MΩ resistance) connected to standard microelectrode equipment. During each measuring session of about 5 min, different muscle fibres were impaled and the steady-state membrane potential recorded. Thereafter, the muscle was washed at least three times with 3 ml of the medium containing 8 mM K_o^+. This took place during a 5 min equilibration period, which is long enough to allow the superficial fibres to reach a new steady state. Subsequently, a new measurement session was performed. Completing the measurements, from 5 to 17 mM K_o^+, took about 1 h.

Table 2. Hyperkalaemia-induced loss of twitch force

$[K^+]_o$ (mM)	% Twitch force after 45 min high K_o^+	% Twitch force after 90 min high K_o^+
5→10	97.6 ± 4.0	70.6 ± 9.2
5→12	88.7 ± 4.4	34.5 ± 7.5
5→12.5	51.2 ± 3.1	3.1 ± 0.1
5→14	18.5 ± 4.3	0

Twitch force was recorded using supramaximal stimuli (1.5 mA). After 45 min of incubation at a $[K^+]_o$ of 5 mM, twitch force was measured and $[K^+]_o$ was increased to 10, 12, 12.5 or 14 mM. Twitch force was again measured after 45 min and 90 min of hyperkalaemia. Force was expressed as a percentage of the original force measured at 5 mM K_o^+. Each value represents the mean of five muscle preparations (±S.E.M.), resulting in a total of 20 muscle preparations. K_o^+ concentrations were increased by equimolar replacement of $NaHCO_3$ with $KHCO_3$.

Calculations and statistics

Results are presented in the figures and text as means ± S.E.M.; n is the number of measurements from different preparations. Statistical analysis was performed on the twitch-curve results as follows. Each individual twitch curve was characterized by curve fitting and the obtained sigmoidal function was used to calculate the current level at which half the maximal twitch force was developed ($I_{1/2}$). These $I_{1/2}$ values were used to compare groups of measurements with one another by two-tailed Student's paired t test. The $I_{1/2}$ values given in the Results represent these averaged group results.

V_m values were measured at a range of $[K^+]_o$. Each muscle was exposed to the full range of $[K^+]_o$, at either low or high Cl_o^-. At each condition, the V_m was obtained from a number of fibres (7–8). Next, these V_m values were averaged ($n = 1$). These averaged values were obtained from a number of muscles ($n = 4$) and used to run the statistical analysis. The analysis of the data was performed with a linear mixed-effect (LME) model with fixed effects: K_o^+ (covariate), Cl_o^- (factor), and their interaction; and rat as a random effect. This approach was chosen to account for the interdependence of observations within and between the two muscles from the same rat.

Results

Effect of hyperkalaemia on twitch force in rat soleus muscle

The first experiment served to establish the relationship between the concentration of K_o^+ and muscle twitch force, at two time points (Table 2). After 45 min at 12.5 mM K_o^+, the muscle preparations had lost around 50% of their twitch force. Steady state had not yet been obtained, as indicated by the total loss of force after 90 min of 12.5 mM K_o^+. The loss of force at 10, 12, 12.5 or 14 mM K_o^+ was completely reversed by reintroducing 5 mM K_o^+ (not shown). Based on these results, we decided to investigate the effect of a reduction in $[Cl^-]_o$ on hyperkalaemic force development after 45 min exposure to 12.5 mM K_o^+. These conditions should allow us to ascertain a dynamic effect of Cl_o^- concentration on force development in either direction.

Effect of low $[Cl^-]_o$ on hyperkalaemia-induced loss of twitch force

Figure 1*A* shows the influence of lowering $[Cl^-]_o$ on twitch-force development during hyperkalaemia. For 55 min twitch force was recorded in modified Krebs–Ringer solution to demonstrate the stability of the preparation. During this period the twitch curve was reconstructed twice, once at the beginning of the experiment at $t = 0$ min and once at $t = 50$ min. As shown in Fig. 1*B* the excitability of the muscles remained stable during this period. The $I_{1/2}$ values from the control situation at $t = 0$ and $t = 50$ min were both 0.38 ± 0.07 mA ($n = 6$). Next, the $[K^+]_o$ was increased to 12.5 mM. After an initial increase, twitch force started to decline. At $t = 160$ min, force was zero and no sign of recovery was present during the final 40 min of the experiment.

This picture changed noticeably when $[Cl^-]_o$ was reduced from 130.2 to 5.2 mM at $t = 105$ min. The ongoing rundown of twitch force was accelerated, leading to a total loss of force in just 5 min. However, hereafter the soleus muscle started to recover gradually, almost reaching control values at $t = 200$ min. At the end of this experiment, twitch force was stable enough to record another twitch curve. As can be seen from Fig. 1*B*, this twitch curve had shifted to the left. The $I_{1/2}$ value from the high-K_o^+/low-Cl_o^- situation was 0.27 ± 0.05 mA ($n = 6$), and differed significantly from the control value of 0.38 ± 0.07 mA ($P < 0.05$). This shift indicates increased membrane excitability with low Cl_o^- levels.

In order to verify that low $[Cl^-]_o$ was responsible for the recovery of twitch force during hyperkalaemia, and not the presence of its substitute isethionate, we performed an additional experiment ($n = 2$) with methanesulphonate as the major anion. The result was identical, i.e. a rapid loss of twitch force followed by complete recovery (not shown).

In an additional set of experiments ($n = 4$) the $[K^+]_o$ was increased to 14 mM. Again, loss of force was accelerated after reducing $[Cl^-]_o$. However, at this slightly higher $[K^+]_o$, no sign of recovery was seen (not shown).

