Journal of Physiology (1992), **455**, *pp.* 663–678
With 8 figures
Printed in Great Britain

Ca^{2+}-DEPENDENT HEAT PRODUCTION UNDER BASAL AND NEAR-BASAL CONDITIONS IN THE MOUSE SOLEUS MUSCLE

BY A. CHINET, A. DECROUY AND P. C. EVEN

From the Department of Physiology, University of Geneva, CMU, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

(*Received 8 October 1991*)

SUMMARY

1. The rate of energy expended for the clearance of sarcoplasmic Ca^{2+} by sarcoreticular Ca^{2+} uptake process(es), plus the concomitant metabolic reactions, was evaluated from measurements of resting heat production by mouse soleus muscle before and after indirect inhibition of Ca^{2+} uptake by sarcoplasmic reticulum (SR).

2. *Direct* inhibition of the Ca^{2+}, Mg^{2+}-ATPase of SR membrane in intact muscle preparations exposed to the specific inhibitor 2,5-di(*tert*-butyl-1,4-benzohydroquinone (tBuBHQ) slowly increased the rate of heat production ($\dot{E}$). *Indirect* inhibition of SR Ca^{2+} uptake was obtained by reducing sarcoplasmic Ca^{2+} concentration (Ca_i^{2+}) as a consequence of reducing Ca^{2+} release from the SR using dantrolene sodium. This promptly decreased $\dot{E}$ by 12%. Exposure of the preparations to an Mg^{2+}-enriched environment (high Mg^{2+}) or to the chemical phosphatase 2,3-butanedione monoxime (BDM), two other procedures aimed at decreasing SR Ca^{2+} release, also acutely decreased $\dot{E}$, by 20 and 24%, respectively.

3. Subthreshold-for-contracture depolarization of the sarcolemma achieved by increasing extracellular K^+ concentration to 11·8 mM induced a biphasic increase of $\dot{E}$: an initial peak to 290% of basal $\dot{E}$, followed by a plateau phase at 140% of basal $\dot{E}$ during which resting muscle tension was increased by less than 3%. Most, if not all, of the plateau-phase metabolic response was quickly suppressed by dantrolene or high Mg^{2+} or BDM. Another means of increasing SR Ca^{2+} cycling was to partially remove the calmodulin-dependent control of SR Ca^{2+} release using the calmodulin inhibitor W-7. The progressive increase in $\dot{E}$ with 30 μM-W-7 was largely reduced by dantrolene or high Mg^{2+} or BDM.

4. In the presence of either dantrolene or BDM to prevent the effect of W-7 on SR Ca^{2+} release, exposure of the muscle to W-7 acutely suppressed about 3% of $\dot{E}$. This and the above results confirm that the plasmalemmal, calmodulin-dependent Ca^{2+}-ATPase, although a qualitatively essential part of the Ca_i^{2+} homeostatic system of the cell, can only be responsible for a very minor part of the energy expenditure devoted to the homeostasis of Ca_i^{2+}. Active Ca^{2+} uptake by SR which, at least in the submicromolar range of Ca_i^{2+}, is expected to be responsible for most of this Ca^{2+}-dependent energy expenditure, might dissipate up to 25–40% of total metabolic energy in the intact mouse soleus under basal and near-basal conditions.

INTRODUCTION

According to quantitative predictions for striated muscles based on experiments on isolated organelles (Carafoli, 1985; Hidalgo, Gonzalez & Garcia, 1986), the main energy dissipating process in the homeostasis of Ca_i^{2+} is Ca^{2+} uptake by SR (see also Ponce-Hornos, 1989). This is also supported by the recent observation that measurable changes of Ca_i^{2+} in cultured muscle cells do not necessarily entail changes of $^{45}Ca^{2+}$ fluxes across the sarcolemma (Jacobs, Benders, Oosterhof, Veerkamp, Wevers & Joosten, 1991). Since under steady-state Ca^{2+} recirculation across the SR membrane the rate of active Ca^{2+} uptake by SR equals that of passive Ca^{2+} release, the rate of energy expenditure associated with Ca_i^{2+} homeostasis should in principle be cut down upon inhibition of the passive, as well as of the active limb of the cycle. Indeed, the rate of Ca^{2+} uptake by SR is governed by Ca_i^{2+} to the fourth power (see Barany, 1987, and recent confirmation by Klein, Kovacs, Simon & Schneider, 1991) so that, basically, Ca_i^{2+} can be considered a tightly controlled variable, and inhibition of SR Ca^{2+} release under these conditions may prove a useful, *indirect* way to inhibit SR Ca^{2+} reuptake and the series of associated metabolic reactions in the cell.

In the intact cell under basal or near-basal conditions, the high-conductance calcium-release channel of SR membrane ought to be blocked by either an increase of sarcoplasmic Mg^{2+} or a decrease of adenine nucleotides (Smith, Coronado & Meissner, 1985). A simple way to increase intracellular Mg^{2+} in the concentration range in which it can inhibit SR Ca^{2+} release (Hymel, Inui, Fleischer & Schindler, 1988) is to increase extracellular Mg^{2+} by 10 mM (see Gonzalez-Serratos, Rasgado-Flores, Sjodin & Montes, 1988). Another way to indirectly inhibit SR Ca^{2+} uptake is to expose muscles to 2,3-butanedione monoxime (BDM) which, at concentrations up to 10 mM, mainly reduces Ca^{2+} release from SR (Fryer, Gage, Neering, Dulhunty & Lamb, 1988) and entails detectable, sustained reductions of Ca_i^{2+} in both skeletal and smooth muscles (van der Bent, Decrouy & Chinet, 1990). The precise mechanism of BDM action on SR Ca^{2+} release is unknown, but an interesting possibility is that BDM acts on the SR calcium channel *via* the reduction of adenine nucleotide concentration, as a consequence of ATP synthetase inhibition (Mojon, Zhang, Schaub & Oetliker, 1991). Finally, Ca^{2+} release through this channel can also be decreased by dantrolene (Ohta, Ito & Ogha, 1990; Jacobs *et al.* 1991, op. cit.). *Direct* inhibition of SR Ca^{2+} uptake with 25 μM-2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), an inhibitor of the Ca^{2+}, Mg^{2+}-ATPases of skeletal muscle SR (Missiaen, Wuytack, Raeymaekers, De Smedt, Droogmans, Declerck & Casteels, 1991) and liver endoplasmic reticulum (Moore, McConkey, Kass, O'Brien & Orrenius, 1987) that can be applied to intact cells, may *a priori* be considered a potential tool to determine the energy cost of Ca_i^{2+} homeostasis. However, the increase of Ca_i^{2+} associated with direct inhibition of the enzyme (see Kass, Duddy, Moore & Orrenius, 1989) produces disturbing secondary effects on cell metabolism.

