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Role of Na^+,K^+ pumps in restoring contractility following loss of cell membrane integrity in rat skeletal muscle

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Abstract

Background and aim: In skeletal muscles, electrical shocks may elicit acute loss of force, possibly related to increased plasma membrane permeability, induced by electroporation (EP). We explore the role of the Na^+,K^+ pumps in force recovery after EP.

Methods: Isolated rat soleus or extensor digitorum longus (EDL) muscles were exposed to EP paradigms in the range 100–800 V cm^{-1}, and changes in tetanic force, Na^+,K^+ contents, membrane potential, ^{14}C-sucrose space and the release of the intracellular enzyme lactic acid dehydrogenase (LDH) were characterized. The effects of Na^+,K^+ pump stimulation or inhibition were followed.

Results: Electroporation caused voltage-dependent loss of force, followed by varying rates and degrees of recovery. EP induced a reversible loss of K^+ and gain of Na^+, which was not suppressed by tetrodotoxin, but associated with increased ^{14}C-sucrose space and release of LDH. In soleus, EP at 500 V cm^{-1} induced complete loss of force, followed by a spontaneous, partial recovery. Stimulation of active Na^+,K^+ transport by adrenaline, the β_2-agonist salbutamol, calcitonin gene-related peptide (CGRP) and dibutyryl cyclic AMP increased initial rate of force recovery by 183–433% and steady-state force level by 104–143%. These effects were blocked by ouabain (10^{-3} M), which also completely suppressed spontaneous force recovery. EP caused rapid and marked depolarization, followed by a repolarization, which was accelerated by salbutamol. Also in EDL, EP caused complete loss of force, followed by a spontaneous partial recovery, which was markedly stimulated by salbutamol.

Conclusion: Electroporation induces reversible depolarization, partial rundown of Na^+,K^+ gradients, cell membrane leakage and loss of force. This may explain the paralysis elicited by electrical shocks. Na^+,K^+ pump stimulation promotes restoration of contractility, possibly via its electrogenic action. The major new information is that the Na^+,K^+ pumps are sufficient to compensate a simple mechanical leakage. This may be important for force recovery in leaky muscle fibres.

Keywords adrenaline, calcitonin gene-related peptide, cyclic AMP, depolarization, electrical shocks, electroporation, lactate dehydrogenase, salbutamol.

In skeletal muscle, the primary function of the Na^+,K^+ pumps is to counterbalance the passive leaks of Na^+ and K^+, thereby allowing the maintenance of transmembrane Na^+,K^+ gradients, excitability and contractility. During electrical stimulation, the excitation-induced influx of Na^+ and efflux of K^+ mediated by

specific channels were found to exceed the capacity of the Na^+,K^+ pumps for compensating the Na^+,K^+ leaks, leading to a net gain of Na^+, a net loss of K^+ and depolarization, both *in vivo* (Sréter 1963, Everts *et al.* 1993) and *in vitro* (for reviews, see Sejersted & Sjøgaard 2000, Clausen 2003). This is associated with a loss of excitability and contractile force (Overgaard *et al.* 1999). Rapid increases in passive unspecific Na^+,K^+ fluxes may also be induced by electroporation (EP), and we have recently demonstrated that EP of isolated rat skeletal muscle leads to a rapid partial loss of cell membrane integrity, accompanied by total loss of contractile force. This was followed by a spontaneous gradual recovery of force (Gissel & Clausen 2003) and activation of the Na^+,K^+ pumps (Buchanan *et al.* 2002). Also in the intact organism, electrical shocks elicit rapid loss of muscle cell membrane integrity (Gehl *et al.* 1999, Lee *et al.* 2000), and electron micrographs have suggested structural defects in the plasma membrane (Bhatt *et al.* 1990). Even more frequently, muscle cells lose integrity by exposure to bruises, mechanical strain or rhabdomyolysis. It is of general interest, therefore, to explore the possible role of the Na^+,K^+ pumps in restoring contractility after loss of plasma membrane integrity, exemplified by EP. This was done using agents known to stimulate (hormones or their second messenger cAMP) or to inhibit (ouabain) the Na^+,K^+ pump and we are testing the following hypotheses:

1. Leaks in sarcolemma induced by EP leads to a reversible loss of contractility, possibly reflecting rundown of Na^+,K^+ gradients, depolarization and ensuing loss of excitability.
2. Acute stimulation of the electrogenic Na^+,K^+ pump favours the repolarization of the plasma membrane allowing restoration of excitability and contractility. Part of the present observations have been presented in a preliminary version (Clausen & Gissel 2004).

Methods

Animals and muscle preparation

All experiments were performed using fed female or male Wistar rats (own breed) weighing 60–70 g (4 weeks old). All handling and use of animals complied with Danish animal welfare regulations. The animals had free access to fodder (Altromin pellets, Nr. 1314; Spezialfutter-Werke, Lage, Germany) and water and were kept in a thermostated environment (21 °C) with constant day length (12 h). Animals of this age were chosen to obtain muscles of sufficiently small size (20–25 mg wet wt.) to improve oxygenation and diffusion of substrate during incubation.