If reduction of $[Cl^-]_o$ can antagonize hyperkalaemia-induced loss of twitch force, then reducing $[Cl^-]_o$ before introducing the hyperkalaemia should prevent loss of force. To test this hypothesis we also performed the high K_o^+/low Cl_o^- experiment in the reverse order, as shown in Fig. 2*A*.

Five minutes after the start of the experiment, $[Cl^-]_o$ was reduced to 5.2 mM by replacing it with isethionate. After a temporary increase, twitch force stabilized at the same level as before the reduction of $[Cl^-]_o$. At $t = 50$ min, twitch force appears to be going down. This was due to the fact that 6 out of the 11 preparations started to show an occasional myotonic contraction after stimulation, i.e. twitch prolongation directly followed by a nonevoked tetanic contraction lasting about 1 min. Consequently, the next evoked twitch response was smaller.

Introducing the high-K_o^+/low-Cl_o^- solution, at $t = 55$ min, resulted in variable twitch force for about 5 min, which explains the increase in S.E.M. During the next 45 min, twitch force remained stable, in contrast to the 50% rundown of twitch force seen with high K_o^+ only (cf. Table 2 and Fig. 1). Next, the modified Krebs–Ringer solution was reintroduced and twitch force stabilized at exactly the same level as 2 h previously.

Replacement of Cl_o^- with isethionate not only prevented hyperkalaemia-induced loss of twitch force, but also induced a leftward shift of the twitch curve (Fig. 2*B*). The calculated $I_{½}$ value from the low-Cl_o^- twitch curve (●) is 0.26 ± 0.03 mA ($n = 11$), which lies 0.08 mA below the calculated $I_{½}$ value from the control twitch curve (○), being 0.34 ± 0.04 mA. This shift was highly significant ($P < 0.0001$) and can be regarded as a 24% increase in membrane excitability. Adding extra K^+ to the low Cl^- medium (▼) did not cause an additional shift of the twitch curve ($I_{½} = 0.24 \pm 0.03$ mA). The leftward shift

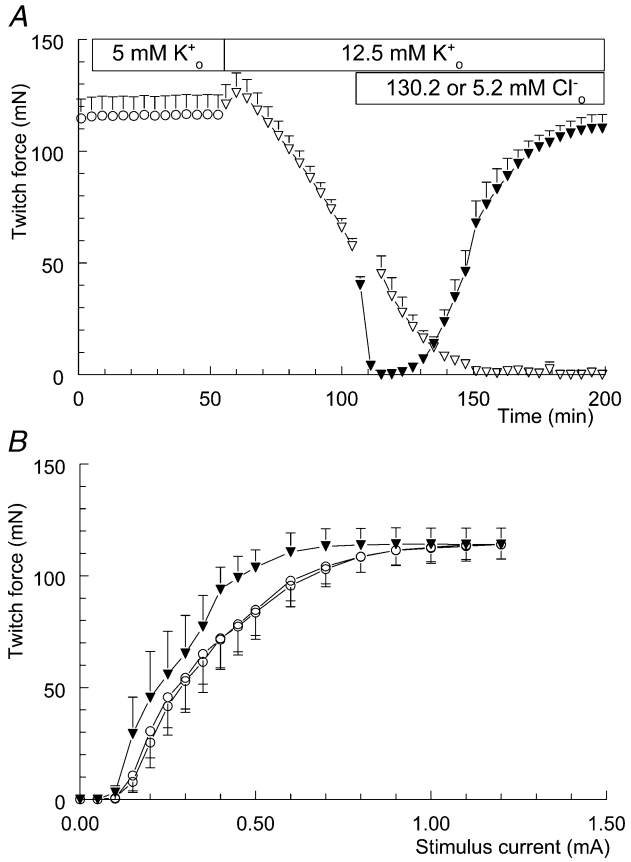

Figure 1. Effect of lowering $[Cl^-]_o$ on twitch-force development during hyperkalaemia
A, twitch force recorded from soleus muscle is shown once every 4 min, although measurements were actually made once every minute. After 55 min, the modified KR solution (○; $n = 10$, +S.E.M.) was replaced by the high-K_o^+ solution (▽; $n = 10$). This 12.5 mM K_o^+ solution was maintained in two preparations (▽; $n = 2$, +S.D.), while the other eight preparations were introduced to the high-K_o^+/low-Cl_o^- solution at $t = 105$ min (▼; $n = 8$, +S.E.M.). *B*, isometric twitch-force–stimulation-strength relationships. These three twitch curves were recorded during the experiment depicted in *A*. Twice during the presence of the control solution, at time points $t = 0$ and $t = 50$ min (○; $n = 6$, −S.E.M.), and once during the presence of the high-K_o^+/low-Cl_o^- solution at $t = 200$ min (▼; $n = 6$, +S.E.M.).