Indirect determination of Ca^{2+}-dependent heat production can also be performed during subthreshold-for-contracture potassium depolarization of the sarcolemma which, in frog muscles, entails a sustained rise of Ca_i^{2+} as measured with a Ca^{2+}-sensitive microelectrode (Lopez, Alamo, Caputo, DiPolo & Vergara, 1983) or aequorin (Snowdowne, 1985), a detectable increase of heat production (Solandt,

1936), and of O_2 consumption which is partly suppressed by dantrolene (Erlij, Shen, Reinach & Schoen, 1982). As an alternative means of enhancing Ca^{2+}-dependent heat production, it was possible to partially suppress the calmodulin-dependent control of SR Ca^{2+} release using the calmodulin antagonist W-7. This procedure was preferred to the progressive locking open of SR Ca^{2+} channels with subcontracture doses of ryanodine (Fryer, Lamb & Neering, 1989).

We report here microcalorimetric estimates of Ca^{2+}-dependent heat production obtained on intact soleus muscles from adult mice during both basal, and slightly enhanced Ca^{2+} release by SR. The results suggest that, under these conditions, as much as 25–45% of muscle cell energy metabolism is devoted to Ca^{2+} recirculation across the SR membrane.

METHODS

Preparations. The non-perfused, intact muscle is a convenient tissue-cell preparation provided muscle size, shape, specific metabolic rate and environmental O_2 availability (therefore temperature) have been adequately selected to ward off the progressive appearance of histochemical alterations, such as those recently described by van Breda, Keizer, Glatz & Geurten (1990) in the core of mouse soleus muscles incubated at 37 °C. The present experiments were performed at 30 °C on soleus muscles from adult, normal mice (C57BL/10, Swiss albino or ICR) weighing 25–50 g. After decapitation of the animal, the muscles were carefully dissected out intact, freed only of loosely attached connective tissue. Both muscles of a pair were held by their tendons at about 130% of relaxation length of the freshly dissected muscle, on a stainless-steel frame. The preparation was then placed in the test chamber of a twin calorimeter perfused with a Krebs–Ringer bicarbonate solution for up to 3 h, the time necessary for stabilization of the thermostat-controlled heat flux microcalorimeter.

Chemicals and solutions. Chemicals, of analytical grade, were purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). The superfusion solution had the following composition (in mM): NaCl, 116·8; $NaHCO_3$, 25; KCl, 5·9; $MgSO_4$, 1·2; NaH_2PO_4, 1·2; $CaCl_2$, 1·27 (added after pre-equilibration at pH 7·4 with a 95% O_2–5% CO_2 gas mixture) and glucose, 5. In the Mg^{2+}-enriched solution, 10 mM-$MgCl_2$ replaced 15 mM-NaCl of the standard solution. To keep the $[K^+][Cl^-]$ concentration product unchanged when extracellular K^+ was increased, part of the NaCl was replaced with sodium isethionate. Sodium dantrolene (a gift from Norwich-Eaton Pharmaceuticals Inc., USA), dissolved in 1 M-NaOH, W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide, Sigma), *N*-ethylmaleimide, dissolved in methanol and tBuBHQ (2,5-di(*tert*-butyl)-1,4-benzohydroquinone; Aldrich-Chemie, Steinheim, Germany), dissolved in dimethyl sulphoxide, were added to solutions immediately before use. It was checked that the solvents *per se*, at the concentration used (1 ml methanol and/or dimethyl sulphoxide per litre of final solution), had no effect on muscle-cell energy metabolism. BDM (2,3-butanedione monoxime, Sigma) was dissolved directly in the superfusion solution.

Heat flow measurements. The calorimetric signal, proportional to total heat production rate ($\dot{E}$) by a pair of soleus muscles (12–26 mg wet weight measured at the end of the experiment after removal of the four tendons and rapid blotting on filter paper), was equal to the voltage difference between two series of six thermal gradient layers surrounding test and control chambers of a twin apparatus (for details, see Chinet, Clausen & Girardier, 1977) minus a blank difference, close to zero, which was recorded before the introduction of the muscles into the test chamber. The proportionality constant between this signal and $\dot{E}$ was determined at various perfusion flow rates using a calibrated field-effect transistor as the heat source. At the flow rate of 2 ml min^{-1}, the constant was 9·9 $\mu W\ \mu V^{-1}$ for the most sensitive and 10·5 $\mu W\ \mu V^{-1}$ for the least sensitive of the calorimeter channels used. The rate of heat production of a muscle pair was calculated as the product of this constant and the recorded voltage difference, and expressed in mW (g wet muscle weight)$^{-1}$.

O_2 consumption measurements. At the outlet of the chambers, an O_2 cathode measured alternately the O_2 partial pressure (P_{O_2}) of the solution from the chamber where the muscle pair was placed (test chamber) and the P_{O_2} of the solution from the control chamber. From the difference between

the two P_{O_2} values (minus a blank P_{O_2} difference), the oxygen consumption was calculated according to the Fick principle and expressed in nanomoles of O_2 per second per gram of wet muscle weight.