The animals were killed by cervical dislocation, then decapitated, and the intact soleus or extensor digitorum longus (EDL) muscles with tendons were dissected out as previously described (Nielsen & Clausen 1997). All incubations took place at 30 °C, and if not otherwise stated, in standard Krebs–Ringer bicarbonate buffer containing (in mM) 120.1 NaCl, 25 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5 D-glucose (pH 7.4). The buffer was gassed continuously with a mixture of 95% O_2 and 5% CO_2.

Measurements of force

Force was measured as previously described in detail (Clausen & Everts 1991). In brief, isolated muscles were mounted vertically with their tendons intact in thermostatically controlled chambers and exposed to direct electrical stimulation via platinum electrodes on either side of the midportion of the muscle. Supramaximal 1 ms pulses of 10–15 V were used. Isometric force was measured using force displacement transducers (Grass FT03; Grass Instrument Co., Quincy, MA, USA) and recorded with chart recorders calibrated with standard weights or with an electronic data acquisition system. After adjustment to optimal resting length, with an average resting tension of 0.015 ± 0.002 N ($n = 8$), muscles were equilibrated for at least 30 min before the onset of experiment. Force was tested at the indicated intervals using trains of 1 ms pulses lasting 2 s at 60 Hz for soleus and 0.5 s at 90 Hz for EDL. This elicited tetanic force amounting to on average 0.345 ± 0.005 N for soleus ($n = 42$) and 0.463 ± 0.019 N for EDL ($n = 18$). In each experiment three tetanic contractions were performed prior to EP to establish the initial force. All data are presented as percentage of this initial level.

Electroporation

Electroporation was carried out by applying a short-lasting intense electric field (100–800 V cm^{-1}) across the muscles. This induces a rapid increase in the permeability of the plasma membrane, but not in the membranes of intracellular organelles. This allows entry of Na^+, loss of K^+ and penetration of extracellular markers into the cytoplasm (Gehl *et al.* 1999, Buchanan *et al.* 2002, Gissel & Clausen 2003). Depending on the intensity and duration of the electric field, most of the pores in the plasma membrane of plated cells reseal spontaneously after 6–30 min (Teissié *et al.* 1999). For the characterization of the effects of EP on contractility following mounting and testing as described above, each muscle was removed from the force transducer, placed in an

EP cuvette containing 1 mL of standard buffer and exposed to a standardized EP paradigm consisting of eight square wave pulses, each lasting 0.1 ms in the range 100–800 V cm^{-1}, applied at a frequency of 1 Hz. Then the muscles were remounted on the transducer and tested at the same length as before EP at the intervals (1–20 min) indicated in the graphs. All experiments were carried out using an Electro Square Porator (ECM 830; BTX, San Diego, CA, USA), and the square shape of the pulses was tested on an oscilloscope. In parallel studies, the effects of EP on the tissue contents of Na^+ and K^+ were assessed. The muscles were removed from the incubation medium at the times indicated and prepared for determination of Na^+ and K^+ contents as described below.

Na^+,K^+ contents and ^{14}C-sucrose space

For the determination of total Na^+,K^+ contents, muscles were blotted, weighed and extracted overnight by soaking in 2 mL of ice-cold 0.3 M trichloroacetic acid (TCA). Next morning 0.5 mL aliquots were taken for flame photometry using Li^+ as internal standard and an FLM3 emission flame photometer (Radiometer, Copenhagen, Denmark). By comparison with standards containing the same concentration of TCA and Na^+ and K^+ in the same concentration range as in the TCA extract, the results were expressed as μmol g wet wt^{-1}. This procedure was shown to give the same values for muscle Na^+,K^+ contents as determined using the clear supernatant obtained after centrifugation of homogenates of the muscles in TCA.

Extracellular volume was determined using ^{14}C-sucrose as a marker. In all experiments muscles were equilibrated for 90 min in standard buffer containing 1 mM sucrose and 0.5 μCi mL^{-1} of ^{14}C-sucrose. The muscles were exposed to EP during equilibration (after 30 or 75 min). After incubation, the muscles were blotted, weighed and soaked overnight in 2 mL of 0.3 M TCA. The next day, ^{14}C-sucrose activity in the TCA extract was determined by liquid scintillation counting (Tri-Carb 2100 TR; Packard Instrument Co., Downers Grove, IL, USA), and the ^{14}C-sucrose space was calculated by comparison with the activity of ^{14}C-sucrose in the incubation medium. The intracellular Na^+ content was determined by subtracting the amount of Na^+ present in the sucrose space and deducting this from the total Na^+ content measured by flame photometry.

