J. Physiol. (1983), **336**, *pp.* 211–228
With 7 *text-figures*
Printed in Great Britain

THE EFFECT OF CELLULAR ENERGY RESERVES AND INTERNAL CALCIUM IONS ON THE POTASSIUM CONDUCTANCE IN SKELETAL MUSCLE OF THE FROG

BY R. FINK, SIGRID HASE, H. Ch. LÜTTGAU AND E. WETTWER
From the Department of Cell Physiology, Ruhr-University, D-4630 Bochum, F.R.G.

(*Received 23 February 1982*)

SUMMARY

1. The increase in K^+ conductance induced by repetitive stimulation in metabolically poisoned sartorius muscle fibres of the frog was investigated, using a two-micro-electrode voltage-clamp technique.

2. After the inhibition of creatine kinase by 0·4 mM-1-fluoro-2,4-dinitrobenzene (FDNB) and a complete and irreversible exhaustion of contractility, a nearly linear current–voltage relation was measured between −100 and 0 mV.

3. In the presence of CN^- (4 mM) an 'intermediate state' could be established by repetitive stimulation towards complete mechanical exhaustion. In this labile state, the high and potential-independent K^+ conductance could be induced by repetitive voltage-clamp pulses (100 ms duration) from −85 to 0 mV membrane potential. After the pulses had ceased, fibres regained their original membrane conductance within several minutes.

4. After the electrophoretic injection of the Ca^{2+}-chelating agent H_2EGTA^{2-} into fibres in the intermediate state, an increase in membrane conductance by repetitive voltage-clamp pulses could no longer be induced.

5. Fibres in the intermediate state into which H_2EGTA^{2-}-buffered Ca^{2+} (free Ca^{2+} $\sim 10^{-5}$ M) was injected, or to which external caffeine (1·5 mM) was applied, showed a spontaneous and reversible increase in membrane conductance.

6. In metabolically poisoned and mechanically exhausted sartorius muscles the concentrations of creatine phosphate (CP) and ATP were estimated using biochemical standard methods.

7. The concentration of CP remained basically unchanged after FDNB poisoning. In solutions containing CN^- plus iodoacetate CP fell below the detectable concentration of about 0·5–1 % of the normal value. ATP decreased to slightly less than 20 % under both conditions.

8. It is concluded that internal free Ca^{2+} promotes the activation of the K^+ conductance in exhausted muscle fibres, and that a shortage of energy reserves increases the 'sensitivity' of K^+ channels to Ca^{2+} ions.

INTRODUCTION

The exhaustion of contractile activity in metabolically poisoned muscle fibres by repetitive stimulation is accompanied by an increase in K^+ conductance of the fibre

membrane (Fink & Lüttgau, 1976). Voltage-clamp experiments revealed a nearly linear current–voltage relation, suggesting a blockade of the potential-sensitive gating mechanism in the open state (Fink & Wettwer, 1978). The structural integrity of the transverse tubular system was maintained in exhausted fibres (Fink, Grocki & Lüttgau, 1980*a*). However, the effect was irreversible with the poisons applied (iodoacetate plus CN^-). Neither contractility nor normal K^+ conductance could be restored.

In the present report we extend the analysis of the fatigue effect. First, we applied 1-fluoro-2, 4-dinitrobenzene (FDNB) as an alternative metabolic poison in order to demonstrate that the increase in K^+ conductance is related to an exhaustion of energy reserves rather than to an unspecific action of iodoacetate. In addition we measured the contents of ATP and creatine phosphate (CP) of normal and exhausted fibres. Secondly we showed that the fatigue effect was reversible when CN^- was used as the sole poison. Finally, we injected Ca^{2+} or the Ca^{2+}-chelating agent H_2EGTA^{2-} into partially exhausted fibres to obtain some information about the role of internal Ca^{2+} in initiating the increase in K^+ conductance. Preliminary reports of this study have been given at the 28th International Congress of Physiological Sciences at Budapest (Wettwer, Hase & Lüttgau, 1980) and a satellite symposium at Debrecen (Wettwer, Hase & Lüttgau, 1981).

METHODS

Preparation and experimental chamber. All experiments were performed with sartorius muscles of the frog (*Rana temporaria*). In the experimental chamber, which had a water-jacket for cooling, the muscles were either stretched to 130% of their slack length and rigidly fixed by needles, or wrapped around a Perspex rod and stretched to about 150% of their slack length (Stefani & Schmidt, 1972). The solution in the chamber (volume *ca.* 5–6 ml) could be exchanged during electrophysiological experiments within 30–45 s (40–45 ml min^{-1}). If not otherwise stated the bath temperature was 10 °C.

Electrodes. Conventional glass micro-electrodes were filled with 3 M-KCl (voltage electrode) or 2 M-K citrate (current electrode). Injection electrodes (see below) were first filled with double-distilled water. A few hours before the experiments started the water was replaced by the injection solution with the help of micropipettes, and the electrodes were afterwards dipped in the same solution.

Voltage-clamp technique. Point voltage-clamp experiments were performed according to a method described by Costantin (1968). For further details see Fink & Wettwer (1978) and Wettwer (1981).

Solutions. Standard Ringer solution (soln. A) was the same as that used by Adrian (1956) and contained (mM): KCl, 2·5; NaCl, 115; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85. In some of the experiments phosphate buffer was replaced by 3 mM-Tris Cl.

The solutions which contained metabolic poisons and other agents had the same composition as standard Ringer. Some of the poisons were added from concentrated stock solutions which diluted the content of standard Ringer by not more than 5%. Soln. B: Ringer plus 4 mM-NaCN; soln. C: Ringer plus 4 mM-NaCN and 1·5 mM-caffeine; soln. D: Ringer plus 2 mM-NaCN and 1 mM-Na iodoacetate; soln. E: Ringer plus 0·38–0·40 mM-1-fluoro-2,4-dinitrobenzene; soln. F: Ringer plus 4 mM-D-glucose and 1 mM-Na pyruvate. The pH of all solutions was 7·2, except soln. E which had a pH of 7·4. In voltage-clamp experiments the regenerative increase in Na^+ conductance was always prevented by the application of 10^{-7} M-tetrodotoxin.

Intracellular injection. H_2EGTA^{2-} and $CaEGTA^{2-}$ were electrophoretically injected into single fibres to modify internal free Ca^{2+}. Conventional glass micro-electrodes for injection were filled with the following solutions. Soln. G: 500 mM-H_2EGTA^{2-} (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid) and 500 mM-TES^- (*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid) as buffer; pH adjusted to 7·2 by KOH. Soln. H: 500 mM-$CaEGTA^{2-}$ buffered with H_2EGTA^{2-} (50:1) to reach a free Ca^{2+} concentration of 1×10^{-5} M ($K'_{CaEGTA^{2-}} = 5{\cdot}0 \times 10^6$ M^{-1}) and 500 mM-

TES^- as pH buffer; adjusted to pH 7·2 by KOH. Soln. I: 500 mM-H_2EGTA^{2-}; adjusted to pH 7·2 by KOH. Soln. K: 500 mM-H_2HDTA^{2-} (hexamethylenediamine *N*, *N*, *N'*, *N'*-tetraacetic acid); adjusted to pH 3·4 by HCl.

During injection the membrane potential was kept constant by voltage clamp. The injection current, which was of the order of 10–100 nA, was measured as the voltage drop across a 10 kΩ resistance and registered continuously.

Estimation of the momentary intracellular concentration of injected substances (H_2EGTA^{2-}/ $CaEGTA^{2-}$). These estimations were based on the direct measurement of the injected charge and on assumptions which had to be made concerning the transport number, the intracellular distribution of injected substances and changes in the free intracellular Ca^{2+} concentration (Wettwer, 1981). In control experiments with $CaEGTA^{2-}$ we tried to estimate the quantities ejected from a pipette with the help of an atomic absorption spectrophotometer. From these direct measurements transport numbers were obtained which were roughly equal to those calculated under the simplifying assumption that all ions in the injection solutions had the same mobility (cf. Wettwer, 1981). The binding capacities of the Ca^{2+}-specific and the Ca^{2+}–Mg^{2+} sites of troponin 140 μM and 140 μM respectively), myosin (0 μM and 140 μM), parvalbumin (0 μM and 140 μM) and calmodulin (20 μM and 0 μM) were recently estimated by Robertson, Johnson & Potter (1981). We made use of these values and also of the corresponding binding constants for Ca^{2+} and Mg^{2+} (free $[Mg^{2+}]_i$, 1 mM) given by the same authors. Some uncertainties exist as to the internal free Ca^{2+} concentration in the intermediate state of exhaustion (see p. 226). In our calculations we used a value of 10^{-7} M.