Tension measurements. In some experiments, tension of a single muscle was measured in an open, temperature-controlled chamber perfused with the effluent from one of the calorimeter chambers. The muscle was placed horizontally and maintained at constant length (130% of relaxed length), with its heel-bone insertion tendon fixed near the upstream extremity of the chamber and its tibial insertion tendon pinned with a stainless-steel needle glued onto the vertical blade of a strain gauge (AE 801, AME, Horten, Norway). To prevent O_2 and CO_2 losses by diffusion, the chamber was covered with a glass slip from its upstream extremity down to the point of attachment of the muscle to the strain gauge.

All results are presented as means ± S.E.M., with the number of experiments in parentheses. Differences were analysed with Student's *t* test.

RESULTS

Direct inhibition of Ca^{2+} uptake processes

Figure 1 illustrates that inhibition of the SR Ca^{2+},Mg^{2+}-ATPase *in situ* by tBuBHQ in the mouse soleus at rest (all three strains of mice) was followed by a slow increase of the heat production rate ($\dot{E}$), of 0·42 ± 0·05 (8) mW g^{-1} in about 90 min (which is 13·8 ± 1·9% of basal $\dot{E}$). Longer exposures to tBuBHQ did not entail much further increase in $\dot{E}$ even when, sometimes, there was a late increase in resting muscle tension (about 10% at 90 min, result not shown). As could be anticipated from the results of experiments on liver microsomes (Moore *et al.* 1987), the effect of tBuBHQ was not reversed by washing.

Suggestions that potassium may act as a counter-ion in SR Ca^{2+} release (Chiu & Haynes, 1980; Abramcheck & Best, 1989) and that a proton gradient across the SR membrane may be responsible for active SR Ca^{2+} reuptake (Madeira, 1978; Wetzel, Liebner & Gros, 1990) are compatible with the idea that a K^+,H^+-ATPase may be active, together with the well-known Ca^{2+}, Mg^{2+}-ATPase, in the process of Ca^{2+} uptake by SR. A functional coupling between a proton pump and transport of calcium which is parallel to that by the Ca^{2+}, Mg^{2+}-ATPase has recently been described by Thévenod, Dehlinger-Kremer, Kemmer, Christian, Potter & Schulz (1989) in a secretory cell. To test whether any compensatory activation of a proton ATPase may have been responsible for the increase in $\dot{E}$ observed under tBuBHQ, we subjected some intact muscles to both tBuBHQ and the H^+-ATPase inhibitor *N*-ethylmaleimide (NEM, 100 μM). Contracture quickly developed under these conditions (tension was more than doubled in 15 min). In spite of this, the rate of heat production slowly decreased (see Fig. 6, lower part). A tentative interpretation of this result was that Ca_i^{2+} was increased, inducing contracture, due to the halting of some overall Ca^{2+} transport ATPase. The relative constancy of $\dot{E}$ probably resulted from the fact that the decrease of energy dissipation by this ATPase was partly compensated by the increase of energy dissipation by the myosin ATPase. After the first 15 min, while the contracture continued to develop for at least 40 min up to a maximum of 7–8 times resting tension, $\dot{E}$ temporarily (re)increased but it always subsided to less than basal $\dot{E}$ after 2 h.

Indirect inhibition of Ca^{2+} uptake processes

Figure 2 illustrates the reversible effects of high Mg^{2+} and BDM on $\dot{E}$, and the non-reversible effect of dantrolene. Exposure of the preparations to an Mg^{2+}-enriched

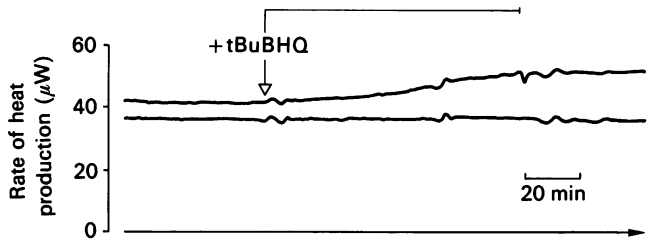

Fig. 1. Upper trace, effect of the Ca^{2+},Mg^{2+}-ATPase inhibitor tBuBHQ (25 μM added to the superfusion solution) on the resting muscle heat production rate ($\dot{E}$) as a function of time. Lower trace, control experiment showing that the solvent of the drug (see Methods) had no effect *per se* on $\dot{E}$ (the oscillations of the calorimeter signal which appear on both traces are thermostatic artifacts). For as long as it was looked for (2 h), no reversal of the effect of tBuBHQ was observed after removal of the drug from the superfusion solution. NB, these graphs and all of the following graphs showing *total* $\dot{E}$ of pairs of adult mouse soleus muscles as a function of time, were redrawn from the original records. A horizontal line attached to a vertical arrow indicates the period of time during which one or several preparations were exposed to drugs and/or otherwise modified superfusion solutions. Mean calorimetric values summarizing the results of series of experiments are given in the text, where they are expressed *per unit muscle weight*, and as *changes* of specific $\dot{E}$ with respect to basal.

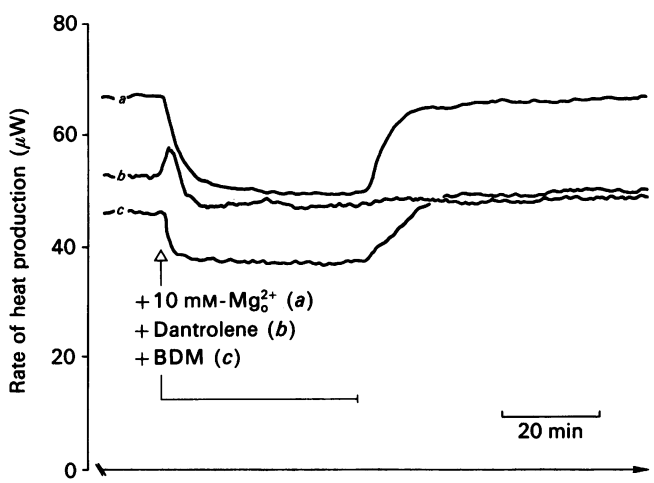

Fig. 2. Acute lowering of muscle heat production rate upon indirect inhibition of SR Ca^{2+} uptake processes by high Mg^{2+} (11·2 mM-extracellular Mg^{2+}), dantrolene (10 μM) and BDM (10 mM). The effect of dantrolene was not reversed by washing.