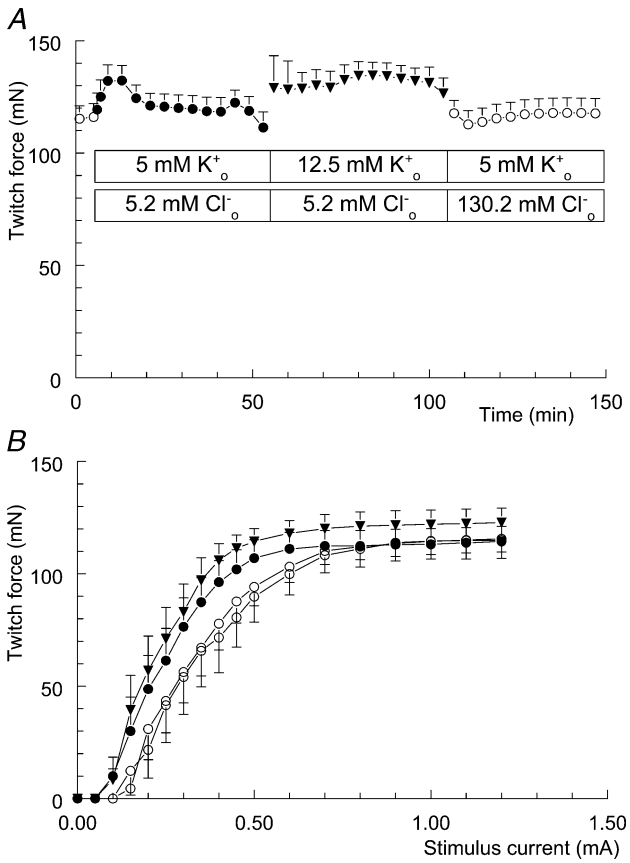

Figure 2. Lowering $[Cl^-]_o$ prevents hyperkalaemia-induced loss of twitch force and affects muscle excitability
A, twitch force recorded from soleus muscle is shown once every 4 min. After 5 min, the modified Krebs–Ringer solution (○; $n = 11$, +S.E.M.) was replaced by the low-Cl_o^- solution (●); at $t = 55$ min the low-Cl_o^- solution was replaced by the high-K_o^+/low-Cl_o^- solution (▼); the modified Krebs–Ringer solution was reintroduced at $t = 105$ min (○). *B*, isometric twitch-force–stimulation-strength relationships. These four twitch curves were recorded during the experiment depicted in *A*. Twice during the presence of the control solution, at time points $t = 0$ (○) and $t = 150$ min (○), once during the presence of the low-Cl_o^- solution at $t = 50$ min (●), and once during the presence of the high-K_o^+/low-Cl_o^- solution at $t = 100$ min (▼).

in the twitch curve, due to reducing $[Cl^-]_o$ to 5.2 mM, was completely reversed after returning to 130 mM Cl_o^- ($I_{½} = 0.35 \pm 0.05$ mA).

Effect of low $[Cl^-]_o$ on hyperkalaemia-induced membrane depolarization

The data presented in the previous sections show that a reduction of $[Cl^-]_o$ modifies the response of rat soleus muscle to hyperkalaemia. Since hyperkalaemia-induced loss of force is due to membrane depolarization (Cairns *et al.* 1997), it is reasonable to assume that lowering $[Cl^-]_o$ causes membrane hyperpolarization. We measured V_m to substantiate our idea that the interaction between the effects of low $[Cl^-]_o$ and high $[K^+]_o$ on twitch force occurs at the level of V_m.

Figure 3 shows the depolarizing response of V_m to hyperkalaemia under two different conditions, 130.2 and 5.2 mM Cl_o^-. The LME model (see Calculations and statistics) showed that there was a significant interaction ($P < 0.001$) between the effects of $[Cl^-]_o$ and $[K^+]_o$ on V_m, i.e. the effect of a change in $[K^+]_o$ on V_m is dependent upon $[Cl^-]_o$, and vice versa. The regression lines at 130 and 5 mM Cl_o^- are $V_m = -85.1 + 27.3 \log[K^+]_o$ and $V_m = -111.2 + 48.8 \log[K^+]_o$, respectively. Therefore, lowering $[Cl^-]_o$ from 130 to 5 mM increased the sensitivity of V_m to changes in $[K^+]_o$ from 27.3 mV per decade $[K^+]_o$ to 48.8 mV per decade $[K^+]_o$. The estimated difference in V_m between high and low Cl^-, at 12.5 mM K_o^+, is 2.5 mV, with V_m being more negative at low Cl_o^-.

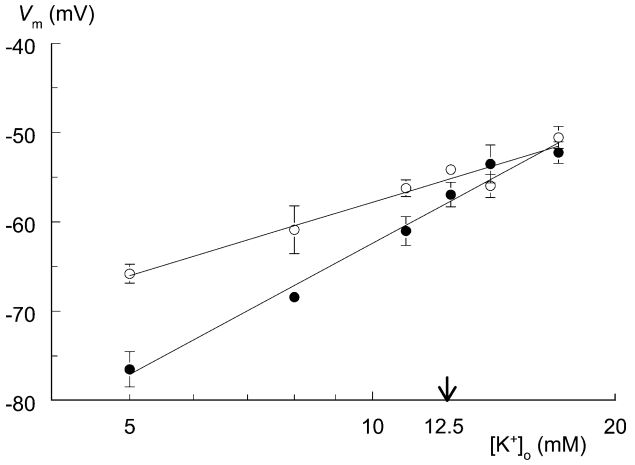

Figure 3. Lowering $[Cl^-]_o$ affects the response of membrane potential (V_m) to changes in $[K^+]_o$
Resting membrane potential was recorded from rat soleus muscle for a range of K_o^+ concentrations (5, 8, 11, 12.5, 14 and 17 mM). The solutions contained either 130.2 mM Cl_o^- (○, control) or 5.2 mM Cl_o^- (●). Extracellular Cl^- was replaced by equimolar amounts of isethionate. Muscles incubated at either 5 or 130 mM Cl^- were subjected to the full range of K_o^+ concentrations, starting at 5 mM. Each point is the mean V_m value of four muscles under steady-state conditions. Error bars represent S.E.M. There is a significant interaction between the effects of $[Cl^-]_o$ and $[K^+]_o$ on V_m ($P < 0.001$, linear mixed-effect model); the regression lines at 130 and 5 mM Cl_o^- are $V_m = -85.1 + 27.3 \log [K^+]_o$ and $V_m = -111.2 + 48.8 \log[K^+]_o$, respectively. These lines intersect at a $[K^+]_o$ of 16.4 mM.