The internal distribution of injected substances was described by a diffusion model, assuming a continuous plane source (Carslaw & Jaeger, 1959, p. 263) and a fibre radius of 50 μm. Taking into account the long injection period (up to several hundred seconds), the three-dimensional distribution within the fibre is negligible compared with the one-dimensional distribution along the fibre axis. Therefore, a one-dimensional model, which assumes the injected quantities to be initially distributed in a plane, seems to be an acceptable approximation. For $CaEGTA^{2-}$ and H_2EGTA^{2-} an intracellular diffusion coefficient of $4·6 \times 10^{-6}$ cm^2 s^{-1} was chosen (Moisescu & Thieleczek, 1978). The intracellular concentrations calculated with these data (cf. Figs. 1 and 6) probably represent an upper limit. The amount of EGTA ions injected might have been smaller than assumed and the diameter of the fibres examined might have been larger than 100 μm. Considering these and further uncertainties and simplifications, it can be assumed that the injection of H_2EGTA^{2-} lowered $[Ca^{2+}]_i$ to values between 10^{-9} and 10^{-8} M and that the injection of $CaEGTA^{2-}$ raised the concentration to between 10^{-6} and 10^{-5} M.

Estimation of ATP and CP in normal and exhausted muscles. Sartorius muscles were tied to two hooks, stretched to 130% of their slack length and placed into standard Ringer or solutions D, E or F. After 1 h of incubation at 0–1 °C the muscles were stimulated to complete exhaustion of force development (see p. 224). They were then placed into standard Ringer to remove the attached poison. After the adhering solution was blotted off by a quick pressing between filter paper, the muscles were ground in liquid N_2 and deproteinized in 0·6 N-$HClO_4$ as described by Lamprecht & Trautschold (1970). After neutralization with KOH and subsequent centrifugation aliquots of the supernatant were taken for the estimation of ATP and CP. The sediment was used for the assay of muscle protein according to Lowry, Rosebrough, Farr & Randall (1951). ATP was assayed by the hexokinase glucose-6-phosphate dehydrogenase method as described by Lamprecht & Trautschold (1970) with the exceptions that the final concentration of $NADP^+$ was changed to 0·18 mM and that the volume of the reaction mixture was 1·0 ml. The increase in optical density at 334 nm after the addition of hexokinase as last component was registered by an automatic recorder. After the complete consumption of ATP, CP was estimated in the same sample after addition of creatine kinase as described by Lamprecht, Stein, Heinz & Weisser (1970). The assay was standardized by the addition of known amounts of ATP. For calculation of muscle ATP and CP the dilutions of the tissue by the procedures of extraction and neutralization were taken into account. Because wet tissue weight and muscle protein were determined, the data were expressed as mmol kg^{-1} wet weighed muscle and as mmol mg^{-1} of protein as well.

RESULTS

The effect of fluorodinitrobenzene-poisoning and mechanical exhaustion upon electrical characteristics of muscle fibres

In earlier measurements we were able to show that repetitive stimulation of metabolically poisoned muscle fibres to complete exhaustion is accompanied by a large increase in membrane conductance (Fink & Lüttgau, 1976). In our experiments

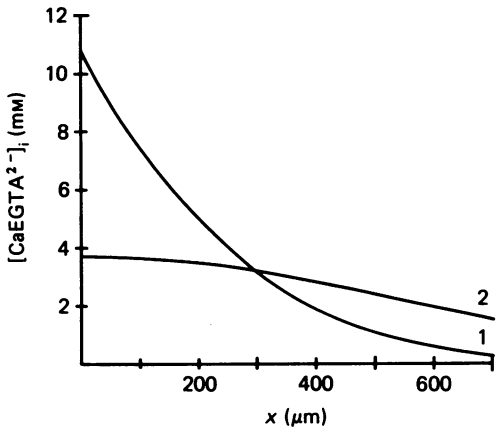

Fig. 1. Example of the intracellular distribution of $CaEGTA^{2-}$ directly after the end of an injection period of 144 s (1) and 4 min after the end of the injection (2). Abscissa: distance x (μm) from site of injection. Ordinate: intracellular $CaEGTA^{2-}$ concentration (mM) according to the following equation of a continuous plane source (Carslaw & Jaeger, 1959, p. 263):

$$C_i(T_i,x) = q\sqrt{\left(\frac{T_i}{\pi . D}\right)} . \exp\left(\frac{-x^2}{4DT_i}\right) - q\,\frac{|x|}{2D} . \operatorname{erfc}\frac{|x|}{2\sqrt{DT_i}}, \quad (1)$$

where q, rate per second and unit area of injected quantities $= 3{\cdot}45 \times 10^{-9}$ mol s^{-1} cm^{-2}; D, diffusion coefficient $= 4{\cdot}6 \times 10^{-6}$ cm^2 s^{-1}; T_i, duration of continuous injection $= 144$ s. To calculate curve 2, the distribution 4 min after the end of the continuous injection, eqn. (1) had to be modified:

$$C_i(T, x) = \sum_{a=1}^{n} \frac{M_i}{n . 2\,(\sqrt{\pi} . \mathrm{D}\,(at + T_w))} . \exp\left(\frac{-x^2}{4D\,(at + T_w)}\right), \quad (2)$$

where T_i, duration of continuous injection $= 144$ s; T_w, time of distrubution after the end of continuous injection $= 240$ s; T, total time of distribution ($T = T_i + T_w$) (s); t, duration of a hypothetical injection interval defined by dividing T_i by the number of intervals n ($10 \leqslant n \leqslant 400$, error $< 1\%$); M_i, amount of injected substance $= 4{\cdot}97 \times 10^{-7}$ mol cm^{-2}. For $n \to \infty$ and $T_w = 0$, eqn. (2) is equal to eqn. (1).

we used CN^- and iodoacetate as metabolic poisons and exhaustion was reached when the available energy reserves (ATP and CP) were to a large extent consumed. It appears, therefore, reasonable to assume that the accompanying increase in membrane conductance was caused in some way by energy deprivation. Alternatively, unspecific effects of the poisons, in particular of the irreversibly acting iodoacetate must be considered. In order to show that energy deprivation rather than an unspecific effect causes the increase in membrane conductance, we applied 1-fluoro-2,4-dinitrobenzene

(FDNB) as an alternative metabolic poison. This substance blocks the enzyme creatine kinase completely and (unfortunately) irreversibly (Cain, Davies & Infante, 1962). In addition, it partially inhibits oxidative phosphorylation and glycolysis (Davies, 1965).

Sartorius muscles were stretched to 1·3 times their resting length and equilibrated for 60 min in normal Ringer plus 0·40 mM-FDNB (soln. E; 1–6 °C). After this period

TABLE 1. Electrical constants of exhausted and normal muscle fibres

Poison	Fibre no.	λ (mm)	R'_0 (kΩ)	R_m (Ω cm²)	D (μm)
FDNB	5	0·53	44·6	207·1	140
(1–3 °C)	6	0·58	107·1	372·2	95
	7	0·54	65·9	257·7	116
	Mean	0·55	72·5	279·0	117
	±S.E. of mean	0·015	18·3	48·8	13·0
FDNB (20 °C) (temperature correction of above data)	Mean	0·46	46	142	
CN^-–IAA (0–1 °C) (Fink & Lüttgau, 1976)	Mean	0·37	56	147	117
CN^-–IAA (23 °C) (Fink & Lüttgau, 1976)	Mean	0·31	31	58	105
Unpoisoned (20 °C) (Hodgkin & Nakajima, 1972)	Mean	1·90	320	3000	85

λ, length constant (mm); R'_0, 'relative' input resistance (kΩ); R_m, membrane resistance (Ω cm²); D, fibre diameter estimated from $D = \sqrt{(2 . \lambda . R_i/R_0 . \pi)}$, in which a constant internal resistance (R_i) of 260 Ω cm at 20 °C was assumed. Mean resting potential, −85±2·1 mV. For temperature correction the following Q_{10} values were applied: $\lambda = 1·1$; $R_0 = 1·3$ and $R_m = 1·5$; $R_i = 0·73$ (Hodgkin & Nakajima, 1972; Fink & Lüttgau, 1976). IAA, iodoacetate.

they were stimulated at 1 Hz to complete exhaustion of contractility. Between 90 and 420 declining twitches were needed until movements were no longer visible under the binocular microscope (×40). This number appears to be somewhat larger than, although comparable with, the equivalent of at least thirty twitches observed by Dydyńska & Wilkie (1966) after FDNB poisoning in a N_2 atmosphere. Since a complete breakdown of ATP should be reached with eight to ten normal twitches (cf. Lüttagau, 1965), the results suggest that more energy remained available and that the ATP store was partly replenished.