environment (high Mg^{2+}, 11·2 mM) produced a sustained and reversible decrease of basal $\dot{E}$ of 0·66±0·05 (7) mW g^{-1} (20·3±1·1 %). Exposure to 10 mM-BDM induced a reversible decrease of basal $\dot{E}$ of 0·79±0·19 (6) mW g^{-1} (24·1±5·0 %), i.e. of the same size as that induced by the Mg^{2+}-enriched solution, and sustained for as long as it was observed (55 min). A dose–response study showed that the maximum inhibitory effect of BDM was practically obtained at the 10 mM concentration (20 mM: −0·84±0·07 (6) mW g^{-1}, no significant difference), and that 2 mM-BDM produced

half-maximum inhibition ($-0{\cdot}41 \pm 0{\cdot}04$ (3) mW g^{-1}). Both heat production and respiration (when measured) were decreased; the mean energetic equivalent of O_2 for this decrease in metabolic rate was 425 ± 52 (14) kJ mol^{-1}, which is not different from the ratio between basal $\dot{E}$ and O_2 consumption (457 ± 51 (14) kJ mol^{-1}). Finally, exposure to 10 μM-dantrolene decreased muscle heat production by $0{\cdot}36 \pm 0{\cdot}08$ (6) mW g^{-1} ($11{\cdot}9 \pm 2{\cdot}1$ % of basal $\dot{E}$) in 5–10 min. Consecutive exposure of the same preparations to tBuBHQ, still in the presence of dantrolene, increased $\dot{E}$ by only $0{\cdot}17 \pm 0{\cdot}05$ mW g^{-1} ($5{\cdot}9 \pm 1{\cdot}5$ %) in 90 min. The difference with respect to 0·42 mW g^{-1} (i.e. to the effect of a 90 min exposure to tBuBHQ in the absence of dantrolene) is statistically significant ($P < 0{\cdot}01$).

Indirect stimulation of Ca^{2+} uptake processes

As illustrated in Fig. 3 (heat and tension traces), the acute elevation of extracellular K^+ concentration (K_o^+) to 11·8 mM (plasmalemmal depolarization of about 10 mV), at constant osmolarity and $[K^+][Cl^-]$ concentration product, induced a transient increase of the heat production rate, followed by a sustained elevation of metabolic rate with respect to basal. $\dot{E}$ peaked to $5{\cdot}04 \pm 0{\cdot}35$ (21) mW g^{-1} ($+191 \pm 15$ % with respect to basal $\dot{E}$) in about 5 min. During the following, sustained phase, $\dot{E}$ and O_2 consumption rate were elevated by $1{\cdot}03 \pm 0{\cdot}07$ (21) mW g^{-1}

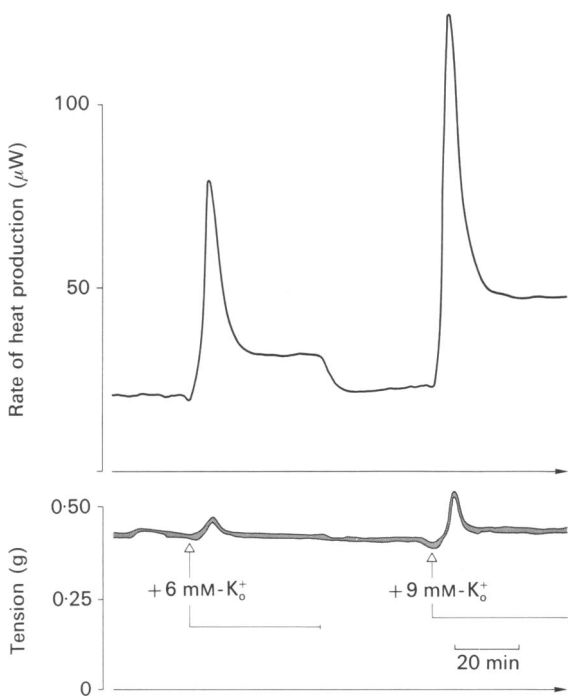

Fig. 3. Effects of 40 min periods of subthreshold-for-contracture elevations of K_o^+ to 11·8 and 14·9 mM (by adding successively 6 and 9 mM-K_o^+) on muscle heat production rate (upper trace) and tension (lower trace). The plateau phase of increased energy metabolism (after the peak) ended immediately upon normalization of K_o^+, after several hours (see text) as well as after 40 min.

and $2{\cdot}50 \pm 0{\cdot}22$ (21) nmol s^{-1} g^{-1} respectively, that is, by as much as $39 \pm 3\%$ and $44 \pm 6\%$ with respect to basal values. In contrast, muscle tension increased by $8{\cdot}2 \pm 1{\cdot}4$ (13)% during the peak phase, and a mere $1{\cdot}9 \pm 0{\cdot}2$ (13)% with respect to resting tension during the sustained phase. As judged from the ratio between the maintained changes of $\dot{E}$ and O_2 consumption rate (470 ± 42 (21) kJ mol^{-1}), the energetic equivalent of O_2 was not modified from its basal value (461 ± 31 (21) kJ mol^{-1}). This suggests that the sustained responses to potassium corresponded to true steady states of elevated oxidative metabolism. With 11·8 mM-K_o^+, the muscles never developed contractures (i.e. progressive and *transitory* increases in tension). These only appeared at, or above, 15 mM-K_o^+, sometimes after a delay. Below 15 mM-K_o^+ the increases of $\dot{E}$ and O_2 consumption rate, and the marginal increase in tension, were steady for as long as they were observed (up to several hours).