Determination of LDH

Muscle cell integrity was examined by measuring the release of LDH from the muscles into the incubation medium using procedures described in detail elsewhere (Gissel & Clausen 2003). In brief, the LDH activity in the incubation medium was determined by spectrophotometric measurement of the decrease in the concentration of the substrate NADH by conversion of pyruvate to lactate. Activity was expressed as units g muscle wet wt^{-1}. A buffer sample of 0.25 mL was mixed with 2.65 mL of a phosphate buffer containing NADH (0.4 mM) and pyruvate (0.4 mM). Absorbance was measured at 340 nm at 30 °C. Soleus muscles were mounted at resting length and allowed to equilibrate in 5 mL standard buffer for 4 × 30 min so as to wash out the LDH associated with the muscles from the dissection. Then the muscles were taken down, exposed to EP (8 × 0.1 ms pulses at 500 V cm^{-1}), remounted and incubated for 225 min, first for 15 min, then in 30-min periods. After each incubation period, the muscles were moved to new tubes and buffer samples were taken for determination of the amount of LDH released from the muscles during that period.

Membrane potential recordings

Resting membrane potentials were recorded using standard electrophysiological techniques as previously described in detail (Overgaard & Nielsen 2001). In brief, each muscle was impaled by a glass microelectrode filled with 1 M KCl (tip resistance 15–35 MΩ) and the potential recorded via an Axoclamp-2A amplifier, was displayed on an oscilloscope and a chart recorder. The muscle bath was grounded with a Ag/AgCl wire. In each muscle around 10 impalements of fibres were made. To avoid measuring from the same fibre twice, the electrode was moved a small distance across the muscle between each impalement. Soleus muscles were mounted at resting length in a perfused chamber containing Krebs–Ringer bicarbonate buffer, which was continuously gassed with a mixture of 95% O_2 and 5% CO_2. The muscles were allowed to rest for 30 min prior to measurement. Muscles exposed to EP were removed from the chamber and placed in a cuvette for EP. Immediately after EP the muscles were remounted in the chamber.

Chemicals and isotopes

All chemicals used were of analytical grade. Salbutamol, ouabain, dibutyryl cyclic AMP (dbcAMP) and tetrodotoxin (TTX) were from Sigma-Aldrich (St Louis, MO, USA), rat calcitonin gene-related peptide (rCGRP) was from Bachem (Bubendorf, Switzerland). [^{14}C] sucrose (0.6 Ci $mmol^{-1}$) was from Amersham International (Aylesbury, Bucks, UK).

Statistics

All data were expressed as means ± SEM. The statistical significance of any difference was ascertained using the two-tailed *t*-test for non-paired observations.

Results

Selection of an EP voltage causing suitable loss of force

Figure 1 shows the time-course of changes in tetanic force of rat soleus muscles before and after exposure to the standard EP paradigm at varying voltages. Whereas 100 V cm^{-1} produced no reduction in force, pulses of 300 V cm^{-1} or more gave a prompt and complete loss of force. After pulses of 300 V cm^{-1}, about 80% force recovery was obtained within 30–50 min. At higher voltages, recovery was much slower and incomplete. After EP at 800 V cm^{-1}, only 3% force recovery was observed, even after 4 h. For the following experiments with soleus, a standardized EP paradigm with a voltage of 500 V cm^{-1} was selected.

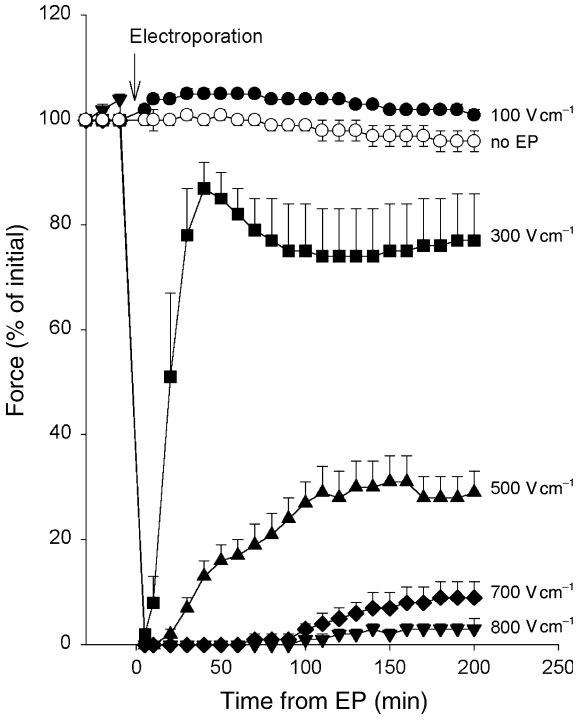

Figure 1 Time-course of the effects of EP on tetanic force in rat soleus. The muscles were mounted for isometric contractions in Krebs–Ringer bicarbonate buffer at 30 °C. Tetanic force was measured using 2 s pulse trains of 60 Hz (1 ms pulses of 10 V). Following an equilibration period of 30 min, tetanic force was recorded three times and all data presented in percentage of this initial level. The muscles were then removed from the transducers and transferred to the EP cuvette and either left unstimulated or exposed to eight EP pulses of 0.1 ms duration at the indicated voltages. Henceforth, the muscles were remounted on the transducer holders and force recorded at the indicated time intervals. Each point indicate the mean of observations on three to 13 muscles with bars denoting SEM.