Cable analysis. After complete exhaustion cable constants were measured by making use of the 'low-frequency cable analysis' (Fatt & Katz, 1951), as described in detail by Fink & Lüttgau (1976) and Fink, Grocki & Lüttgau (1980*a*,*b*). The data are collected in Table 1. We included in addition those obtained from fibres poisoned with CN^- and iodoacetate (Fink & Lüttgau, 1976) and from normal ones (Hodgkin & Nakajima, 1972). It can be seen that exhaustion from poisoning with FDNB causes a drastic decrease in membrane resistance, as was observed with CN^- and iodoacetate. The values with FDNB appear to be somewhat larger. However, the difference rests mainly on deviating data from one fibre in a small sample. To clarify this point we

measured in addition the relative input resistance R_0' (i.e. neglecting the potential decay between current and potential electrode; $x = \sim 30\ \mu m$) and obtained $51{\cdot}5 \pm 5{\cdot}3$ kΩ (1–3 °C). According to former calculations (Fink & Lüttgau, 1976) this value is no more than 10% smaller than the absolute membrane input resistance R_0 (at $x = 0$). Therefore, it corresponds well with the value for R_0 of 56·1 kΩ obtained by Fink & Lüttgau (1976) in fibres poisoned with CN^- plus iodoacetate. Consequently,

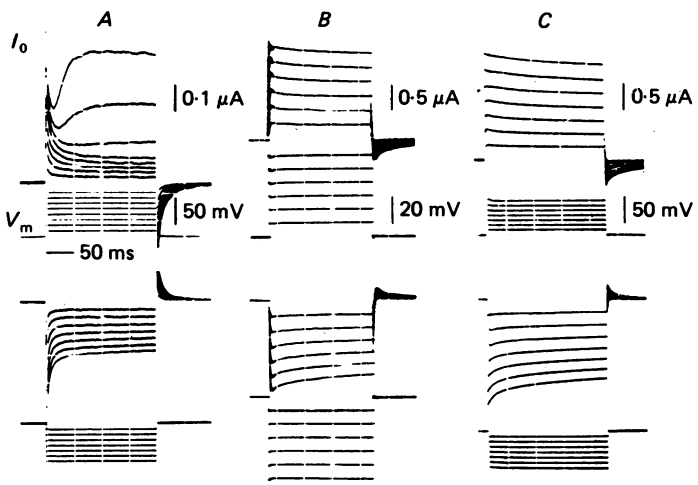

Fig. 2. Depolarizing (above) and hyperpolarizing (below) voltage-clamp pulses (V_m) together with the total current (I_0) under normal conditions in Ringer solutions plus 350 mM-sucrose (*A*) and after mechanical exhaustion in the presence of CN^- plus iodoacetate (*B*; soln. D) and FDNB (*C;* soln. E). *A:* resting potential (r.p.), −87 mV; holding potential (h.p.) −90 mV; temperature, 3·2 °C. *B:* r.p., −85 mV; h.p., −85 mV; temperature, 2·7 °C. *C:* r.p., −81 mV; h.p., −85 mV; temperature, 2·4 °C.

we conclude that the decrease in membrane resistance observed after mechanical exhaustion in solutions with different metabolic poisons is due to a shortage in available energy reserves and not to unspecific actions of the poisons applied. Since the poisons block different enzymatic reactions, it also appears rather unlikely that the effect is caused by the accumulation of a specific metabolic end-product or alterations in pH.

Current–voltage relations. Earlier measurements showed that the decrease in membrane resistance of exhausted fibres is due mainly to a large and rather specific increase in K^+ conductance (Fink & Lüttgau, 1976). The following point voltage-clamp experiments were performed in order to obtain further information about the gating behaviour of these channels. They may be regarded as an extension of related measurements by Fink & Wettwer (1978). In Fig. 2 currents induced by negative and positive voltage steps in exhausted fibres can be compared with those from a normal fibre (*A*). The poisons applied were CN^- plus iodoacetate in *B* and FDNB in *C*. Both fibres were stimulated to complete exhaustion. The currents from the exhausted fibres were very much alike. They were larger than those from the normal fibre and revealed a different time characteristic. With depolarizing voltage steps the exhausted fibres responded with proportionally increasing currents. A threshold for the increase in K^+

conductance and the characteristic delay in activation were absent. Repolarization was followed by a large inward tail current, probably caused by a K^+ enrichment in the transverse tubular system. The opposite, namely a tubular K^+ depletion, might explain the slow decrease in inward current during hyperpolarizing steps (cf. Almers, 1972*a*, *b*; Fink, 1978; R. Fink & E. Wettwer, unpublished).

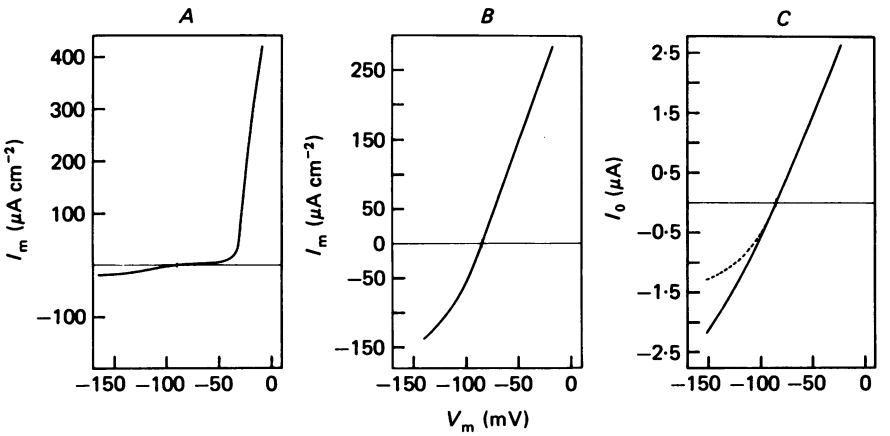

Fig. 3. Current–voltage relationships taken from the voltage-clamp experiments shown in Fig. 2. Abscissa: membrane potential (V_m) in mV. Ordinate: current density (I_m). Total current (I_0) was read 150 ms after the onset of the clamp pulses and converted into current density (I_m) by applying Coles's theorem:

$$I_m = \frac{R_i}{\pi^2 . D^3} . I_0 . \frac{dI_0}{dV_m} .$$

R_i (the resistivity of the sarcoplasm) was assumed to be 400 Ω cm in *A* (hypertonic solution) and 310 Ω cm in *B*. *D* (fibre diameter) was calculated from cable constants (for further details see Costantin, 1968; Fink & Wettwer, 1978). In *C*, I_0 and I_m (in arbitrary units) were plotted on the ordinate, because the length constant was not measured.

The 'steady-state' current–voltage relations of the fibres from Fig. 2 are shown in Fig. 3. In exhausted fibres these relations are nearly linear between −100 and 0 mV. A threshold for the activation of K^+ channels appears to be absent. The absolute current density upon depolarization to 0 mV is smaller in the exhausted fibres (*B*) than in the normal one (*A*), but of the same order of magnitude. Comparable data were obtained by Fink & Wettwer (1978).