Effect of 9-AC on hyperkalaemia-induced loss of twitch force

The results presented above show the influence of Cl^- on hyperkalaemia-induced membrane depolarization and loss of twitch force. In these experiments we lowered $[Cl^-]_o$ to a level comparable with the low intracellular concentration. This should have diminished the influence of Cl^- on V_m. Another way to reduce the influence of Cl^- on V_m is by blocking specific Cl^- conductance. We used 9-AC to block the ClC-1 Cl^- channel.

Figure 4 shows the absence of an effect of 100 μM 9-AC on force decline during hyperkalaemia. Introducing the high-K_o^+ solution at $t = 55$ min resulted in a gradual loss of twitch force. Addition of 9-AC to the high-K_o^+ solution at $t = 105$ min had no effect on this ongoing loss of force, as stated above. The time course was similar to the hyperkalaemia-induced loss of twitch force shown in Fig. 1*A*.

Reversing the order of changing the solutions did reveal an effect of 9-AC on hyperkalaemia-induced loss of twitch force (Fig. 5*A*). In this series of experiments 9-AC was added to half of the preparations at $t = 5$ min (filled symbols), while the other half received the solvent ethanol (0.5% v/v). Introducing the high-K_o^+ solution at $t = 55$ min revealed a difference between the preparations with and without 100 μM 9-AC. The loss

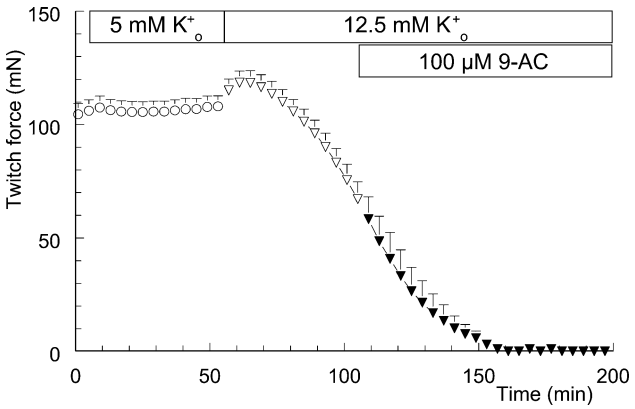

Figure 4. No influence of 9-anthracene carboxylic acid (9-AC) on hyperkalaemia-induced loss of twitch force
Twitch force recorded from soleus muscle is shown once every 4 min. At $t = 5$ min, the modified Krebs–Ringer (KR) solution (○; $n = 6$, +S.E.M.) was replaced by modified KR containing 0.5% v/v ethanol (○). At $t = 55$ min this solution was replaced by the high-K_o^+ solution, again containing 0.5% v/v ethanol (▽). This latter fluid was replaced by the high-K_o^+ solution containing 100 μM 9-AC and 0.5% v/v ethanol at $t = 105$ min (▼).

of force due to 50 min of hyperkalaemia was reduced in the presence of 100 μM 9-AC. Changing the solutions back to modified Krebs–Ringer solution, including 0.5% v/v ethanol, quickly restored full muscle force. This experiment was repeated with a concentration of 200 μM 9-AC (Fig. 5*B*). To this end DMSO was used to dissolve 9-AC. The experimental solutions contained 0.05% v/v DMSO. Figure 5*B* shows that 9-AC has reversed the hyperkalaemia-induced loss of twitch force into a situation with increased twitch force. At $t = 100$ min the difference in force has grown to 100 mN. Changing the solutions back to modified Krebs–Ringer solution, including 0.05% v/v DMSO, quickly reduced the difference in force between the two groups.