Indirect inhibition of stimulated Ca^{2+} uptake

Comparisons of the effects of high Mg^{2+}, dantrolene and BDM under basal conditions and during subthreshold-for-contracture potassium depolarization (see Figs 4 and 5) suggested that most of the maintained increase in metabolic rate due to potassium was related to the Ca_i^{2+} homeostatic activity of SR. Indeed, the sustained $\dot{E}$ response to potassium was decreased by $79 \pm 5\%$ (14), with respect to control, under inhibition of SR Ca^{2+} release.

Direct inhibition of stimulated Ca^{2+} uptake

It apparently took a long time for Ca^{2+}-dependent heat under 11·8 mM-K_o^+ to be

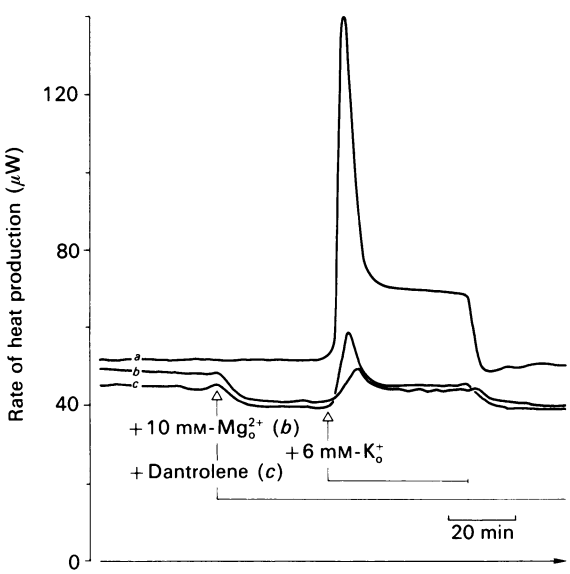

Fig. 4. Metabolic responses of three pairs of non-contracting soleus muscles to a 40 min exposure to 11·8 mM-total K_o^+: control (*a*), under high Mg^{2+} (11·2 mM-extracellular Mg^{2+}, *b*), in the presence of 10 μM-dantrolene (*c*). Note that dantrolene was less effective than high Mg^{2+} in suppressing the response to potassium.

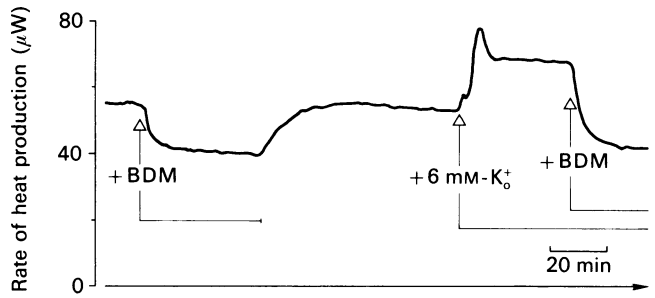

Fig. 5. Effects of BDM (10 mM added to the superfusion solution) on the rate of muscle heat production, under basal conditions and during the plateau phase of a subthreshold-for-contracture potassium depolarization (11·8 mM-total K_o^+).

suppressed by direct inhibition of the SR calcium uptake processes with tBuBHQ + NEM. Figure 6 shows samples of the time courses of $\dot{E}$ and tension under 11·8 mM-total K_o^+, and of $\dot{E}$ under basal conditions. Again, the reason for this apparent slowness was evident: as energy dissipation by one or several Ca^{2+} uptake processes was decreased, dissipation by the myosin ATPase was increased (contracture). When after a 2 h exposure to tBuBHQ + NEM total heat production rate had come back to 88 ± 11 (6) % of basal $\dot{E}$ there was still, under 11·8 mM-K_o^+ as under normal K_o^+, a significant residue of the contracture which had quickly started to develop upon exposure of the muscles to the drugs. The impossibility of separating in time the opposite effects on $\dot{E}$ of two events (i.e. the direct inhibition of ATP-consuming processes and the increase of Ca_i^{2+}) clearly disqualified the *direct* approach, as opposed to the *indirect* one, in our attempts to quantify cellular energy expenditure for calcium homeostasis in the non-contracting skeletal muscle.

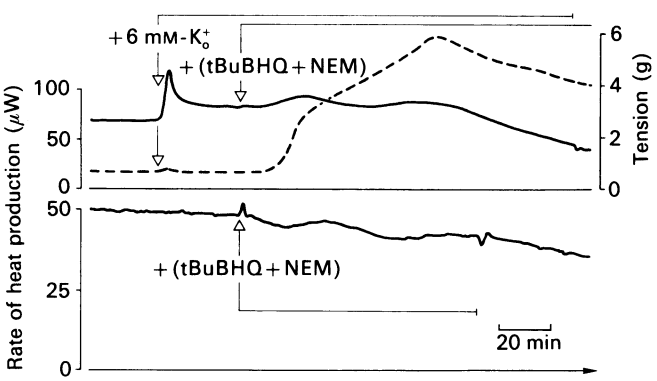

Fig. 6. Effects of tBuBHQ (25 μM) + NEM (100 μM) on muscle heat production (continuous line) and tension (dashed line) as a function of time. Upper part, the drugs were applied during the plateau phase of the metabolic response to a subthreshold-for-contracture potassium depolarization (11·8 mM-total K_o^+). Lower part, effect of the drugs on basal heat production rate; the contracture induced by tBuBHQ + NEM under these control conditions was similar to that shown in the upper part of the figure.