Reversibility of the exhaustion-induced decrease in membrane resistance: the intermediate state

The energy reserves in frog skeletal muscle fibres render it feasible to perform more than 20000 single twitches. Hence it follows that long-lasting stimulation periods are needed for metabolic exhaustion which fibres only rarely survive (Lüttgau, 1965). Therefore, we applied metabolic poisons and reduced the exhaustion procedure to 10^2–10^3 declining twitches. Unfortunately, the most effective poisons, which block the replenishment of the stores of ATP and CP, act more or less irreversibly. Using iodoacetate and FDNB, restoration of contraction or membrane resistance after

removal of the poison was minimal or entirely absent. Even the extra- or intracellular application of ATP, glucose and further metabolites did not restore the normal state. We finally concentrated our interest on CN^-, which is known to act reversibly (Lüttgau, 1965). For the exhaustion of the energy reserves resulting from glycolysis and the breakdown of ATP and CP, which are still available under this treatment (equivalent to about 2000 twitches), we developed the following method.

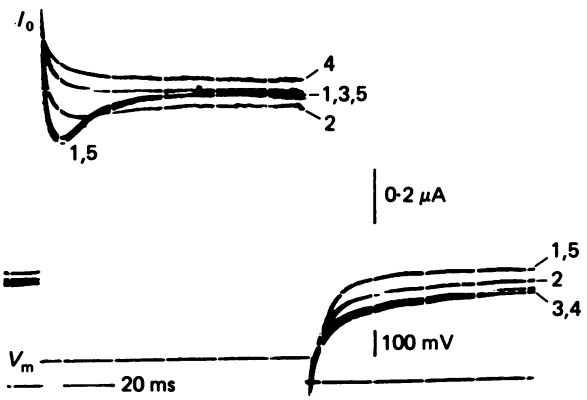

Fig. 4. The reversible transition from delayed rectification in the intermediate state to an exhaustion-induced potential-independent activation of the K^+ conductance. Depolarizing voltage-clamp steps of 100 ms duration were induced from a holding potential of −80 mV to +15 mV at a frequency of 2 Hz. The Figure shows the first (1), 10th (2), 20th (3) and 30th (4) voltage-clamp steps together with an additional one (5) after an interruption in stimulation for 3 min. V_m, membrane potential; I_0 total current; CN^- Ringer solution (soln. B); temperature, 10 °C.

The sartorius muscles were equilibrated in Ringer solution plus 4 mM-CN^- for 40–60 min and subsequently stimulated with extracellular electrodes. At the beginning fibres were continuously stimulated at frequencies between 1 and 4 Hz and after twitch height had declined to low values the final exhaustion of contraction was achieved by series of short tetani at 10–20 Hz. When no or only minimal twitching could be observed under the binocular microscope (× 40) after a rest of 5 min, current and voltage electrodes were inserted and the exhaustion procedure was resumed with depolarizing voltage-clamp pulses from the resting potential to zero or positive values. In the example shown in Fig. 4 a depolarizing step of 100 ms duration from a holding potential of −80 to +15 mV was repeated at a frequency of 2 Hz. Superimposed are the first (1), the 10th (2), the 20th (3) and the 30th (4) voltage steps of one series, together with an additional step (5) which shows the recovery after an interruption in stimulation of 3 min. The Na^+ current was blocked by tetrodotoxin and the first and last run thus show the delayed activation of the K^+ current. The subsequent alterations are due to the development of two processes. First, an inactivation (see Almers, 1976) of the K^+ conductance prevails (2). It is finally surmounted by the progress in exhaustion-induced activation of the K^+ conductance. Both effects are fully reversible within a few minutes, as is proven with the final run (5), and the procedure could be repeated several times.

After the exhaustion of contraction with extracellular electrodes the fibres existed in a labile phase which we called the 'intermediate state'. The absence of strong movements allowed experiments with micro-electrodes. A full and reversible activation of the exhaustion-induced K^+ channels could be attained with a few voltage steps. Unfortunately, this state was reached only with a relatively small number of fibres. Many others were simply not exhaustible under these conditions; they reached an irreversibly activated state or showed signs of damage. In addition, those in a plain 'intermediate state' became very sensitive to further stress, as for example the impalement of additional electrodes. Only in a limited number of trials was it possible to work for a longer period of time with several repetitions of the activation cycle. These measurements, however, revealed interesting details about the exhaustion-induced activation of the K^+ conductance which will be presented in the following section. The principal effects shown in Figs. 5–7 were in each case observed in at least three different experiments.

The effect of the injection of H_2EGTA^{2-} and $CaEGTA^{2-}$ into fibres in the 'intermediate state'

Test experiments. In order to find out whether in our experiments sufficient H_2EGTA^{2-} entered muscle fibres to depress the internal free Ca^{2+} significantly, we used the threshold for mechanical activation in normal untreated fibres as a sensitive indicator. The fibres were depolarized by short voltage-clamp steps of 100 ms duration. Mechanical artifacts were prevented by avoiding depolarizing voltage steps beyond the mechanical threshold. The latter was defined as that potential at which a contraction first became visible under the binocular microscope (magnification $\times 100$). From measurements with skinned fibres it is known that contraction activation starts at an internal free Ca^{2+} concentration of 10^{-8} to 10^{-7} M and reaches its maximum between 10^{-6} and 10^{-5} M (see Lüttgau & Moisescu, 1978). After the normal threshold had been estimated, H_2EGTA^{2-} (soln. I) was injected in a series of six pulses, each lasting for about 300 s (Fig. 5), and the threshold was estimated consecutively. It shifted from $-42{\cdot}5$ mV to $+5$ mV. During a recovery period of 44 min it returned to $-34{\cdot}5$ mV. The large shift clearly demonstrates the effectiveness of the method. Interestingly, an alteration in the threshold for the activation of the delayed rectifier was not observed (Fig. 5). Ultimately the activation threshold for contraction was about 37 mV more positive than that of the delayed rectifier. Our findings argue against a functional interdependence of the two processess. This possibility had formerly been the object of several investigations (Costantin, 1968; Kao & Stanfield, 1968, 1970), since under normal conditions the threshold of both processes is close to -50 mV.

During each injection period, which lasted for about 300 s, 8×10^{-6} to 2×10^{-5} Ampere seconds (A s) were injected, which reduced $[Ca^{2+}]_i$ to about $4{\cdot}4 \times 10^{-9}$ M. The latter probably increased to $1{\cdot}6 \times 10^{-8}$ M during the recovery period of 44 min after the fourth injection ($x = 100$ μm; for details see Methods).

In three additional experiments of this kind similar threshold shifts were observed. Occasionally, mechanical activity was first detected at a distance of about 500 μm from the electrodes, suggesting that transiently the spatial distribution of the membrane potential was less steep than that of the threshold for mechanical activation.

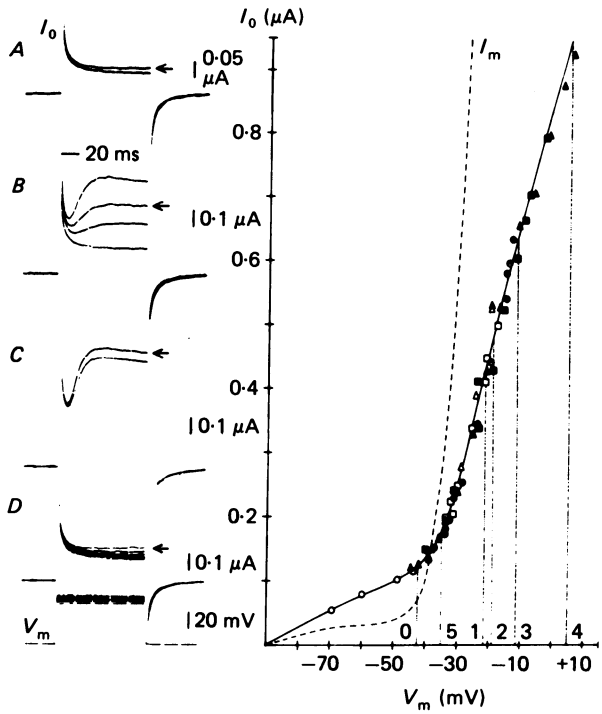

Fig. 5. The effect of injecting H_2EGTA^{2-} upon the activation of contraction (observed with a microscope) and delayed rectification.