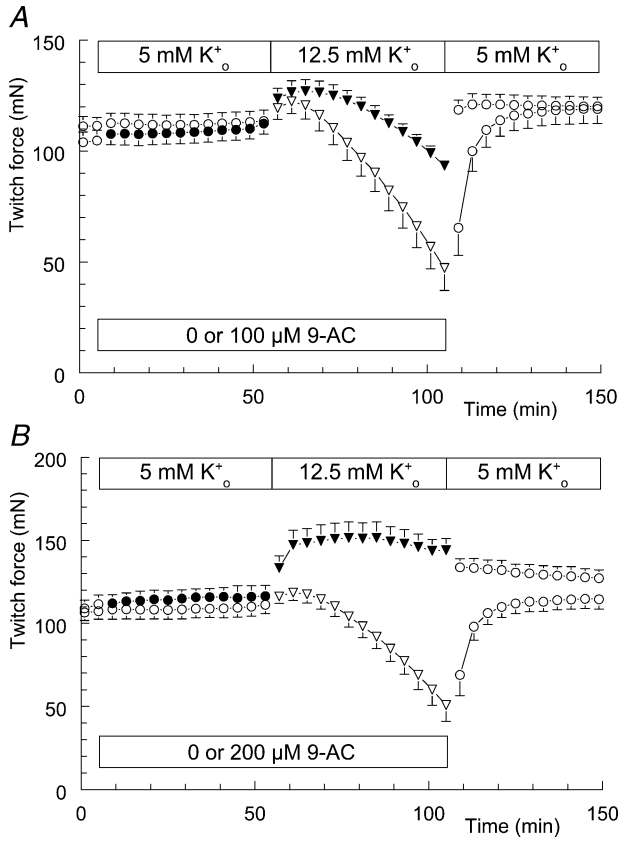

Figure 5. Effect of pre-incubating soleus muscle with 9-AC on hyperkalaemia-induced loss of twitch force
The effect of hyperkalaemia on twitch force in the absence (open symbols) and in the presence of 100 or 200 μM 9-AC (filled symbols, *A* and *B*, respectively). Four animals were used in the experiment depicted in *A*, and each animal contributed to both groups (with and without 9-AC). Muscles from the right and left legs were evenly distributed between the two groups. The same holds for the experiment depicted in *B*. The 9-AC groups were exposed to 100 or 200 μM 9-AC at $t = 5$ min (●; $n = 4$, ±S.E.M.), while the control groups received only the solvent (○; $n = 4$, ±S.E.M.). In the experiment with 100 μM 9-AC, the solutions contained 0.5% v/v ethanol. In the experiment with 200 μM 9-AC, the solutions contained 0.05% v/v DMSO. In addition, all groups were exposed to 12.5 mM K_o^+ at $t = 55$ min (▼,▽). After the measurement of $t = 105$ min fluids were replaced by modified Krebs–Ringer solution, still containing the solvent (○).

Effect of bumetanide on hyperkalaemia-induced loss of twitch force

The results presented above show that a hyperkalaemia-induced loss of twitch force can be prevented or antagonized by a reduction of either $[Cl^-]_o$ or specific Cl^- conductance. It has been reported that both these manipulations can cause hyperpolarization, and that this hyperpolarization depends on intracellular accumulation of Cl^- via the bumetanide-sensitive NKCC1 (Betz *et al.* 1984; Harris & Betz, 1987; Aickin *et al.* 1989; van Mil *et al.* 1997). In order to see whether we could trace back our results to this transporter-mediated influx of Cl^-, the experiments were repeated in the presence of bumetanide.

No effect of bumetanide on hyperkalaemia-induced loss of twitch force was found, either at a concentration of 100 μM or at a concentration of 400 μM. The result of one series of experiments is shown in Fig. 6*A*. During these experiments half of the preparations received 400 μM bumetanide before $[K^+]_o$ was raised to 12.5 mM (filled symbols). Irrespective of the presence of bumetanide, both groups responded to the hyperkalaemia with a 40% drop in twitch force. Twitch force was completely restored after changing the solutions back to modified Krebs–Ringer solution. In addition, 400 μM bumetanide had no effect on the left–right position of the twitch curves (Fig. 6*B*). These three twitch curves belong to the bumetanide group and were recorded in the absence ($t = 0$ min), presence ($t = 50$ min) and again in the absence ($t = 150$ min) of bumetanide. At the end of these experiments twitch force had increased by about 10 mN, most likely because of the presence of 0.4% v/v methanol.

Discussion

This study shows that reducing Cl^- conductance, either by lowering $[Cl^-]_o$ or blocking specific Cl^- conductance with 9-AC, can have two distinct effects on hyperkalaemia-induced loss of twitch force. On one hand it was found that lowering $[Cl^-]_o$ during hyperkalaemia accelerates ongoing rundown of twitch force. Unexpectedly, 9-AC did not produce this effect, probably because it diffuses too slowly into the core of the muscle to affect the ongoing rundown of twitch force. The rapid decline of force after lowering $[Cl^-]_o$ most likely reflects both the loss of a stabilizing influence of Cl^- on V_m and a temporary change in equilibrium potential of Cl^- (Hodgkin & Horowicz, 1959; Dulhunty, 1978). The increased membrane excitability with low $[Cl^-]_o$ clearly shows the stabilizing influence of Cl^- on

V_m. As such, this result is consistent with the general notion that the negative E_{Cl}, in combination with a large specific membrane conductance, stabilizes V_m during depolarization (Hodgkin & Horowicz, 1959; Dulhunty, 1978, 1979; McCaig & Leader, 1984; Wallinga *et al.* 1999). On the other hand we found that blocking Cl^- channels or lowering $[Cl^-]_o$ could completely prevent hyperkalaemia-induced loss of force. The range of $[K^+]_o$ associated with loss of force was shifted to higher values after lowering $[Cl^-]_o$. This was due to a low-Cl_o^--induced hyperpolarization of the membrane. This result is in line with studies showing that E_{Cl} is less negative than V_m (Dulhunty, 1978; Aickin *et al.* 1989). Since the NKCC1 transporter is held responsible for the difference between E_{Cl} and V_m, we expected that blocking this transporter would also prevent hyperkalaemia-induced loss of force. This was not the case. The most significant conclusion to be drawn from this study is that the normal high Cl^- conductance of rat soleus muscle can add to the loss of force during hyperkalaemia.

Methodology and limitations

We studied twitch contractions instead of tetanic contractions because it has been demonstrated that hyperkalaemia-induced fatigue can be overcome by high-frequency stimulation, probably by activation of the electrogenic Na^+,K^+-ATPase (Overgaard & Nielsen, 2001). Another aspect of high-frequency stimulation is that the amount of K^+ released from the muscle fibres can cause a significant rise in $[K^+]_o$ (Juel, 1986). To prevent these confounding influences on our hyperkalaemia experiments we used single twitches.

