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(Ca^{2+} + Mg^{2+})-ATPase activity associated with the maintenance of a Ca^{2+} gradient by sarcoplasmic reticulum at submicromolar external [Ca^{2+}]. The effect of hypothyroidism

Warner S. Simonides and Cornelis Van Hardeveld

Laboratory for Physiology, Free University, Amsterdam (The Netherlands)

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The formation and maintenance of Ca^{2+}-filling levels by sarcoplasmic reticulum vesicles from euthyroid (control) and hypothyroid skeletal muscle were investigated using the Ca^{2+}-indicator quin-2, at [Ca^{2+}] in the medium ([Ca_o^{2+}]) of 0.05–0.3 μM. Rapid ATP-dependent Ca^{2+} uptake resulted in a steady-state Ca^{2+}-filling level, Ca_i^{2+}, within one minute. This Ca^{2+} gradient was maintained for at least three minutes, during which < 20% of the ATP was consumed. Ca_i^{2+} was maximal (120 nmol/mg) for [Ca_o^{2+}] > 0.3 μM and decreased to 40 nmol/mg at [Ca_o^{2+}] of 0.05 μM. Preparations from both experimental groups showed qualitatively and quantitatively the same relationship between Ca_i^{2+} and [Ca_o^{2+}] at steady state, despite a significantly lower Ca^{2+}-pump content of hypothyroid sarcoplasmic reticulum, which resulted in a 25% lower maximal (Ca^{2+} + Mg^{2+})-ATPase activity. Maintenance of the steady state, at all levels of Ca_i^{2+}, was associated with net ATP consumption by the Ca^{2+} pump and cycling of Ca^{2+}, which processes were 30% slower in the hypothyroid group as compared to the control group. Determination of the passive efflux of Ca^{2+}, as well as the fraction of leaky or unsealed sarcoplasmic reticulum fragments, excluded either of these possibilities as an explanation for the relatively high (Ca^{2+} + Mg^{2+})-ATPase rates at steady state. On the basis of these and previously reported results, it is concluded that the maintenance of a Ca^{2+} gradient by sarcoplasmic reticulum under physiological conditions with respect to external [Ca^{2+}] and the concentrations of ATP, ADP and P_i, is associated with the cycling of Ca^{2+} coupled to net ATP hydrolysis. Using the obtained data it is calculated that the sarcoplasmic reticulum may account for 20% of the resting metabolic rate in skeletal muscle. Consequently, together with the previously reported lower sarcoplasmic reticulum content of skeletal muscle in hypothyroidism, we calculate that about one third of the decrease in basal metabolic rate in this thyroid state can be related to the alterations of the sarcoplasmic reticulum.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; [Ca_o^{2+}], concentration of free Ca^{2+} in the assay medium; Ca_i^{2+}, total amount of Ca^{2+} sequestered by sarcoplasmic reticulum.

Correspondence: W.S. Simonides, Laboratory for Physiology, Free University, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

Introduction

Depletion of thyroid hormone leads to a lower metabolic rate of resting and active skeletal muscle [1,2], and recent studies have also shown a decrease in both the content and specific Ca^{2+}-transport activity of sarcoplasmic reticulum in this tissue in hypothyroidism [3,4]. Using heat measurements of contracting muscles, Leijendekker et al. integrated these observations by showing that the reduced energy turnover in hypothyroidism could largely (slow muscle) or even completely (fast muscle) be accounted for by the reduced sarcoplasmic reticulum activity in these muscle types [5]. That this may also play a role in the 40% reduction of the metabolic rate at rest is suggested by an earlier study in which Dantrolene, a drug that interferes with the Ca^{2+}-regulatory properties of the sarcoplasmic reticulum, significantly reduced the difference in metabolic rate between euthyroid (normal) and hypothyroid fast muscle [2].