Right side: current–voltage relationship of an unexhausted fibre in normal Ringer (soln. A). The total clamp current (I_0), read at the end of a 100 ms pulse, was plotted on the ordinate against the corresponding membrane potential (V_m) on the abscissa. From this curve membrane current density (I_m in arbitrary units) was derived using Cole's theorem (dashed line). The Figure shows current measurements before and after six subsequent injections of H_2EGTA^{2-}. The mechanical thresholds (indicated by dotted lines in the Figure) are given in brackets together with the corresponding symbols. 0: control (○; −42·5 mV); 1: first injection (△; −21·5 mV); 2: second injection (●; −19 mV); 3: third injection (□; −11·5 mV); 4: fourth injection (▲; +5 mV); 5: recovery 44 min after the fourth injection (▲; −35 mV). The mechanical thresholds after the fifth (◒; −17 mV) and sixth injections (■; −10 mV) are not indicated by a dotted line.

Left side: a selection of voltage-clamp registrations of the experiment evaluated on the right. The mechanical threshold is indicated by arrows. I_0, total current; V_m membrane potential (only shown for the currents in *D;* duration of the voltage step, 100 ms; Ringer (soln. A); temperature, 3 °C. *A:* before the first injection; steps to −44 and −39 mV. *B:* after the first injection; steps to −39, −29, −24·5 and −19·5 mV. *C:* after the second injection; steps to −15 and −13 mV. *D:* 44 min after the fourth injection; steps towards −44·5 to −34·5 mV (threshold).

In further experiments H_2HDTA^{2-} (soln. K) was injected instead of H_2EGTA^{2-}. This substance has comparable binding characteristics for Mg^{2+} but possesses an absolute binding constant for Ca^{2+} 10^6 times smaller than H_2EGTA^{2-}. We injected up to 1×10^{-5} As and observed no alteration in mechanical threshold. Since the structure of this substance is very similar to that of H_2EGTA^{2-} these experiments confirm that the shift in mechanical threshold observed after H_2EGTA^{2-} injections is due to a specific removal of free Ca^{2+} rather than to side-effects of this substance.

Ca^{2+} ions set free H^+ from H_2EGTA^{2-}. The following experiment, however, suggests that the internal buffer capacity is sufficient to suppress to a large extent alterations in pH. From a pipette filled with H_2HDTA^{2-} (pH 3·4) we injected H^+ by simply altering polarity. An injection of 6×10^{-6} A s remained without effect upon the mechanical threshold. Nevertheless, in the experiments described below we always injected H_2EGTA^{2-} or $CaEGTA^{2-}$ together with the pH buffer TES^- (solns. G and H).

The injection of H_2EGTA^{2-}. If an increase in internal free Ca^{2+} is made responsible for the activation of K^+ channels, the activation procedure in the intermediate state as described above should be suppressed after the injection of the Ca^{2+}-chelating agent H_2EGTA^{2-}. The corresponding experiment is shown in Fig. 6 (*A–C*). The fibre had already been brought into the intermediate state. Subsequently, repetitive pulses at 2 Hz induced the known activation of the high membrane conductance (Fig. 6*A*) and its reversal during a rest of 3 min. Thereupon H_2EGTA^{2-} ($+TES^-$: soln. H) was injected and the next stimulation period brought about a complete inactivation of the delayed rectifier but not the exhaustion-induced activation (Fig. 6*B*). A test about 20 min after a further injection of H_2EGTA^{2-} shows the disappearance of the suppression (Fig. 6*C*).

Applying the plane source diffusion model (see Methods) the injection of $4{\cdot}6 \times 10^{-5}$ A s raised internal H_2EGTA^{2-} to 12·7 mM (100 μm away from the site of injection; Fig. 6*B*). Consequently, $[Ca^{2+}]_i$ decreased from the original value of 1×10^{-7} to 4×10^{-9} M. The reversibility is shown in Fig. 6*C*. After a rest of 20 min, $[H_2EGTA^{2-}]$ had fallen to 2·5 mM from the original value of 8·6 mM (as a consequence of a second injection of H_2EGTA^{2-} which reached $2{\cdot}4 \times 10^{-5}$ A s). This amount corresponds to an internal Ca^{2+} concentration of $1{\cdot}4 \times 10^{-8}$ M. Although our calculations probably give the lower limit, $[Ca^{2+}]_i$ had certainly not yet reached the original order of magnitude. Therefore, we must assume that during repetitive stimulation the sarcoplasmic reticulum releases a sufficient amount of Ca^{2+}, which even in the presence of the H_2EGTA^{2-} buffer tends to increase $[Ca^{2+}]_i$ above the value before the injection of H_2EGTA^{2-}.

The injection of H_2EGTA^{2-} into fibres in the intermediate state produced a normalization of the current–voltage characteristics by itself, i.e. without further stimulation. The resting conductance decreased, and the onset of the activation of the delayed rectifier became visible.

The injection of $CaEGTA^{2-}$. Since H_2EGTA^{2-} inhibited the exhaustion-induced conductance activation, it is to be expected that the injection of Ca^{2+} produces the opposite effect. In our experiments we avoided injecting an unbuffered Ca^{2+} solution since this would have caused an irreversible clotting of the contractile material. Instead we injected a mixture of $CaEGTA^{2-}$ and H_2EGTA^{2-} with a free Ca^{2+} concentration of 10^{-5} M (soln. H). In Fig. 6 (*D–F*) currents before and a few seconds after the injection of $CaEGTA^{2-}$ can be seen (Fig. 6*D*). The injection caused a drastic increase at both potential steps. The input resistance at -55 mV dropped from 417 to 192 kΩ, which corresponds to a 5-fold decrease in membrane resistance. The effect was largely reversible (Fig. 6*E*) and a second injection caused an even larger effect (Fig. 6*F*).

The injection amounted to $1{\cdot}3 \times 10^{-5}$ A s and $1{\cdot}0 \times 10^{-5}$ A s, respectively (Fig. 6*D* and *F*). We calculated $CaEGTA^{2-}$ concentrations of 7·4 mM (Fig. 6*D*), 3·6 mM (Fig. 6*E*) and 8·4 mM (Fig. 6*F*) ($x = 100$ μm) and internal free Ca^{2+} concentrations of $5{\cdot}6 \times 10^{-6}$ M (Fig. 6*D*), $3{\cdot}0 \times 10^{-6}$ M (Fig. 6*E*) and $6{\cdot}2 \times 10^{-6}$ M (Fig. 6*F*). The remaining $CaEGTA^{2-}$ from the first injection was responsible for the larger increase in conductance during the second injection, even though the applied amount of $CaEGTA^{2-}$ was smaller in the latter case.

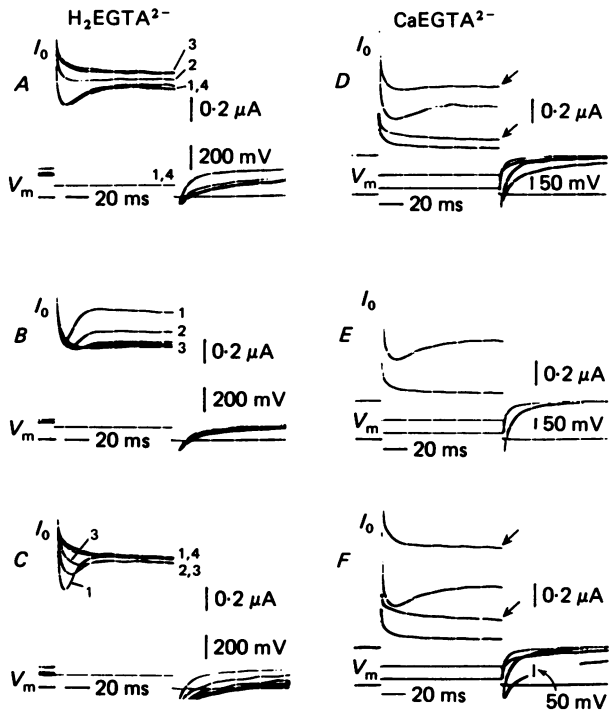

Fig. 6. *Left side:* The effect of H_2EGTA^{2-} injection upon the membrane current during voltage-clamp steps of 100 ms duration from −80 mV to +25 mV at a frequency of 2 Hz. The fibre was in the intermediate state. V_m, membrane potential; I_0 total current; CN^- Ringer solution (soln. B); temperature, 10 °C. *A:* control before the injection of H_2EGTA^{2-}. Superimposed are the first (1), 10th (2), 30th, 50th and 70th steps (3) together with a further one (4) after a rest of 5 min (recovery). Subsequently H_2EGTA^{2-} (soln. G) was injected. *B:* stimulation period, starting 3 min after the end of the injection, with the first (1), 10th, 30th, 50th, 70th, and 90th pulses (3). *C:* control 20 min after the last injection of H_2EGTA^{2-} with the first (1), 10th (2), 30th (3), 50th, 70th and 90th (4) pulses.