Hyperkalaemia-induced loss of twitch force in the near absence of extracellular Cl^-

In order to investigate the influence of Cl^- on the loss of twitch force during hyperkalaemia, $[Cl^-]_o$ was reduced to 5 mM. This value was chosen because it lies at the bottom of the range of values reported for intracellular Cl^- activity and, as such, is nearly identical to the intracellular Cl^- activity associated with passive Cl^- distribution (Donaldson & Leader, 1984; McCaig & Leader, 1984; Aickin *et al.* 1989; Cairns *et al.* 1997). As a consequence, Cl^- should rapidly lose its influence on V_m. Lowering $[Cl^-]_o$ from 130 to 5 mM accelerated the ongoing loss of twitch force around 10 times (Fig. 1*A*). Our result is consistent with results from mouse EDL and soleus muscle (Cairns *et al.* 2004). It was shown that low $[Cl^-]_o$ mainly increased the rate of fatigue with tetanic stimulation. Whether an increase in $[K^+]_o$ caused the tetanus depression was not examined.

The rapid loss of force shown in Fig. 1 indicates that normal $[Cl^-]_o$ exerts a stabilizing influence on V_m during hyperkalaemia. It has been shown that membrane depolarization during hyperkalaemia is sped up in the absence of extracellular Cl^-, most likely due to the loss of an influx of Cl_o^- (see also Introduction) (Hodgkin & Horowicz, 1959; Dulhunty, 1978, 1979; McCaig & Leader, 1984). Dulhunty (1978) showed that the depolarization proceeded around 10 times faster in the absence of

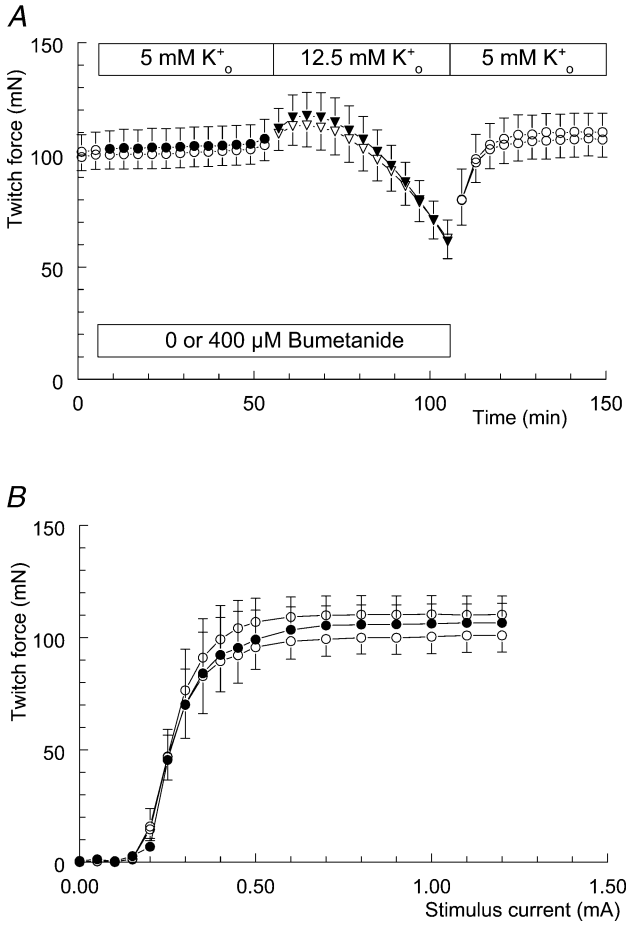

Figure 6. No effect of pre-incubating soleus muscle with bumetanide on hyperkalaemia-induced loss of twitch force
A, the effect of hyperkalaemia on twitch force in the absence (open symbols) and in the presence of 400 μM bumetanide (filled symbols). The bumetanide group was exposed to 400 μM bumetanide at $t = 5$ min (●; $n = 4$, +S.E.M.), while the control group only received the solvent methanol (0.4% v/v) (○; $n = 4$, −S.E.M.). In addition, both groups were exposed to 12.5 mM K_o^+ at $t = 55$ min (▼,▽). After the measurement of $t = 105$ min, fluids were replaced by modified Krebs–Ringer solution, still containing 0.4% v/v methanol (○). *B*, isometric twitch-force–stimulation-strength relationships from the bumetanide group. These three twitch curves were recorded during the experiment depicted in *A* at time points $t = 0$ (○, lower curve; modified Krebs–Ringer solution), $t = 50$ (●; modified Krebs–Ringer solution containing 400 μM bumetanide and 0.4% v/v methanol) and $t = 150$ min (○, upper curve; modified Krebs–Ringer solution containing 0.4% v/v methanol).

Cl^- conductance. So, it appears that the unusual rapid loss of force during hyperkalaemia reflects the changes occurring in V_m without the stabilizing influence of Cl^- conductance.