Effects of EP on Na^+,K^+ contents

As shown in Figure 2, EP induced an increase in total Na^+ content of around 19 μmol g wet wt^{-1}. within 5 min and an almost equivalent decrease in K^+ content. Both changes were reversible and within 40 min, the Na^+,K^+ contents returned to the same levels as those of the untreated control muscles.

In other experiments, the EP-induced changes in intracellular Na^+,K^+ contents were determined by deducting the Na^+ and K^+ confined to the water space available to ^{14}C-sucrose. As shown in Table 1, EP increased the intracellular Na^+ content by 15.2 μmol g wet wt^{-1}. and decreased intracellular K^+ by 11.9 μmol g wet wt^{-1}., i.e. changes in the same order of magnitude

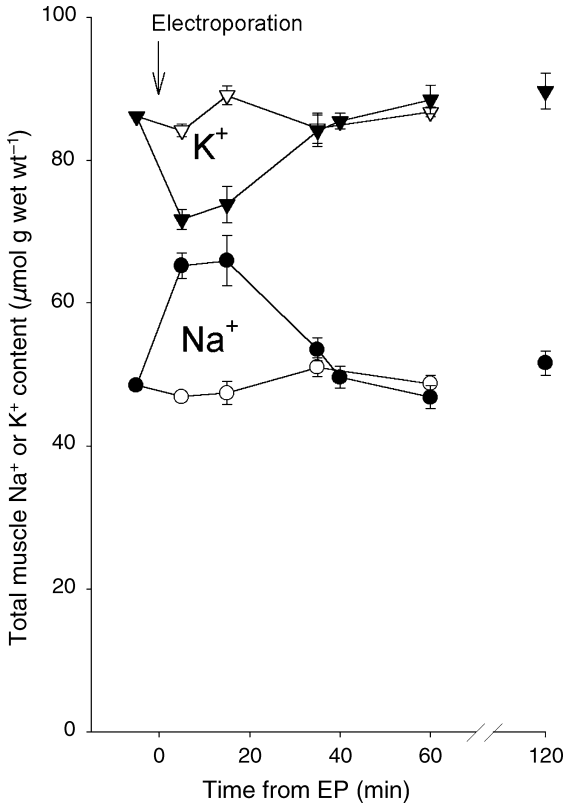

Figure 2 Effects of EP on the time course of changes in Na^+,K^+ contents in rat soleus. Experimental conditions as described in the legend to Figure 1. Untreated controls (open symbols) or muscles exposed to EP (8 × 0.1 ms pulses at 500 V cm^{-1} – closed symbols) were taken at the times indicated and prepared for flame photometric determination of Na^+,K^+ contents. Each point represents the mean of observations on three to six muscles with bars denoting 2× SEM.

Table 1 Effects of EP and TTX on intracellular Na^+,K^+ contents in rat soleus

	Intracellular Na^+ (μmol g wet wt^{-1})	Intracellular K^+ (μmol g wet wt^{-1})
Controls	11.1 ± 0.4	85.6 ± 0.9
15 min after EP	26.3 ± 1.2; $P < 0.001$	73.7 ± 1.8; $P < 0.001$
TTX (10^{-7} M)	9.7 ± 0.6	85.3 ± 1.6
TTX (10^{-7} M) 15 min after EP	26.5 ± 0.9; $P < 0.001$	68.2 ± 1.1; $P < 0.001$

Soleus muscles were mounted at resting length and incubated in Krebs–Ringer bicarbonate buffer containing ^{14}C-sucrose (0.1 μCi mL^{-1}) and 1 mM unlabelled sucrose. After 75 min of equilibration, the muscles were electroporated (8 × 0.1 ms pulses of 500 V cm^{-1}) in an EP cuvette containing buffer with ^{14}C-sucrose, returned to the ^{14}C-sucrose containing buffer and 15 min later, prepared for determination of ^{14}C-sucrose space and Na^+,K^+ contents. TTX (10^{-7} M), when present, was added to the incubation medium 15 min before EP. The amount of Na^+ and K^+ confined to the sucrose space was calculated and deducted from the total Na^+,K^+ contents of the muscles, and the intracellular Na^+ and K^+ contents expressed as μmol g^{-1} wet wt. ± SEM ($n = 6$).

as those shown in Figure 2. In order to test whether these changes were induced by excitation and opening of Na^+ channels, the experiment was also performed in the presence of the Na^+ channel blocker TTX (10^{-7} M). As shown in Table 1, this gave no reduction in the effects of EP on Na^+,K^+ contents, but rather an increase, indicating that they were not elicited by opening of Na^+ channels, but reflects a general leakage of the plasma membrane.

Effects of EP on LDH-release and ^{14}C-sucrose space

We have already shown that EP (three pulses of 0.2 ms at 300 V cm^{-1}) induces an increase in the loss of LDH from rat EDL muscles (Gissel & Clausen 2003). In soleus muscles exposed to the standard EP paradigm used in the present study, the release of LDH showed an initial 11-fold increase (within 30 min) as compared with untreated muscles ($P < 0.001$), followed by a 4–5-fold increase lasting for up to 225 min (Fig. 3).