Right side: The effect of $CaEGTA^{2-}$ injection upon the membrane current during voltage-clamp steps of 100 ms duration from −80 mV to −55 and −2·5 mV. The fibre was in the intermediate state. V_m, membrane potential; I_0, total current; CN^- Ringer solution (soln. B); temperature, 10 °C. *D:* before and 5 s after (arrows) the injection of $CaEGTA^{2-}$ (soln. H). *E:* control of reversibility 4 min after the injection of $CaEGTA^{2-}$. *F:* before and after (arrows) a second injection of $CaEGTA^{2-}$. The injection caused a considerable increase in tail current.

In our calculations we ignored the activity of the Ca^{2+} pump of the sarcoplasmic reticulum. Even if this pump worked only at a reduced rate due to the preceding exhaustion procedure, it is reasonable to assume that it lowered free Ca^{2+} by a significant amount during the minutes following the injection of $CaEGTA^{2-}$.

The action of caffeine in the intermediate state

In these experiments internal free Ca^{2+} was increased by another method, namely the application of caffeine. This substance easily crosses the muscle membrane and in some way causes the release of Ca^{2+} from the terminal cisternae (cf. Endo, 1977).

In our experiments we applied 1·5 mM-caffeine (soln. C), which is close to the threshold concentration for the initiation of a contracture under normal conditions. In Fig. 7*A* and *B* it can be seen that the application of the drug to a fibre in the intermediate state caused the expected increase in membrane conductance and also in 'tail' current. In another fibre the input resistance decreased continuously without

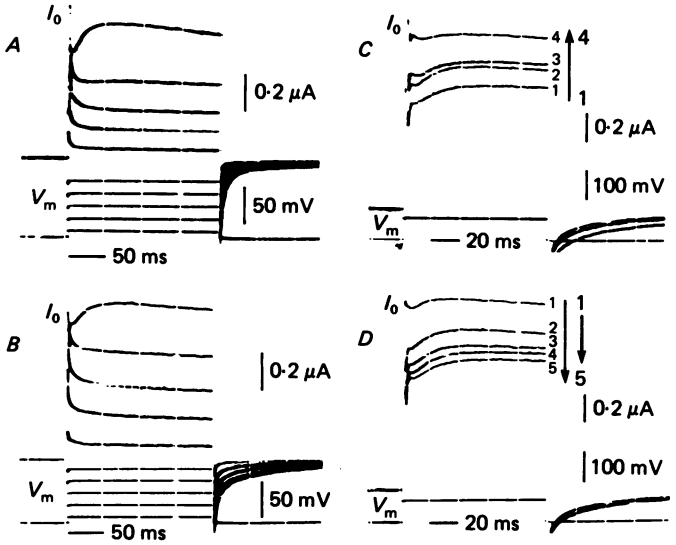

Fig. 7. The effect of caffeine upon the membrane current during voltage-clamp steps of 100 ms duration. The fibres were in the intermediate state. V_m, membrane potential; I_0, total current; CN^- Ringer solution (soln. B); temperature, 10 °C. *A:* control; voltage steps to −71, −55, −39, −23 and −6 mV. *B:* same fibre, shortly after the application of soln. B plus 1·5 mM-caffeine. Steps to −71, −55, −39, −21 and −5 mV. *C.* Voltage-clamp steps to −5 mV before (1) and 3 (2), 6 (3) and 9 (4) min after the application of 1·5 mM-caffeine. Fibre from a new preparation. *D:* reversibility of the caffeine effect. Voltage-clamp steps to −2·5 mV before (1) and 5 (2), 10 (3), 15 (4) and 20 (5) min after removal of the drug. Same fibre as in *C.*

stimulation from 90 kΩ to 64 kΩ, which corresponds roughly to a reduction in membrane resistance of 50 % (Fig. 7*C*). The input resistance recovered after removal of the drug and reached the original value within 20 min (Fig. 7*D*).

The energy reserves in metabolically poisoned and mechanically exhausted sartorius muscles

Since experiments with erythrocytes suggest that ATP might play a direct role in the regulation of the gating mechanism of Ca^{2+}-activated K^+ channels (see Discussion), we measured the ATP and CP contents of muscle in the state of complete mechanical exhaustion. The experiments were performed with whole sartorius muscles. Two muscles were used as controls, i.e. they were not metabolically poisoned and not stimulated but otherwise treated in exactly the same way as test muscles. After equilibration for 1 h in the poisoned Ringer solution, muscles were stimulated to complete mechanical exhaustion (controlled under the binocular microscope), first

at 1 Hz and in the final stage at higher frequencies (10–20 Hz). The final estimation of ATP and CP followed slightly modified standard procedures which are described under Methods.

The results are collected in Table 2. In normal muscles the ATP concentration was comparable with earlier data of other authors (3·4 mmol kg^{-1} wet wt: Infante & Davies, 1965; 2·87 mmol kg^{-1} wet wt: Dydyńska & Wilkie, 1966). The CP concen-

TABLE 2. Mean concentrations of ATP and CP in normal (control) and mechanically exhausted sartorius muscles

Solution	I (mmol kg^{-1} wet wt)	II (mmol kg^{-1} protein)	III (mmol l^{-1} FW)
		ATP	
Solns. A and F (control)	2·69	15·76	4·12
$\pm S_{\bar{x}}$ ($n = 5$)	0·43	1·96	0·65
Soln. D ($CN^- + IAA$)	0·51	3·32	0·73
$\pm S_{\bar{x}}$ ($n = 6$)	0·08	0·60	0·11
Soln. E (FDNB)	0·44	3·02	0·64
$\pm S_{\bar{x}}$ ($n = 4$)	0·05	0·63	0·08
		CP	
Solns. A and F (control)	12·38	76·16	19·07
$\pm S_{\bar{x}}$ ($n = 5$)	1·27	11·42	2·01
Soln. D ($CN^- + IAA$) (n = 6)	*	*	*
Soln. E (FDNB)	14·73	96·08	21·32
$\pm S_{\bar{x}}$ ($n = 4$)	0·92	5·83	1·33

The concentrations of ATP and CP in millimoles refer per wet weight (I, wet wt), protein content (II, protein), and litre fibre water (III, l^{-1} FW). For calculating fibre water content a decrease in extracellular space and a slight swelling of the fibres in exhausted muscles were taken into consideration, assuming that these alterations occur not only in fibres poisoned with $CN^- + IAA$ but also in those poisoned with FDNB (see Fink, *et al.* 1980*b* for further details). *n* gives the number of measurements, with two muscles in each case, and $S_{\bar{x}}$ presents the standard error of the means; temperature, 0–1 °C. IAA, iodoacetate.

* In fibres poisoned with $CN^- + IAA$ CP dropped below the traceable concentration of about 0·5–1 % of the normal value.

trations, however, reached only about half of the expected normal value given in the literature (e.g. 16–26 mmol kg^{-1} wet wt: Dydyńska & Wilkie, 1966). The low values found in our experiments seem to be due to a partial breakdown of CP during the analytical procedure. From known amounts of ATP and CP added to the preparation before freezing nearly 100 % of ATP but only 69·5 % of CP (mean of six estimations) was recovered in the final estimation. The CP values, therefore, have to be multiplied by a factor of 1·4. This would raise the CP level to 17·33 and 21·19 mmol kg^{-1} wet wt in the control and the FDNB-poisoned muscles, respectively.