Increase of SR Ca^{2+} release by calmodulin antagonist

It has been proposed that SR Ca^{2+} release is regulated not only by cytoplasmic Ca^{2+}, Mg^{2+} and ATP, but also by the ubiquitous calcium-binding protein calmodulin, whose direct effect on the Ca^{2+}-release channel may be to control the mean duration of its opening time (Smith, Rousseau & Meissner, 1989). Exposure of

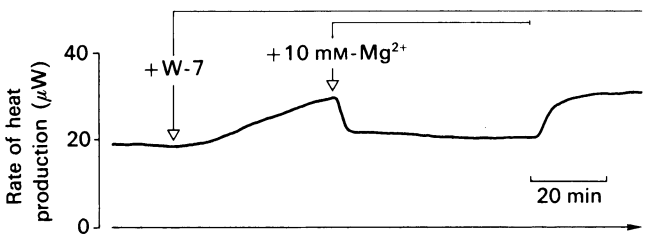

Fig. 7. Effect of calmodulin inhibition (30 μM-W-7 added to the superfusion solution) on the non-contracting muscle metabolic rate. A large part of the progressive, non-saturating increase of the rate of heat production was suppressed by high Mg^{2+} (11·2 mM-extracellular Mg^{2+}).

muscles to 30 μM of the calmodulin antagonist W-7 induced a slowly developing and non-saturating increase of $\dot{E}$ ($+2{\cdot}80 \pm 0{\cdot}41$ (7) mW g^{-1} after 60 min), 83 ± 4 (6)% of which was quickly and reversibly suppressed by high Mg^{2+} (Fig. 7) or BDM. The non-reversible suppression or prevention by dantrolene was somewhat less (about 60% in two experiments). The slow development of the metabolic response to W-7, and its slow disappearance upon removal of the drug from the superfusion solution (not shown), suggested that penetration of the cell by the calmodulin antagonist was slow. On the other hand, the residual positive slope of $\dot{E}$, observed for as long as W-7 was present, may have resulted from the loss of the calmodulin dependent, sarcolemmal Ca^{2+}-ATPase activity (see below) which normally compensates for Ca^{2+} influx across plasma membrane during steady states.

Acute inhibition of the plasma membrane Ca^{2+}-ATPase in the presence of dantrolene or BDM

No specific inhibitor of the sarcolemmal Ca^{2+}-ATPase is known. However, since the activity of this enzyme is strictly calmodulin dependent in skeletal muscle (Michalak, Famulski & Carafoli, 1984), the calmodulin inhibitor W-7 was used in experiments aimed at evaluating the overall contribution of this Ca^{2+}-ATPase to basal $\dot{E}$. This was done in the presence of either dantrolene or BDM to prevent the already described consequence of a loss of the calmodulin-dependent control of SR Ca^{2+} release. In the presence of 10 μM-sodium dantrolene, exposure to W-7 (30 μM) induced an immediate decrease of $\dot{E}$ of $0{\cdot}12 \pm 0{\cdot}02$ (8) mW g^{-1}. This is illustrated in Fig. 8, lower trace. The same drop of $\dot{E}$ was observed in the presence of 10 mM-BDM ($0{\cdot}09 \pm 0{\cdot}01$ (4) mW g^{-1}. These acute changes are very small fractions of total cell metabolic rate ($3{\cdot}2 \pm 0{\cdot}3$ (12)%). Among other non-specific inhibitors of the plasmalemmal Ca^{2+}-ATPase, sulphydryl reagents, and in particular NEM (Richards,

Rega & Garrahan, 1977) were used instead of the above combination of an inhibitor of SR Ca^{2+} release plus an anticalmodulin. As opposed to the long-term effect of NEM, the acute effect of 100 μM-NEM in the standard, calcium-containing solution was a drop of $\dot{E}$ of the same size as that observed with W-7 in the presence of dantrolene or BDM (about 0·1 mW g^{-1}, see Fig. 8, upper trace).

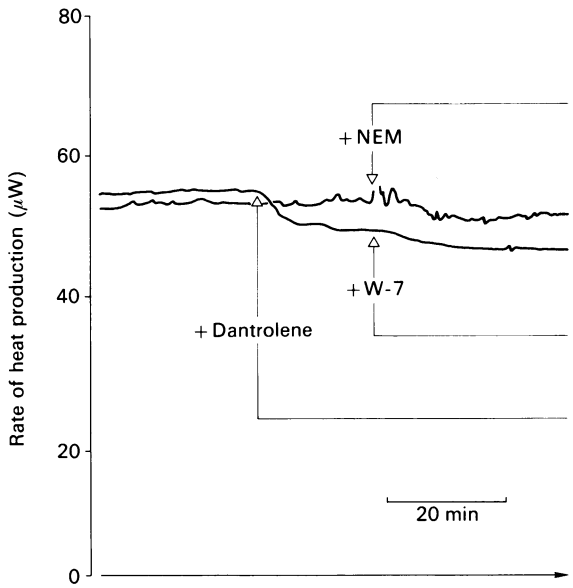

Fig. 8. Acute effects of NEM (100 μM), and of W-7 (30 μM) in the presence of dantrolene (10 μM), on the rate of energy dissipation by resting soleus muscle pairs.

DISCUSSION

The present attempts to estimate in intact tissue-cell preparations the energy cost of homeostasis of the sarcoplasmic free calcium ion concentration (Ca_i^{2+}) using microcalorimetry was justified for two reasons. Firstly, one cannot calculate the reversible work of active Ca^{2+} transport by SR, because it is not possible to measure unidirectional Ca^{2+} fluxes across the SR membrane *in situ*, and it is also not possible to predict their value during any steady state without detailed knowledge of the *in situ* conductance of the Ca^{2+} efflux pathway on the one hand, and the electrochemical difference for Ca^{2+} between SR matrix and sarcoplasm on the other. Secondly, it is not possible to calculate Ca^{2+}-dependent heat from the reversible work of active Ca^{2+} transport because the overall energetic efficiency of the Ca^{2+}-uptake processes is not known. The original finding, with the present experiments, is that the energy cost of Ca_i^{2+} homeostasis under resting conditions appears to be more than twice as large as that of active Na^+–K^+ transport, which was measured in the past under identical conditions and in the same preparation (Biron, Burger, Chinet, Clausen & Dubois-Ferrière, 1979). It was also found that this Ca^{2+}-dependent heat production in a mammalian skeletal muscle largely increases under marginal, if not undetectable increases of Ca_i^{2+} with respect to basal.