This evidence of cell membrane leakage suggested that the water space available to an extracellular marker would increase. Measurements of ^{14}C-sucrose space showed that 60 min after exposure to the standard EP paradigm there was an increase from 21.1 ± 1.1 to 28.6 ± 0.9% ($n = 6$ vs. 6 and $P < 0.001$). Other experiments showed that a similar increase in ^{14}C-sucrose space was seen for up to 120 min after EP.

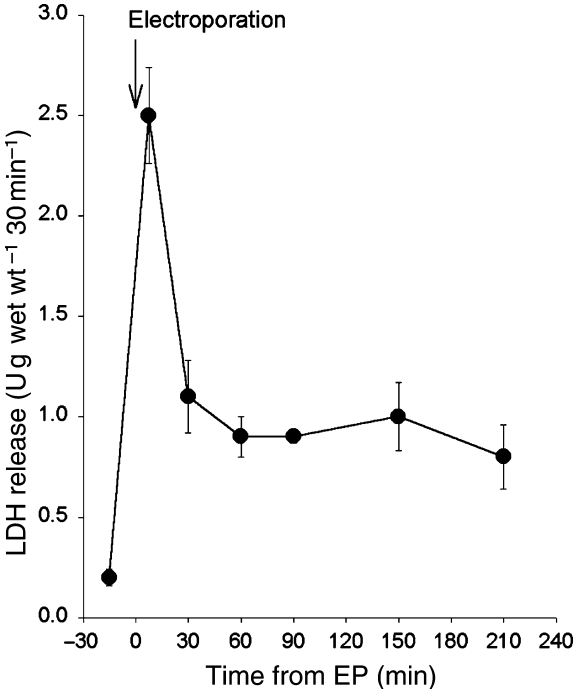

Figure 3 Effect of EP on the release of LDH from rat soleus. Soleus muscles were mounted at resting length and allowed to equilibrate in 5 mL standard buffer for 4 × 30 min so as to wash out the LDH associated with the muscles from the dissection. Then the muscles were taken down, exposed to EP (8 × 0.1 ms pulses at 500 V cm^{-1}), remounted and incubated for 225 min, first for 15 min, then in 30-min periods. After each incubation period, the muscles were moved to new tubes and buffer samples were taken for determination of the amount of LDH released from the muscles during that period. Each point represents the mean of observations on three muscles with bars denoting 2× SEM.

Role of Na^+,K^+ pumps in restoring EP-induced loss of tetanic force

The significance of the Na^+,K^+ pumps in restoring contractility was evaluated by comparing the effects of inhibition or stimulation of active Na^+,K^+ transport.

As shown in Figure 4, the spontaneous force recovery seen after the standard EP paradigm was clearly inhibited by 10^{-5} M ouabain and completely blocked by 10^{-3} M ouabain.

It is well-documented that salbutamol and rat calcitonin gene-related peptide (rCGRP) stimulate the Na^+,K^+ pump. When added immediately after EP at concentrations earlier shown to elicit maximum effect on active Na^+,K^+ transport (Clausen & Flatman 1980, Andersen & Clausen 1993), both agents produced almost the same improvement of the force recovery after EP (Fig. 5). We also tested the effects of simultaneous addition of salbutamol (10^{-5} M) and rCGRP (10^{-7} M). As measured 150 min after EP, salbutamol,

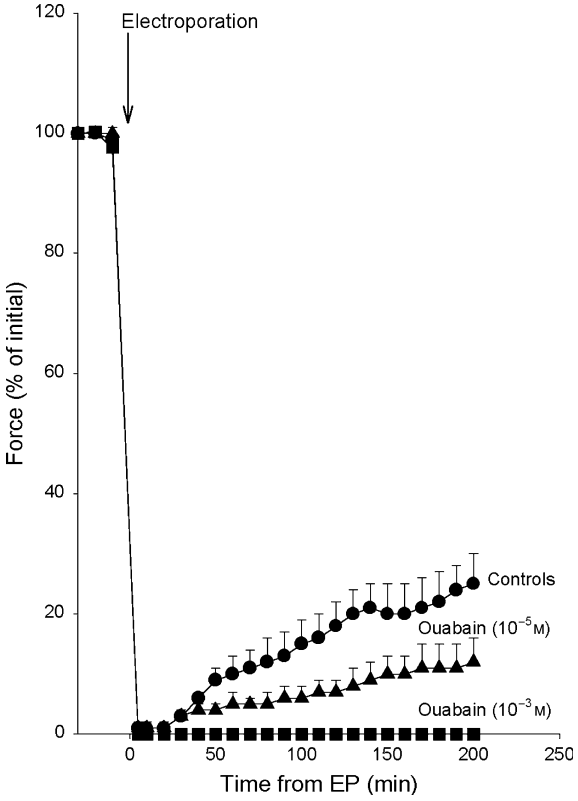

Figure 4 Effects of ouabain on the response to EP in rat soleus. Experimental conditions as described in the legend to Figure 1. Muscles were exposed to EP (8 × 0.1 ms pulses at 500 V cm^{-1}) in the absence or the presence of ouabain. Each point represents the mean of observations on three to six muscles with bars denoting SEM.