In muscles poisoned with CN^- plus iodoacetate, CP fell below a detectable value. This is of special interest in so far as it shows that the number of damaged or depolarized fibres, which cannot be exhausted by stimulation, was small. FDNB-poisoned fibres had a slightly larger concentration of CP. This increase, which is not

easy to understand, has formerly been seen and discussed by Dydyńska & Wilkie (1966).

The level of ATP dropped to 18·9 % in muscles poisoned with CN^- plus iodoacetate and to 16·3 % in FDNB-poisoned ones, but not to zero. This explains the observation that exhausted fibres were usually plastic and not in rigor. In earlier publications (Grabowski, Lobsiger & Lüttgau, 1972) we were able to show that at least part of the remaining energy reserves in exhausted fibres could apparently be utilized for contractile activity if a subthreshold concentration of caffeine was applied. It is known that caffeine causes the release of Ca^{2+} from the sarcoplasmic reticulum. Contractile fatigue in this stage thus appears to be mainly due to a deficiency in excitation–contraction coupling or Ca^{2+} release (Eberstein & Sandow, 1963; Grabowski *et al.* 1972; Nassar-Gentina, Passonneau & Rapoport, 1981). During repetitive activity twitch height falls to low values long before the level of ATP decreases (Nassar-Gentina *et al.* 1981). The occasionally observed failure to attain the increase in K^+ conductance in CN^--poisoned fibres during repetitive stimulation may thus be explained by an 'escape' from exhaustion through an early interruption in excitation– contraction coupling.

The activation of K^+ channels occurs during a more advanced stage of exhaustion. In fibres poisoned with CN^- plus iodoacetate it develops in a sigmoid time curve during the short period of only forty to fifty declining twitches (Fink & Lüttgau, 1976). It probably starts only after CP is completely used up, and the ATP level begins to fall. The remaining ATP content (15–20 % of the original value) on the other side appears to be sufficient to maintain ATP-driven ion pumps (e.g. Nelson & Blaustein, 1980) unless secondary metabolites interfere by product inhibition or secondary reactions (see Dawson, Gadian & Wilkie (1978) for further details).

DISCUSSION

The present experiments, together with those published earlier (Fink & Lüttgau, 1976), show that, independent of the poison used, the increase in K^+ conductance develops when twitches have ceased and the level of ATP has fallen to about 20 % of the normal value. Consequently, the same effect can be reached with a poison which blocks oxidative metabolism (CN^-) or one which inhibits the enzyme creatine kinase (FDNB). From these findings it is likely that the effect is not dependent on the accumulation of intermediate metabolites resulting from interference with energy metabolism. The reversibility of the conductance increase shows that the membrane is not simply 'impaired'. It could be explained by a biochemical reaction, e.g. phosphorylation, depending in some way on the internal concentration of Ca^{2+} and ATP (Lüttgau, 1977; Peyer, Cachelin, Levitan & Reuter, 1982). The intermediate state, defined in Results, may be characterized as follows: (1) the internal free Ca^{2+} concentration is only slightly increased because fibres are not in rigor; (2) the store of CP is depleted or blocked and the concentration of ATP begins to fall.

The activation of the K^+ conductance in exhausted fibres occurs during only a few short voltage-clamp steps. Which factor is ultimately responsible for this drastic alteration in membrane characteristics? Depolarization induces both a release of Ca^{2+} from the sarcoplasmic reticulum and a faster consumption of ATP. The latter

probably also occurs after the injection of $CaEGTA^{2-}$ and the application of caffeine. It appears, therefore, difficult to discern whether an elevation of $[Ca^{2+}]_i$ or a fall in ATP, or both, initiates the increase in K^+ conductance during repetitive stimulation in the intermediate state. The suppression of conductance increase after the injection of H_2EGTA^{2-}, i.e. after a reduction in $[Ca^{2+}]_i$, is probably the most convincing result in favour of a $Ca^{2+}{}_i$-induced activation of the K^+ conductance. This phenomenon is well known (cf. Meech, 1978). It was first detected in erythrocytes, and recently it was also demonstrated in fragments of muscle fibres (patch clamp: Pallotta, Magleby & Barrett, 1981; vesicles from the transverse tubular system incorporated in planar bilayers: Latorre, Vergara & Hidalgo, 1982). It seems reasonable to relate the increase in K^+ conductance observed in whole fibres to these findings in muscle fragments. Detailed models concerning the activation and inactivation kinetics of Ca^{2+}-activated K^+ channels were presented by Methfessel & Boheim (1982).

The role of metabolic energy in this context appears to be more complex and less well understood. Shortage of energy must in the end lead to an increase in internal free Ca^{2+} since ATP is needed for the reaccumulation of Ca^{2+}. In this way the promoting effect of exhaustion could be explained. The following observations suggest, however, that metabolic energy appears to be more directly involved in this effect.

(1) In normal fibres the Ca^{2+}-induced increase in K^+ conductance was not seen during twitches and short tetani, although $[Ca^{2+}]_i$ may reach a concentration between 10^{-6} and 10^{-5} M (Grabowski, *et al.* 1972).

(2) The concentration of free Ca^{2+} in fibres with a high K^+ conductance is, if at all, only slightly larger than that in normal ones, since mechanically exhausted fibres were usually not in rigor, at least in experiments at low temperatures (Fink & Lüttgau, 1976).

(3) In the experiments in which a mixture of $CaEGTA^{2-}$ and H_2EGTA^{2-} with a free Ca^{2+} concentration of 10^{-5} M was injected we observed a distinct increase in conductance but not a contracture.

(4) In normal fibres caffeine in relatively high concentrations caused a contracture but only a relatively small increase in membrane conductance (Axelsson & Thesleff, 1958; Fink & Lüttgau, 1976). In exhausted fibres low caffeine concentrations drastically increased the membrane conductance without inducing a contracture.

There remain some uncertainties as to the Ca^{2+} threshold for the activation of force or rigor. It appears to be slightly larger in exhausted fibres. Over-all, however, the cited observations no longer contradict each other, if the assumption is made that the sensitivity of the K^+ conductance towards $[Ca^{2+}]_i$ increases during exhaustion. Related observations of low and high Ca^{2+}-sensitive states of the K^+ channel and their direct dependence on the concentration of metabolic energy (ATP), and probably other factors, have been described in red blood cells (cf. Lew, Bookchin, Brown & Ferreira, 1980; Lassen, Pape & Vestergaard-Bogind, 1980).

The experiments, however, do not exclude a less direct action of metabolic energy. It is conceivable that an alteration of the internal fluid in the final stage of exhaustion facilitates in some unknown way the action of internal Ca^{2+}. A fall in ATP, for example, should be accompanied by an increase in Mg^{2+}, AMP or inorganic phosphate. In addition, internal free Ca^{2+} for contraction activation may not increase

uniformly throughout the cross-section of the fibre. A metabolically driven Ca^{2+} pump may normally lower the Ca^{2+} level near surface and tubular membranes. Thus, further experiments are needed to clarify the role of metabolism in this phenomenon.

This research was supported by SFB 114 'Bionach'. The concentrations of ATP and CP were measured in co-operation with Dr D. Busse and Dr R. Thieleczek who took part in the calculation of internal Ca-concentrations. We wish to thank both for their help and stimulating discussions.

REFERENCES

ADRIAN, R. H. (1956). The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* **133**, 631–658.

ALMERS, W. (1972*a*). Potassium conductance changes in skeletal muscle and the potassium concentration in the transverse tubules. *J. Physiol.* **225**, 33–56.

ALMERS, W. (1972*b*). The decline of potassium permeability during extreme hyperpolarization in frog skeletal muscle. *J. Physiol.* **225**, 57–83.

ALMERS, W. (1976). Differential effects of tetracaine on delayed potassium channels and displacement currents in frog skeletal muscle. *J. Physiol.* **262**, 613–637.

AXELSSON, J. & THESLEFF, S. (1958). Activation of the contractile mechanism in striated muscle. *Acta physiol. scand.* **44**, 55–66.