Direct inhibition of Ca^{2+} uptake processes

Although based on the reasonable assumption that energy metabolism is tightly controlled by the rate of overall ATPase processes, our attempts to use direct inhibition of SR Ca^{2+} uptake to evaluate Ca^{2+}-dependent heat met with two obstacles. First, as it appears on comparing the initial effects of tBuBHQ alone (very slow increase of $\dot{E}$, late contracture) with those of NEM + tBuBHQ (early contracture, no concomitant increase and even a decrease of $\dot{E}$ with respect to basal), the Ca^{2+}, Mg^{2+}-ATPase of SR might not be the only process responsible for the uptake of sarcoplasmic calcium by SR. The results of our attempts to directly inhibit the active limb of the SR Ca^{2+} cycle using a combination of two inhibitors (tBuBHQ and NEM) cannot be taken as decisive arguments to support the hypothesis of a coupling between proton and calcium transports (see Thévenod *et al.* 1989) in skeletal muscle, if only because one cannot ascertain that the only direct effect of NEM was to inhibit a proton ATPase. Thus, it is not possible to decide whether it is a proton ATPase or a novel ATP-dependent calcium pump, such as that whose mRNA was recently shown to be expressed in skeletal muscle (Burk, Lytton, MacLennan & Shull, 1989), that may have been both sensitive to NEM and responsible for the increase in $\dot{E}$ observed under tBuBHQ alone. The second obstacle was that the direct inhibition of SR Ca^{2+} uptake was not quick enough for its primary energetic consequences to be separated in time from those of an associated increase of Ca_i^{2+}. Indeed, the early appearance of a contracture in all experiments where NEM was applied, either in combination with tBuBHQ (e.g. Fig. 6) or alone (results not shown), precluded any quantitative evaluation of the acute energetic consequences of the blocking of one or several SR Ca^{2+} uptake processes. On the other hand, the long-term consequences of this blocking must be interpreted with caution. Indeed, whereas muscles seemed to recover their initial, control heat production rate after a 30 min exposure to NEM and the contracture, they did not recover it after exposures long enough (2 h) for $\dot{E}$ to become lower than basal in spite of the residual contracture.

Indirect inhibition of Ca^{2+} uptake processes

In contrast with the direct inhibition of SR Ca^{2+} uptake, measures taken to indirectly arrest it by restricting Ca^{2+} release from SR induced acute, clear-cut suppression of a significant part of basal $\dot{E}$. This was observed with each of the three different means used to block Ca^{2+} release from the SR.

The neutral, lipophilic molecule dantrolene sodium (dantrolene) was used here at a concentration (10 μM, about a quarter of the maximum that can be dissolved in physiological solutions) which does not guarantee that the degree of hydrophobic interactions of the molecule with lipophilic SR membrane constituents had reached the maximum. However, the long duration of exposure to the drug, together with the irreversibility of its effect, make it likely that this was a saturating effect at 30 °C. On the other hand, it should be noted that the maximum effect of dantrolene, even in experiments at 38 °C, may well correspond to a less than 50% decrease of SR Ca^{2+} release (see Ohta *et al.* 1990). This may explain why the 12% decrease of $\dot{E}$ with dantrolene was only about half that observed under high Mg^{2+} or BDM. There is another possible explanation for this difference, or at least for part of it: both high

Mg^{2+} and BDM might have inhibited cellular processes other than passive Ca^{2+} release from SR. At a physiological concentration of intracellular Mg^{2+} which might be as low as 0·4 mM (Nishimura, Matsubara, Nakayama, Nonoda, Yoshida, Terada, Kanashiro, Itoh & Sakamoto, 1991), an increase of the concentration of this ion could have somewhat decreased the electrophoretic entry of Ca^{2+} into the mitochondrial matrix, and therefore decreased the rate of energy dissipation associated with Ca^{2+} recirculation across the mitochondrial inner membrane (McCormack & Denton, 1986). As for BDM, although in skeletal muscle its main effect at concentrations up to 10 mM is to inhibit Ca^{2+} release from SR, the occasional detection of a slight decrease of resting muscle tension (result not shown) suggested that a slight part of its effect may have resulted from some inhibition of the basal, 'resting' rate of cross-bridge turnover.

Basal activity of SR Ca^{2+} uptake processes in terms of percentage V_{max} activity of the Ca^{2+}, Mg^{2+}-ATPase

There is no doubt that Ca_i^{2+} in the intact, non-stimulated muscle fibre may be very low, perhaps as low as 10 nM or even lower (Mommaerts, 1987). This can be achieved at low energy cost if the permeability of SR membrane for Ca^{2+} is low under low Ca_i^{2+}, or alternatively at high energy cost if this permeability is not much reduced in the absence of stimulation. One can predict from literature data that maximum turnover rates of the SR Ca^{2+}, Mg^{2+}-ATPase *in situ* are of the order of 100 nmol ATP s^{-1} (g wet muscle weight)$^{-1}$ in a slow-twitch mammalian muscle, at Ca_i^{2+} values $\geqslant$ 100 nM (Haynes & Mandveno, 1987). Assuming 1 mole of ATP turnover dissipates at least 80 kJ, as a mean, of substrate oxidation enthalpy (see Prusiner & Poe, 1968), the rate of basal energy expenditure associated here to SR Ca^{2+} uptake (i.e. 0·4–0·8 mW g^{-1}, which is the range of the effects of dantrolene, high Mg^{2+} and BDM under basal conditions) would then correspond to at most 5–10 % of maximum ATPase activity.

Indirect stimulation of Ca^{2+} uptake processes

In early descriptions of potassium-induced increases of metabolic rate in the absence of contraction in frog muscles (Solandt, 1936; Novotny & Vyskočil, 1966), very small changes of muscle length or tension, such as those that were detected in the present experiments on mammalian muscles, may have been overlooked. Also, experimental results may have been complicated by changes in extracellular medium tonicity and/or $[K^+][Cl^-]$ product, concomitant with the imposed changes of K_o^+. But more important, it should be stressed that the metabolic changes described by Solandt (1936) were in fact slow transients, as opposed to the steady-state changes observed at or below 20 mM in the frog sartorius (Novotny & Vyskočil, 1966) or below 15 mM in the mouse soleus (present study).