Whether this is directly related to the diminished $(Ca^{2+} + Mg^{2+})$-ATPase activity (Ca^{2+} pump, EC 3.1.6.38) of the sarcoplasmic reticulum would seem questionable, since it is estimated that only 7% of the resting ATP consumption is associated with this organel [6]. This low value reflects the generally held view, that the Ca^{2+}-transport process responsible for the steep Ca^{2+} gradient between the lumen of the sarcoplasmic reticulum and the cytosol, is close to thermodynamic equilibrium and hence consumes little or no ATP [6–8,20]. Only a small $(Ca^{2+} + Mg^{2+})$-ATPase activity is thought to remain, compensating for a minor passive leak of Ca^{2+} [6,9]. However, in vitro studies have shown that vesicular sarcoplasmic reticulum preparations can sequester Ca^{2+} and maintain a gradient as in vivo, but when maximally filled, these preparations show a net ATP consumption by the Ca^{2+} pump coupled to cycling of Ca^{2+}, that exceeds the activity required for the compensation of passive Ca^{2+} efflux [10–12,29]. These results were obtained with $[Ca^{2+}]$ in the medium of 50–100 μM, but an unexplained $(Ca^{2+} + Mg^{2+})$-ATPase activity was also reported by Feher and Briggs, who examined the steady-state behaviour of heart sarcoplasmic reticulum down to 0.3 μM Ca^{2+} [11]. It can therefore not be excluded that the $(Ca^{2+} + Mg^{2+})$-ATPase activity of Ca^{2+}-loaded sarcoplasmic reticulum under in vivo conditions is in fact higher than expected. Apart from this possible direct contribution to the energy turnover, the alterations of this organel may affect its role in the homeostasis of the low cytosolic $[Ca^{2+}]$, which in turn could affect the metabolic activity [13]. Therefore, using fast-muscle sarcoplasmic reticulum from euthyroid and hypothyroid rats, we examined the formation of a Ca^{2+} gradient as well as the $(Ca^{2+} + Mg^{2+})$-ATPase activity and Ca^{2+} fluxes associated with the maintenance of the gradient. The experiments were performed at $[Ca^{2+}]$ in the medium from 0.3 μM down to the value thought to prevail in the cytosol of resting muscle, i.e., 0.05 μM, in order to assess the possible involvement of the sarcoplasmic reticulum in the thyroid-hormone regulated basal metabolic rate in muscle.

Materials and Methods

Animals

Male rats of the Wistar strain were used. Hypothyroidism was induced in animals kept on a low-iodine diet (Hope Farms, Linschoten) by injection of ^{131}I or addition of $KClO_4$ to the drinking water as described previously [3,4]. The hypothyroid condition was maintained for six weeks. Control (euthyroid) animals were maintained on a normal diet and were weight-matched at the time of killing; 240–280 g.

Ca^{2+}-uptake measurement

Sarcoplasmic reticulum was isolated from the M. gastrocnemius-plantaris by the method of Meissner [14]. For details on the purified sarcoplasmic reticulum see Ref. 3.

Ca^{2+} uptake was determined firstly by the Millipore filtration method using $^{45}Ca^{2+}$. The reaction medium of 10 ml contained 100 mM KCl, 10 mM Mops, 5 mM $MgCl_2$, 10 mM NaN_3, 100 μM $CaCl_2$ and 1 mg sarcoplasmic reticulum protein, pH 6.9, 25°C. The uptake reaction was started by addition of 100 μM ATP and an ATP-regenerating system (400 μM phospho*enol*pyruvate/0.2 mg/ml pyruvate kinase) to an otherwise complete reaction medium. Aliquots of 200 μl were taken at timed intervals, diluted in 2.5 ml ice-cold 10 mM $LaCl_3$ in the same buffer and filtered through 0.45 μm Millipore filters. The $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation counting and blanks were subtracted for Ca^{2+} binding in the absence of ATP.

Fluorescence measurements of Ca^{2+} uptake at low external $[Ca^{2+}]$ used medium of the following composition: 2 mM $MgCl_2$, 100 mM KCl, 10 mM NaN_3 and 10 mM Mops (pH 6.9). The compound quin-2 was present at a final concentration of 40 μM. Quin-2, derived from EGTA, is a Ca^{2+} buffer with Ca^{2+}-dependent fluorescent properties enabling its use as a sensitive indicator of the $[Ca^{2+}]$ in the nanomolar range [15]. Like EGTA it does not permeate through biological membranes. Fluorescence measurements of the free $[Ca^{2+}]$ in the medium were performed with an Aminco-Bowman Spectrofluorometer equipped with a magnetic stirrer and thermostatted cuvet holder. The extinction and emission wavelengths were 332 and 498 nm, respectively, at 2 mm slit-aperture and 10 nm band width for both. The incubation volume was 2.0 ml and the sarcoplasmic reticulum concentration was routinely 0.1 mg/ml. After adjustment of the $[Ca^{2+}]$ to the desired level the uptake reaction was started by addition of ATP (1 mM). Additions to the incubation medium during measurements were given through an injection port in the cuvet housing. The output of the fluorometer was constantly monitored by a chart recorder. Calibration at the end of each experiment was achieved by determination of the maximal fluorescence (F_{max}) in the presence of 0.5 mM Ca^{2+} and the minimal fluorescence (F_{min}) by subsequent addition of EGTA (5 mM) and adjustment of the pH to > 8.3. The concentration of Ca^{2+} in the medium, $[Ca^{2+}]$ free, for a given fluorescence value F is calculated with the following equation [15]:

$$[Ca^{2+}]_{free} = K_d(F - F_{min})/(F_{max} - F)$$

where $K_d = 120$ nM [15,16].