CAIN, D. F., DAVIES, R. E. & INFANTE, A. A. (1962). Adenosine triphosphate and phosphorylcreatine, as energy supplies for single contractions of working muscle. *Nature, Lond.* **196**, 214–217.

CARSLAW, H. S. & JAEGER, J. C. (1959). *Conduction of heat in Solids*, 2nd edn. Oxford: Clarendon Press.

COSTANTIN, L. L. (1968). The effect of calcium on contraction and conductance threshold in frog skeletal muscle. *J. Physiol.* **195**, 119–132.

DAVIES, R. E. (1965). On the mechanism of muscular contraction. *Essays in Biochemistry* **1**, 29–55.

DAWSON, M. J., GADIAN, D. G. & WILKIE, D. R. (1978). Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature, Lond.* **274**, 861–866.

DYDYŃSKA, M. & WILKIE, D. R. (1966). The chemical and energetic properties of muscles poisoned with fluorodinitrobenzene. *J. Physiol.* **184**, 751–769.

EBERSTEIN, A. & SANDOW, A. (1963). Fatigue mechanism in muscle fibres. In *The Effect of Use and Disuse on Neuromuscular Functions*, pp. 515–526. Prague.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**, 71–107.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

FINK, R. (1978). Die Modifizierung aktivierbarer Kaliumkanäle bei Erschöpfung der Stoffwechselenergie. Thesis, Faculty of Biology, Ruhr-University, Bochum.

FINK, R., GROCKI, K. & LÜTTGAU, H.Ch. (1980*a*). The effect of energy deprivation and hyperosmolarity upon tubular structures and electrophysiological parameters of muscle fibres. *Eur. J. Cell Biol.* **21**, 101–108.

FINK, R., GROCKI, K. & LÜTTGAU, H.Ch. (1980*b*). Na/K selectivity, ion conductances and net fluxes of K^+ and Na^+ in metabolically exhausted muscle fibres. *Eur. J. Cell Biol.* **21**, 109–115.

FINK, R. & LÜTTGAU, H.Ch. (1976). An evaluation of the membrane constants and the potassium conductance in metabolically exhausted muscle fibres. *J. Physiol.* **263**, 215–238.

FINK, R. & WETTWER, E. (1978). Modified K-channel gating by exhaustion and the block by internally applied TEA^+ and 4-aminopyridine in muscle. *Pflügers Arch.* **374**, 289–292.

GRABOWSKI, W., LOBSIGER, E. A. & LÜTTGAU, H.Ch. (1972). The effect of repetitive stimulation at low frequencies upon the electrical and mechanical activity of single muscle fibres. *Pflügers Arch.* **334**, 222–239.

HODGKIN, A. L. & NAKAJIMA, S. (1972). The effect of diameter on the electrical constants of frog skeletal muscle fibres. *J. Physiol.* **221**, 105–120.

INFANTE, A. A. & DAVIES, R. E. (1965). The effect of 2, 4-dinitrofluorobenzene on the activity of striated muscle. *J. biol. Chem.* **240**, 3996–4001.

KAO, C. Y. & STANFIELD, P. R. (1968). Actions of some anions on electrical properties and mechanical threshold of frog twitch muscle. *J. Physiol.* **198**, 291–309.

KAO, C. Y. & STANFIELD, P. R. (1970). Actions of some cations on the electrical properties and mechanical threshold of frog sartorius muscle fibres. *J. gen. Physiol.* **55**, 620–639.

LAMPRECHT, W., STEIN, P., HEINZ, F. & WEISSER, H. (1970). Creatinphosphat. In *Methoden der emzymatischen Analyse*, 2nd edn, ed. BERGMEYER, H. U., pp. 1729–1733. Weinheim/Bergstr., F.R.G.: Verlag Chemie.

LAMPRECHT, W. & TRAUTSCHOLD, I. (1970). ATP. Bestimmung mit Hexokinase und Glucose-6-phosphat Dehydrogenase. In *Methoden der enzymatischen Analyse*, 2nd edn., ed. BERGMEYER, H. U., pp. 2024–2033. Weinheim/Bergstr., F.R.G.: Verlag Chemie.

LASSEN, U. V., PAPE, L. & VESTERGAARD-BOGIND, B. (1980). Calcium related transient changes in membrane potential of red cell. In *Membrane Transport in Erythrocytes*, Alfred Benzon Symposium no. **14**, ed. LASSEN, U. V., USSING, H. H. & WIETH, J. O., pp. 255–268. Copenhagen: Munksgaard.

LATORRE, R., VERGARA, C. & HIDALGO, C. (1982). Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. natn. Acad. Sci. U.S.A.* **79**, 805–809.

LEW, V. L., BOOKCHIN, R. M., BROWN, A. M. & FERREIRA, H. G. (1980). Ca-sensitive modulation. In *Membrane Transport in Erythrocytes*, Alfred Benzon Symposium no. **14**, ed. LASSEN, U. V., USSING, H. H., WIETH, J. O., pp. 196–207. Copenhagen: Munksgaard.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

LÜTTGAU, H.Ch. (1965). The effect of metabolic ihibitors on the fatigue of the action potential in single muscle fibres. *J. Physiol.* **178**, 45–67.

LÜTTGAU, H.Ch. (1977). New trends in membrane physiology of nerve and muscle fibres. *J. comp. Physiol.* **120**, 51–70.

LÜTTGAU, H.Ch. & MOISESCU, G. D. (1978). Ion movements in skeletal muscle in relation to the activation of contraction. In *Physiology of Membrane Disorders*, ed. ANDREOLI, T. E., HOFFMAN, J. F. & FANESTIL, D., pp. 493–515. New York: Plenum Publishing Corporation.

MEECH, R. W. (1978). Calcium-dependent potassium activation in nervous tissues. *A. Rev. Biophys. Bioeng.* **7**, 1–18.

METHFESSEL, Ch. & BOHEIM, G. (1982). The gating of single calcium-dependent potassium channels is described by an activation/blockade mechanism. *Biophys. Struct. Mech.* **9**, 35–60.

MOISESCU, D. G. & THIELECZEK, R. (1978). Calcium and strontium concentration changes within skinned muscle preparations following a change in the external bathing solution. *J. Physiol.* **275**, 241–262.

NASSAR-GENTINA, V., PASSONNEAU, J. V. & RAPOPORT, S. I. (1981). Fatigue and metabolism of frog muscle fibers during stimulation and in response to caffeine. *Am. J. Physiol.* **241**, C160–166.

NELSON, M. T. & BLAUSTEIN, M. P. with the technical assistance of E. M. SANTIAGO (1980). Properties of sodium pumps in internally perfused barnacle muscle fibers *J. gen. Physiol.* **75**, 183–206.

PALLOTTA, B. S., MAGLEBY, K. L. & BARRETT, J. N. (1981). Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature, Lond.* **293**, 471–474.

PEYER, J. E. de, CACHELIN, A. B., LEVITAN, I. B. & REUTER, H. (1982). Ca^{2+}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4207–4211.

ROBERTSON, S. P., JOHNSON, D. J. & POTTER, J. D. (1981). The time-course of Ca^{2+} exchange with calmodulin, troponin, parvalbumin, and myosin in response to transient increases in Ca^{2+}. *Biophys. J.* **34**, 559–569.

STEFANI, E. & SCHMIDT, H. (1972). A convenient method for repeated intracellular recording of action potentials from the same muscle fibre without membrane damage. *Pflügers Arch.* **334**, 276–278.

WETTWER, E. (1981). Voltage-clamp Untersuchungen zur Kaliumleitfähigkeit der Skelettmuskelfaser des Froches. Thesis, Faculty of Biology, Ruhr-University, Bochum.

WETTWER, E., HASE, S. & LÜTTGAU, H.Ch. (1980). The increase in potassium conductance in metabolically poisoned skeletal muscle fibres. *Proc. Int. Union Physiol. Sci.* **14**, 785.

WETTWER, E., HASE, S. & LÜTTGAU, H.Ch. (1981). The increase in potassium conductance in metabolically poisoned skeletal muscle fibres. *Adv. Physiol. Sci.* **5**, 263–269.