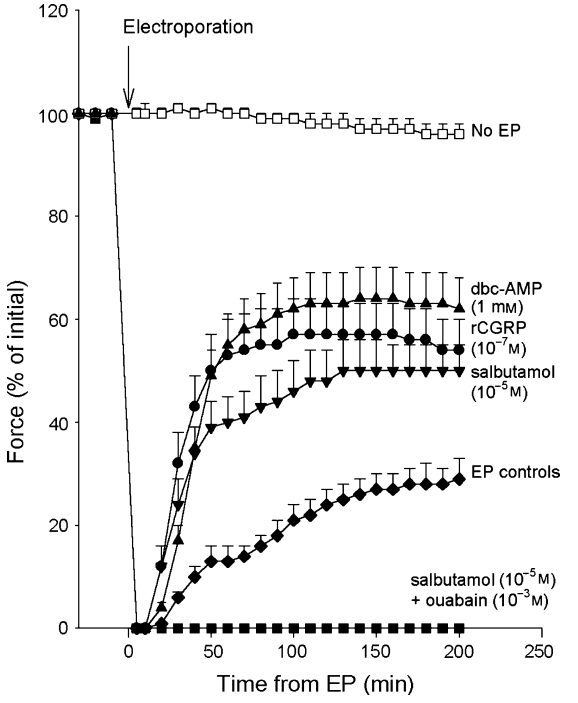

Figure 5 Effects of salbutamol, rCGRP, dbcAMP and ouabain on the response of tetanic force to EP in rat soleus. Experimental conditions as described in the legend to Figure 1. After EP, force recovery was followed in buffer without additions ($n = 13$) or in buffer containing salbutamol (10^{-5} M) ($n = 9$), rCGRP (10^{-7} M) ($n = 7$), dbc-AMP (1 mM) ($n = 7$) or salbutamol (10^{-5} M) with ouabain (10^{-3} M) ($n = 4$). Each point represents the mean of observations on four to 13 muscles with bars denoting SEM.

rCGRP or the combination of both agents induced peak force recoveries of 57 ± 6% ($n = 9$), 50 ± 5% ($n = 7$) and 57 ± 4% ($n = 4$), respectively. These values were not significantly different.

The effects of salbutamol and rCGRP on the Na^+,K^+ pumps are both elicited via stimulation of the adenylate cyclase and mediated by cyclic AMP (Clausen 2003). As shown in Figure 5, dbcAMP induced a marked, albeit slower increase in the force recovery after EP, but reaching a level slightly higher than that seen after the addition of salbutamol or rCGRP. Ouabain (10^{-3} M) completely suppressed the stimulating effect of salbutamol on force recovery (Fig. 5) as well as the effects of 10^{-7} M rCGRP (force development 0 N, $n = 2$) and 1 mM dbcAMP (force development 0 N, $n = 3$).

As shown in Table 2, salbutamol, CGRP, adrenaline and dbcAMP increased the initial rate of force recovery as recorded 30 min after EP by between 183 and 433%.

Experiments with rat EDL muscles

Since fast-twitch fibres constitute about half the total mass of skeletal muscles, it was of interest to assess the effects of eletroporation and salbutamol on EDL muscles. As shown in Figure 6, EP at 350 V cm^{-1} induced total suppression of tetanic force, followed by partial spontaneous recovery. This force recovery was considerably improved (117%) by salbutamol (10^{-5} M). Ouabain (10^{-3} M) completely suppressed the spontaneous force recovery ($n = 1$) as well as that induced by salbutamol ($n = 1$).

Effects of EP on membrane potentials

In cardiac myocytes, EP was found to elicit depolarization (Tung *et al.* 1994). Figure 7 shows that also in the isolated rat soleus, EP caused a prompt and marked depolarization of around 50 mV, followed by a spontaneous repolarization which developed with a rapid early phase and a later slower phase. Measurements performed 5 min after the addition of salbutamol (10^{-5} M) showed a significantly smaller depolarization. Furthermore, the initial rate of repolarization as measured over the first 30 min increased 1.5-fold, followed by a 10–18 mV larger repolarization than the controls.

Table 2 Rate of force recovery in soleus muscle following EP at 500 V cm^{-1}

	Initial force recovery (%/30 min)	Percentage increase compared with controls
Controls	6 ± 1 (13)	–
Salbutamol	32 ± 6 (6)	433
CGRP	24 ± 5 (7)	300
Adrenaline	32 ± 6 (7)	433
dbc-AMP	17 ± 3 (7)	183
Ouabain	0 ± 0 (5)	–
Salbutamol + ouabain	0 ± 0 (4)	–

Soleus muscles were electroporated using eight pulses with an amplitude of 500 V cm^{-1} and a duration of 100 μs given at a frequency of 1 Hz. Following EP the muscles were remounted in the force displacement transducer and force recovery was followed. Force was normalized with regard to maximal force output before EP. The effects of salbutamol (10^{-5} M), rCGRP (10^{-7} M), adrenaline (10^{-5} M), dbc-AMP (1 mM) and ouabain (10^{-3} M), on the initial rate of force recovery was tested. Mean values are given ± SEM with the number of muscles in parentheses.