The amount of Ca^{2+} bound to quin-2, $[Ca^{2+}]_{bound}$, is then given by:

$$[Ca]_{bound} = [quin\text{-}2]/(1 + K_d/[Ca]_{free})$$

where [quin-2] = 40 μM.

$(Ca^{2+} + Mg^{2+})$-ATPase activity at steady state

Ca^{2+} uptake and establishment of steady state was determined as described above. During steady state, five samples of 100 μl each were drawn from the cuvet at 30 s intervals followed by the addition of EGTA (2 mM) after which again five samples were taken. These were processed immediately for determination of P_i by the method of Black and Jones [17]. The $(Ca^{2+} + Mg^{2+})$-ATPase activity was calculated from the difference between the rate of P_i liberation before and after the addition of EGTA.

Passive Ca^{2+} efflux

When Ca^{2+} uptake, determined as described above had reached steady state a single addition of quench reagent, 12.5 mM glucose and 25 μg/ml hexokinase (final concentration), was followed by efflux of calcium which started within 5 s. The efflux curve was recorded and the graph was analyzed as described in the text.

Ca^{2+} cycling at steady state

Ca^{2+} uptake and establishment of steady state was determined as described above and during the steady state a tracer amount of $^{45}Ca^{2+}$ (0.24 μCi) was added to the cuvet and samples of 150 μl each were immediately drawn at 10 s intervals, diluted in 2.5 ml ice-cold incubation buffer containing 10 mM $LaCl_3$ and no quin-2 and filtered through 0.45 μm Millipore filters. The $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation counting and correction was made for $^{45}Ca^{2+}$ bound to the outside of the sarcoplasmic reticulum. Analysis of the data was as follows [11]. At steady state approx. 30% of the total amount of Ca^{2+} in the incubation is sequestered by the sarcoplasmic reticulum and the added $^{45}Ca^{2+}$ first equilibrates, instantaneously, with the Ca^{2+} in the medium. The rate with which the $^{45}Ca^{2+}$ then equilibrates between the sarcoplasmic reticulum- and the medium Ca^{2+}-pool can be used to calculate the unidirectional Ca^{2+} flux at steady state.

If A and B are the Ca^{2+}-pools of the medium and the sarcoplasmic reticulum, respectively (both expressed as nmol/mg sarcoplasmic reticulum), which are determined experimentally and A^* and

B^* the amounts of $^{45}Ca^{2+}$ (cpm) in both pools at any time, then we can write:

$$-\frac{dA^*}{dt} = J\frac{A^*}{A} - J\frac{B^*}{B}$$

where $A^* + B^* = A_o^*$, the total amount of added $^{45}Ca^{2+}$ (cpm) and J is the unidirectional flux of Ca^{2+} at steady state (influx = efflux). The following equation can then be derived:

$$\ln\left(\frac{A^*}{A} - \frac{B^*}{B}\right) = \ln\frac{A_o^*}{A} - \frac{A+B}{A\cdot B}\cdot J\cdot t$$

and by plotting the left-hand part of the equation against time, a line is obtained with a slope equal to $((A+B)/A\cdot B)\cdot J$ from which J (nmol/mg per min) can be calculated.

Separation of sealed and leaky membrane fragments

Sarcoplasmic reticulum, 1.0 mg, was incubated at 25 °C in 6 ml reaction medium containing: 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes, 20 μM free Ca^{2+}, 10 mM sodium oxalate and 5 mM ATP (pH 6.9). Ca^{2+} uptake was followed with the Ca^{2+}-stat method which maintains a constant $[Ca^{2+}]$ in the medium [3]. Following a 30 min incubation during which the Ca^{2+}-uptake reached a plateau, the reaction mixture was cooled in ice and transferred to a rotor 40 tube (Beckman) onto two layers of sucrose; 1 ml 30% and 1 ml 50% in 1 mM Hepes (pH 7.4). In a 45 min run at 117000 × g the calcium oxalate-loaded vesicles formed a pellet while the remaining material banded at the 30/50% interface. Control experiments were performed in which ATP was omitted from the incubation mixture.

Other determinations

The steady-state level of the phosphoprotein was determined as described before [3] under the conditions of the $(Ca^{2+} + Mg^{2+})$-ATPase activity measurements.

Protein was measured by the method of Lowry et al. [18] using bovine serum albumin as a standard. Student's *t*-test was used in the statistical analyses.