The hypothesis that the stimulation of active sodium transport across the sarcolemma which occurs during potassium depolarization might be responsible for the increase in metabolic rate (Horowicz & Gerber, 1965) was ruled out by two subsequent observations on frog muscles: (a) prevention of Na^+ extrusion, using strophanthidin to specifically inhibit active Na^+–K^+ transport, does not measurably affect the metabolic response to potassium (see Clinch, 1968), and (b) dantrolene, which in frog muscles largely inhibits the sustained metabolic response, does not

block the stimulation of Na^+ efflux (Erlij *et al.* 1982). More recently, it was shown that sustained sarcolemmal depolarizations of only a few millivolts produce sustained, albeit small increases of Ca_i^{2+} in frog skeletal muscle (Snowdowne, 1985) and in rat ventricular myocytes (Talo, Stern, Spurgeon, Isenberg & Lakatta, 1990). This, together with the present results showing that high Mg^{2+}, dantrolene or BDM suppressed most of the increase in $\dot{E}$ and O_2 consumption induced by relatively small changes of K_0^+ (Figs 3–5), strongly suggests that the sustained increase in metabolic rate during subthreshold-for-contracture depolarizations is essentially caused by an increased energy cost of Ca_i^{2+} homeostasis. No change of Ca_i^{2+} could be detected in the mouse soleus (V. van der Bent, A. Decrouy & A. Chinet, unpublished) or in cultured human muscle cells (Jacobs *et al.* 1991) under high extracellular potassium. This raises the question of whether in muscle cells, as in other cell types (see Muallem, Beeker & Fimmel, 1987), cytoplasmic Ca^{2+} sequestration processes might be stimulated by some other factor than a rise of Ca_i^{2+}. In the absence of further evidence, however, very small or even undetectable steady-state changes of Ca_i^{2+} and tension concomitant with significant steady-state changes of Ca^{2+}-dependent energy dissipation can simply be taken to reflect the existence of a tight control of Ca_i^{2+}, the energy cost of which increases significantly as soon as the rate of SR Ca^{2+} release increases.

The present results show that the main route of Ca^{2+} entry into sarcoplasm during the sustained phase of subthreshold-for-contracture depolarization of mammalian skeletal muscle fibres is the SR Ca^{2+} channel. Similarly, in isolated cardiomyocytes, it was recently shown that the sustained increase of Ca_i^{2+} observed under subthreshold-for-twitch depolarization requires functional SR Ca^{2+} channels (Talo *et al.* 1990). Since the absolute rate of Ca^{2+} entry across the plasmalemma probably remained very low during subthreshold depolarization, even if it was doubled under 15 mM-extracellular K^+ as in smooth muscle (Cattaneo, Gende, Cingolani & Venosa, 1991), these results strongly support the idea that Ca^{2+} recirculation occurs at much larger rates across the SR membrane than across the sarcolemma during near-basal and basal steady states. In a wide variety of cell types regulated by calcium-mobilizing receptors or voltage-sensitive calcium channels, Ca_i^{2+} and the activity of Ca_i^{2+}-dependent processes follow a characteristic biphasic pattern upon cell stimulation, qualitatively similar to the one illustrated by upper traces in Figs 3 and 4. It is generally admitted that the source of the mobilized Ca^{2+} is mainly an intracellular calcium pool during the initial phase (calcium redistribution), whereas during the plateau phase it is Ca^{2+} from the extracellular pool that directly enters the cytoplasm, even according to the capacitative calcium entry hypothesis (Putney, 1990). Whether striated muscles undergoing subthreshold potassium stimulation are a unique exception to this scheme awaits further evidence.

The results of experiments in which the calmodulin inhibitor W-7 was used as another means to increase Ca^{2+} cycling between SR and sarcoplasm confirm both the calmodulin dependence of SR Ca^{2+} release and the possibility that intracellular Ca^{2+} recycling, which is already responsible for a significant fraction of maintenance energy expenditure under basal conditions, may dissipate almost half of the total energy flow rate under steady states of subthreshold-for-contracture release of SR calcium.

Direct inhibition of the plasmalemmal Ca^{2+}-ATPase

As already mentioned in Results one of the effects of NEM is to inhibit the activity of the plasmalemmal Ca^{2+}-ATPase. Such an inhibition may have caused the very initial but modest drop of $\dot{E}$ (3% of basal $\dot{E}$ as a mean) observed before the appearance of contracture upon exposure to NEM under basal conditions (Fig. 8; compare also Fig. 6, lower part, to Fig. 1: tBuBHQ administered alone does not cause any initial drop of $\dot{E}$). This interpretation is in keeping with our observation of an acute drop of $\dot{E}$ of the same size caused by calmodulin inhibition with W-7 in preparations pretreated with BDM or dantrolene (Fig. 8). Comparison of this 3% effect with the much larger drop of $\dot{E}$ (up to 24%) observed upon indirect inhibition of the activity of the basal Ca_i^{2+} homeostatic process confirms that, under basal steady-state conditions in the mouse soleus muscle, the activity of the plasmalemmal Ca^{2+}-ATPase is responsible for but a very minor part of what was referred to here as the *Ca^{2+}-dependent heat production* (i.e. the overall rate of energy dissipation associated with Ca_i^{2+} homeostasis).

The present quantitative determinations of Ca^{2+}-dependent heat production under basal and subthreshold-for-contracture steady states of SR Ca^{2+} release support the conclusion that, in the submicromolar range of sarcoplasmic Ca^{2+}, up to 25–45% of steady-state energy expenditure in a mammalian, slow-twitch muscle at rest is related to Ca^{2+} recirculation between sarcoplasm and sarcoplasmic reticulum.

This work was supported by grants Nos. 3.356.086 and 31-9467.88 from the Swiss National Science Foundation.

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