However, lowering $[Cl^-]_o$ is impossible without inducing a concomitant change in the E_{Cl}. It has been shown that the V_m of muscle fibres is transiently depolarized when $[Cl^-]_o$ is reduced and Cl^- loses its influence on V_m (Hodgkin & Horowicz, 1959; Dulhunty, 1978; Aickin *et al.* 1989). Aickin *et al.* (1989) reported 20 mV depolarizations, lasting about 5 min, when $[Cl^-]_o$ was reduced to zero. Certainly, the fibres will experience a change in E_{Cl} when we quickly reduce $[Cl^-]_o$ from 130 to 5 mM. E_{Cl} will temporarily become less negative due to the reduction in the chemical gradient. This change in E_{Cl} will depolarize the muscle fibre and add to the rapidly developing hyperkalaemia-induced depolarization. So, the rapid decline of twitch force during high $[K^+]_o$ and low $[Cl^-]_o$ most likely reflects both the loss of a stabilizing influence of Cl^- on V_m and the temporary change in E_{Cl}.

The rapid loss of twitch force was followed by a remarkable recovery, despite the high concentration of 12.5 mM K_o^+ (Fig. 1). The experiment shown in Fig. 2 also shows that low $[Cl^-]_o$ protects muscle twitch force against hyperkalaemia. This is most likely to be due to the depolarizing influence of Cl^- on V_m, under normal resting conditions. This became clear when we recorded V_m. The regression lines in Fig. 3 show that low $[Cl^-]_o$ produced steady-state hyperpolarizations at $[K^+]_o$ below 16.4 mM (the intersect of the regression lines). As such, our results are in line with studies showing that E_{Cl} is less negative than V_m and, thereby, exerts a depolarizing influence on V_m under normal resting conditions (Dulhunty, 1978; Aickin *et al.* 1989). Lowering $[Cl^-]_o$ at 5 mM K_o^+ produced a hyperpolarizing response of 11.1 mV. This falls within the range of values reported in literature. Hyperpolarizations ranging from a few to 20 mV have been measured after reducing $[Cl^-]_o$ or blocking Cl^- channels (Dulhunty, 1978; Aickin *et al.* 1989; van Mil *et al.* 1997). However, our V_m data seem in conflict with data presented by Cairns *et al.* (2004) who showed no change in the resting V_m of mouse soleus muscle following equilibration at 10 mM $[Cl^-]_o$. Perhaps the absence of an effect in their study was due to the less pronounced reduction in $[Cl^-]_o$ at 25°C.

Although the estimated difference in V_m between high and low Cl_o^-, at 12.5 mM K_o^+, is only 2.5 mV, we still consider it feasible that this small change in V_m is responsible for the recovery of force. Cairns *et al.* (1995, 1997) showed that the dependence of twitch force on V_m is very steep. While depolarizations up to around −60 mV induce twitch potentiation, all force is lost when fibres are depolarized above −55 mV. Thus, a substantial recovery of twitch force is possible with a hyperpolarization of just a few millivolts. In addition, the small hyperpolarization occurs within the narrow range of V_m values associated with loss of twitch force (Fig. 3).

Figure 2*A* shows a 15% increase in peak twitch force when the soleus muscle is exposed to a combination of low Cl_o^- and high K_o^+ (middle segment). Therefore, lowering $[Cl^-]_o$ has reversed the hyperkalaemia-induced loss of twitch force into a situation with increased twitch force (cf. Figs 1 and 2). Twitch potentiation is known to occur at values of V_m, which are slightly more negative than the values needed to depress twitch force (Cairns *et al.* 1997; Yensen *et al.* 2002). Therefore, this result is in line with our data on V_m.

Since no sign of force recovery was seen at 14 mM K_o^+, we conclude that low $[Cl^-]_o$ shifts the range of $[K^+]_o$ associated with loss of twitch force to higher values.

Finally, we point out that the interactive effect of $[Cl^-]_o$ and $[K^+]_o$ on V_m, as shown in Fig. 3, has been previously reported by Geukes Foppen *et al.* (2002). Those authors showed that decreasing the accumulation of Cl^- in lumbrical muscle of mice, by decreasing medium osmolality, decreases $\Delta V_m/\Delta[K^+]_o$ from 50 to 31 mV per decade between 5.7 and 22.8 mM K_o^+. Concomitantly, the membrane was hyperpolarized by 18 mV at 5.7 mM K_o^+. Consequently, the relationships between V_m and $[K^+]_o$ appear to intersect at an estimated value of 20 mM K_o^+.

Hyperkalaemia-induced loss of twitch force in the presence of 9-AC

Next, we wanted to test the effect of 9-AC on hyperkalaemia-induced loss of twitch force. Since 9-AC is a potent blocker of Cl^- channels (Palade & Barchi, 1977), it should eliminate the influence of Cl^- on V_m. The results obtained using 9-AC are in line with those we obtained with low $[Cl^-]_o$.

The ongoing loss of twitch force was unaffected when 9-AC was applied 45 min after the onset of hyperkalaemia (Fig. 4). However, when 9-AC was already in the bath for 45 min when hyperkalaemia was induced, we did find an effect of 9-AC on the development of hyperkalaemia-induced loss of twitch force (Fig. 5). This indicates that 9-AC diffuses slowly into the core of the soleus muscle.

At a concentration of 100 μM, 9-AC caused a delay in the development of hyperkalaemia-induced loss of twitch force, while 200 μM 9-AC completely prevented the loss of force. This latter result is identical to the effect of low $[Cl^-]_o$ on hyperkalaemia-induced loss of twitch force (cf. Figs 2 and 5). Moreover, the combination of 200 μM 9-AC and high $[K^+]_o$ produced twitch potentiation, similar to the effect of low $[Cl^-]_o$ and high $[K^+]_o$ on twitch force (cf. Figs 2 and 5).