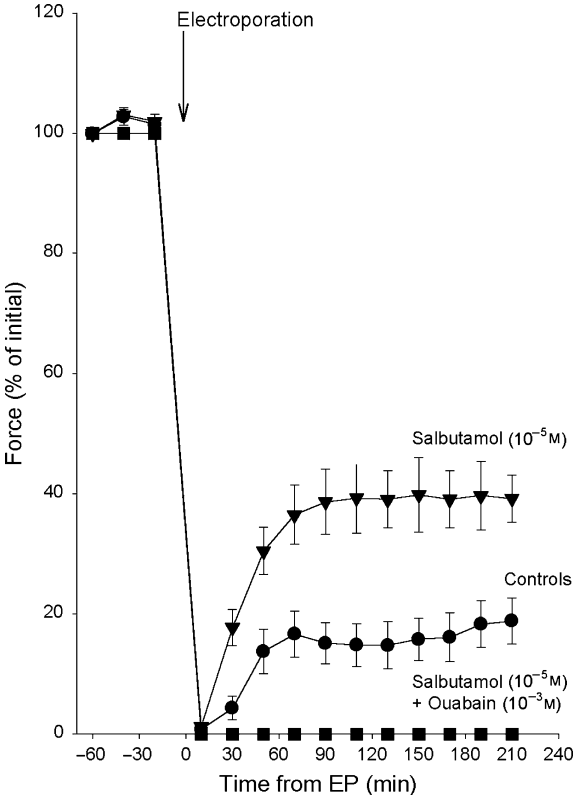

Figure 6 Effects of salbutamol and ouabain on the response of tetanic force to EP in rat EDL. EDL muscles were mounted for isometric contractions in Krebs–Ringer bicarbonate buffer at 30 °C. Tetanic force was elicited using 0.5 s pulse trains of 90 Hz (1 ms pulses of 10 V). Following an equilibration period of 60 min, tetanic force was recorded three times and all data are presented in percentage of this initial level. The muscles were then removed from the transducers, transferred to the EP cuvette and exposed to eight 0.1 ms EP pulses of 350 V cm^{-1}. Henceforth, the muscles were remounted on the transducer holders and force recorded at the indicated time intervals. Each point indicates the mean of observations ± SEM on six vs. six contralateral muscles without or with the addition of 10^{-5} M salbutamol. One muscle was exposed to 10^{-3} M ouabain from 5 min before the EP, followed by the addition of 10^{-5} M salbutamol.

This improvement of the repolarization is likely to reflect stimulation of the electrogenic Na^+,K^+ transport which would be expected to reduce intracellular Na^+. This was examined by measuring the Na^+ content of the water space not available to ^{14}C-sucrose over the time-interval where salbutamol produced a stable improvement of force recovery (120–210 min after EP. Intracellular Na^+ content of the controls and the salbutamol treated muscles was 9.3 ± 0.8 and 7.2 ± 0.5 μmol g wet wt^{-1}, respectively ($n = 14$ vs. 11, $P < 0.05$), documenting that a net increase in Na^+ extrusion had taken place, presumably via the Na^+,K^+ pumps.

Discussion

The present study has explored a model for graded loss of muscle cell membrane integrity, allowing rapid induction of increased permeability to Na^+,K^+, sucrose and LDH, depolarization and loss of contractility. This functional impairment underwent spontaneous recovery, and we obtained evidence that the Na^+,K^+ pump is an essential mechanism for the spontaneous restoration of contractility. This was seen both in soleus and EDL muscles, indicating that the response is general for the two major types of muscle fibres.

In soleus muscles exposed to EP (8 × 0.1 ms pulses) it could be observed that at 100 V cm^{-1} no loss of force was observed suggesting that the EP threshold had not been reached. However, at 300 V cm^{-1} there was a complete loss of force immediately after EP. This was taken as an indication of that the threshold had been exceeded and permeabilization had occurred. The relatively fast and large recovery (80%) suggests that the pores reseal spontaneously and the membrane regains excitability. At the higher voltages an increasing percentage of irreversible membrane leakage would be expected and we do observe increasingly smaller force recovery in the muscles, even after several hours. Thus, at 800 V cm^{-1} only 3% recovery was obtained. In experiments performed using a standardized EP paradigm (8 × 0.1 ms pulses of 500 V cm^{-1}), the early rapid phase of force decline was associated with a highly significant gain of Na^+ and an almost equivalent loss of K^+ as well as a depolarization of around 50 mV. This

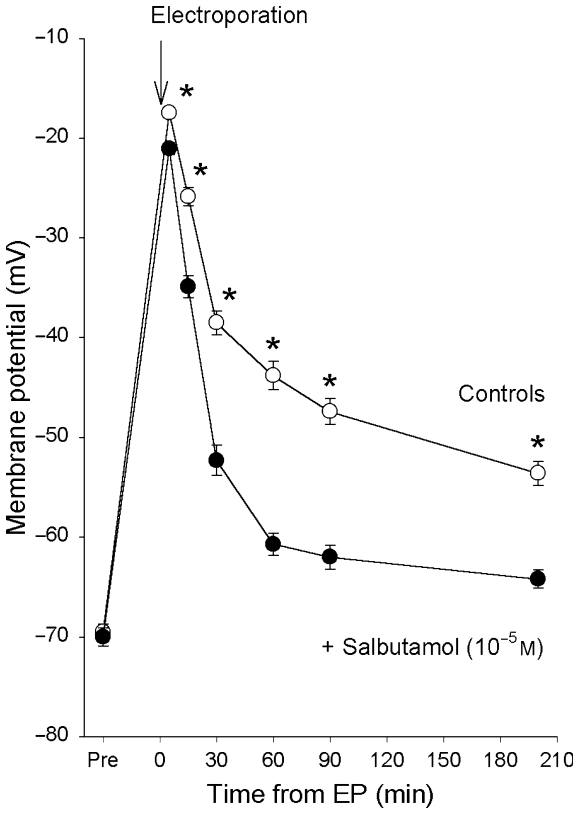

Figure 7 Effects of EP on the time-course of changes in resting membrane potential in soleus muscles exposed to EP without or with salbutamol (10^{-5} M). Soleus muscles were mounted at resting length in a perfused chamber containing Krebs–Ringer bicarbonate buffer, which was continuously gassed with a mixture of 95% O_2 and 5% CO_2. Using standard KCl-filled microelectrodes, resting membrane potentials were recorded from the muscle fibres at the indicated time intervals. Each point represents the mean ± SEM of 34–44 observations collected in four muscles by repeated penetrations in individual cells across the muscle belly. The significance of the difference between the salbutamol-treated and the controls is indicated by asterisks (*$P < 0.001$).

redistribution of total Na^+ and K^+ contents underwent spontaneous restoration within 40 min, a process accompanied by around 20 mV repolarization and 15% force recovery. Henceforth, both repolarization and force recovery proceed at a slower rate than in the early phase. Two hundred minutes after EP the membrane potential had recovered by an additional 15 mV and force recovery was around 30%.

The early Na^+,K^+ redistribution was not suppressed by TTX, indicating that it is not due to activation of voltage sensitive Na^+ channels, but rather reflects an unspecific loss of cell membrane integrity. We found a minor but significant increase in ^{14}C-sucrose space indicating loss of cell membrane integrity in a fraction of the cells. This confirms previous observations *in vivo* with the extracellular marker Cr-EGTA (Gehl *et al.* 1999) and *in vitro* with ^{14}C-sucrose (Gissel & Clausen 2003). Moreover, EP induced a rapid and sustained loss of LDH, further supporting the conclusion that the cell membrane undergoes an unspecific persisting leakage at the chosen EP paradigm (500 V cm^{-1}). This is in keeping with the returning clinical experience that electrical shocks elicit rhabdomyolysis and hyperkalaemia (Bhatt *et al.* 1990, Brumback *et al.* 1995).

The rapid increase in membrane permeability is evidently associated with depolarization and it seems reasonable to assume that this is the cause of the loss of excitability and ensuing loss of tetanic force. Moreover, the time-course of force recovery was similar to that of the repolarization. The rapid spontaneous repolarization is likely to reflect spontaneous resealing of the reversible pores combined with a stimulation of the electrogenic active Na^+,K^+ transport, elicited by the increase in intracellular Na^+. It is well-established that β_2-agonists produce hyperpolarization in resting isolated skeletal muscle (Tashiro 1973, Clausen & Flatman 1980). We here show that the depolarization elicited by EP is reduced and to some extent reversed by salbutamol. It seems reasonable to assume that a further stimulation of the Na^+,K^+ pump causes the acceleration of force recovery.

As adrenaline and CGRP also induce hyperpolarization in rat soleus (Clausen & Flatman 1980, Andersen & Clausen 1993), the improved force recovery elicited by these hormones may have a similar mechanism. The stimulating effects of catecholamines and CGRP on the Na^+,K^+ pumps are mediated by the intracellular messenger cAMP. The similarity in mechanism is also evident from the observation that the effects of salbutamol and rCGRP on force recovery were not addictive and not very different from that elicited by dbcAMP.

The major new information gained from the present study is that the Na^+,K^+ pumps in a skeletal muscle are sufficient to compensate the Na^+,K^+ leaks arising from a simple mechanical damage of the plasma membrane. This further supports the idea that the Na^+,K^+ pumps contribute efficiently to the maintenance of contractile force.

Perspectives

In electroporated muscles, inhibition of the Na^+,K^+ pump with ouabain prevents the spontaneous restoration of contractility. Conversely, stimulation of the Na^+,K^+ pump with different agents elicits a considerable acceleration of force recovery. This indicates that Na^+,K^+ pump activation is one of the basic physiological mechanisms for the recovery of function in leaky skeletal muscle cells, possibly also following mechanical trauma or work. It is interesting that it has recently been

shown that a β_2-agonist hastens force recovery after chemical injury in rat EDL muscle (Beitzel *et al.* 2004).

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