The concentration of 200 μM 9-AC is twice as high as the concentration that is commonly used (Fahlke & Rudel, 1995; Coonan & Lamb, 1998). The need for this

high concentration could be related to the presence of two different Cl^- channels in mammalian muscle fibres (Gurnett *et al.* 1995). ClC-1 is blocked by 9-AC from the extracellular side (Steinmeyer *et al.* 1991), but the T-tubular Cl^- channel is probably blocked by 9-AC from the myoplasmic side (Ide *et al.* 1995; Coonan & Lamb, 1998). The fact that several authors have reported a partial effect of 9-AC on V_m might be due to the use of 9-AC at concentrations of 75–100 μM (Aickin *et al.* 1989; Geukes Foppen *et al.* 2002).

The protective effect of 9-AC on twitch force during high $[K^+]_o$ is most likely to be due to membrane hyperpolarization because: (1) low $[Cl^-]_o$, which also eliminates the influence of Cl^- on V_m, produced membrane hyperpolarization and protected twitch force against hyperkalaemia; (2) 9-AC is known to produce membrane hyperpolarization in rat and mouse lumbrical muscle (Betz *et al.* 1984; Aickin *et al.* 1989; Geukes Foppen *et al.* 2002); (3) the combination of 9-AC and high $[K^+]_o$ produced substantial twitch potentiation. Twitch potentiation is known to occur at values of V_m that are slightly more negative than the values needed to depress twitch force (Cairns *et al.* 1997; Yensen *et al.* 2002).

Hyperkalaemia-induced loss of twitch force in the presence of bumetanide

Thus far, we showed that our results give evidence of a depolarizing influence of Cl^- on V_m. To exert such an influence the $[Cl^-]_i$ needs to be above that predicted by a passive Donnan type of equilibrium. The transporter held responsible for the uphill transport of Cl^- into the muscle fibre is the NKCC1. This conclusion comes from experiments with rat lumbrical muscle, in which it was shown that the internal Cl^- activity can be reduced by addition of furosemide or lowering either the $[Na^+]_o$ or the $[K^+]_o$ (Harris & Betz, 1987; Aickin *et al.* 1989) even at room temperature. Blocking the Cl^-/HCO_3^- antiporter was of no consequence to the accumulation of Cl^-. Moreover, both the reduction of Cl^- conductance and the presence of loop diuretics can lead to membrane hyperpolarization in lumbrical muscle of rat or mouse (Betz *et al.* 1984; Aickin *et al.* 1989; van Mil *et al.* 1997; Geukes Foppen *et al.* 2002). Therefore, we investigated whether the effect of lowering Cl^- conductance on hyperkalaemia-induced loss of force could be mimicked by blocking the NKCC1.

We found that this was not the case. Addition of bumetanide at a concentration of 100 or 400 μM had no effect on either the developmental course or the extent of the hyperkalaemia-induced loss of twitch force (Fig. 6). This result is not easily explained, but several possibilities will be discussed.

Whether or not there is a bumetanide-sensitive Cl^- influx in rat soleus muscle has not yet been verified by radioisotope measurements, but a few studies indicate that it is present. These studies demonstrated the presence of either a Cl^--dependent, Na^+-dependent, bumetanide-sensitive $^{86}Rb^+$ influx (Wong *et al.* 1999) or a Cl^--dependent, K^+-independent, bumetanide-sensitive $^{22}Na^+$ influx (Dørup & Clausen, 1996). In order to obtain complete inhibition of the NKCC1, it was necessary to use concentrations of at least 100 μM (Dørup & Clausen, 1996).

Thus, the absence of an effect of bumetanide on hyperkalaemia-induced loss of twitch force in rat soleus muscle indicates that the NKCC1 is not involved in setting V_m through accumulation of Cl^-. But, blocking the NKCC1 is likely to disturb the influx of Na^+ and K^+. Since the NKCC1 is held responsible for 15% of the influx of K^+ at rest, at either 30 or 37 °C (Wong *et al.* 1999, 2001; Lindinger *et al.* 2001, 2002), there might be a small increase in $[K^+]_o/[K^+]_i$, leading to a depolarization of a few millivolts. Likewise, a bumetanide-related change in $[Na^+]_i$ might depolarize V_m and, thereby, oppose a bumetanide-induced Cl^--related hyperpolarization. More specifically, since the activity of the electrogenic Na^+,K^+-ATPase is dependent upon $[Na^+]_i$, although in a complex way, a reduction in total Na^+ influx is likely to reduce pump activity (Nielsen & Clausen, 1997; Sejersted & Sjøgaard, 2000; Buchanan *et al.* 2002). A reduced activity of the Na^+,K^+-ATPase causes membrane depolarization, since blocking the Na^+,K^+-ATPase with ouabain in rat soleus muscle causes 8 mV depolarization, at 30 °C (Clausen & Flatman, 1977).

In conclusion, the present study suggests that the $[Cl^-]_i$ in rat soleus muscle is above that predicted by a passive Donnan type of equilibrium and, as a consequence, exerts a depolarizing influence on V_m. The accumulation of Cl^- is most likely to be related to the regulation of the volume of the muscle fibres (Chinet, 1993; van Mil *et al.* 1997; Russell, 2000; Lindinger *et al.* 2002; Gosmanov *et al.* 2003). A side effect of the accumulation of Cl^- is that muscle fibres can become more prone to losing their excitability during periods of hyperkalaemia. It was shown that the range of $[K^+]_o$ associated with loss of twitch force is shifted to higher values during periods of reduced Cl^- conductance.

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