Results

Ca^{2+}-pump content and maximal Ca^{2+} filling

Sarcoplasmic reticulum isolated from the M. gastrocnemius-plantaris of euthyroid (control) and hypothyroid rats was purified to a similar degree, with less than 10% contaminating material as judged by marker enzyme analysis and polyacrylamide gel electrophoresis. The yield of sarcoplasmic reticulum protein per g muscle was 30% less in the hypothyroid as compared to the control group confirming previous results [3]. Determination of the maximal specific $(Ca^{2+} + Mg^{2+})$-ATPase activity of the preparations indicated a significantly lower value for hypothyroid material, which is primarily due to a lower number of active Ca^{2+} pumps (^{32}P-enzyme. Table I). Comparison of protein profiles obtained by electrophoresis confirmed the decreased $(Ca^{2+} + Mg^{2+})$-ATPase content relative to other sarcoplasmic reticulum proteins in the hypothyroid group

TABLE I

MAXIMAL $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY, ^{32}P-ENZYME CONTENT AND MAXIMAL Ca^{2+} FILLING OF EUTHYROID AND HYPOTHYROID SARCOPLASMIC RETICULUM

Maximal $(Ca^{2+} + Mg^{2+})$-ATPase activity was determined in the presence of ionophore A23187 and 100 μM Ca^{2+} and the maximal filling level was determined by the Millipore filtration technique at 100 μM Ca^{2+}, as described in Materials and Methods. The $(Ca^{2+} + Mg^{2+})$-ATPase content of sarcoplasmic reticulum was determined by measuring the steady-state incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$. Data represent the means ± S.E. of (*n*) individual determinations.

	$(Ca^{2+} + Mg^{2+})$-ATPase (μmol/mg per min)	^{32}P-enzyme (nmol/mg)	Ca^{2+} filling (nmol/mg)
Euthyroid	4.03 ± 0.16 (7)	4.51 ± 0.09 (5)	118 ± 5 (6)
Hypothyroid	3.11 ± 0.28 (8) *	3.85 ± 0.23 (7) *	122 ± 5 (5)

* $2P < 0.05$.

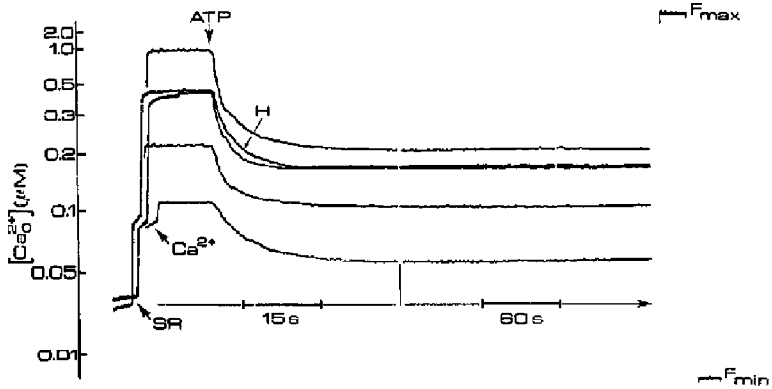

Fig. 1. Ca^{2+}-progress curves of assays started at various levels of $[Ca_o^{2+}]$, using euthyroid and hypothyroid (H) sarcoplasmic reticulum. The uptake medium was composed as described in Materials and Methods. Addition of sarcoplasmic reticulum (SR) (0.1 mg/ml) resulted in a fluorescence signal which indicated that 100–130 nmol Ca^{2+}/mg was released to the medium. This amount correlates with the value of Ca^{2+}-bound obtained by flame photometric determination, i.e., 118 ± 15 and 113 ± 13 nmol/mg protein (±S.D.) for euthyroid and hypothyroid sarcoplasmic reticulum, respectively. Sarcoplasmic reticulum previously washed with EGTA to remove Ca^{2+} did not produce any signal. $[Ca^{2+}]$ is adjusted by addition of $CaCl_2$ and the uptake was started by addition of 1 mM ATP. ATP alone did not affect the fluorescence signal. Calibration was performed by measurement of F_{max} (0.5 mM Ca^{2+}) and F_{min} (excess EGTA). The Ca^{2+}-filling level of sarcoplasmic reticulum was calculated from the difference between the amount of Ca^{2+} bound to quin-2 + free Ca^{2+} at the time of ATP addition and when net uptake had ceased. The inclusion of an ATP-regenerating system did not affect the Ca^{2+}-filling level of the sarcoplasmic reticulum.

(not shown). Determination by $^{45}Ca^{2+}$-Millipore filtration of the maximal Ca^{2+}-filling level yielded the same value for both groups (Table I).

Steady-state Ca^{2+} filling at external $[Ca^{2+}]$ below 0.5 μM

Because the maximal filling level was determined at a $[Ca^{2+}]$ in the medium, $[Ca_o^{2+}]$, of 100 μM, we next examined the uptake of Ca^{2+} under more physiological conditions where the Ca^{2+}-filling level may be determined by the $[Ca_o^{2+}]$, i.e., below 0.5 μM. The conditions were furthermore such, that less than 20% of the 1 mM ATP present in the medium was consumed during the assay.

The fluorescent Ca^{2+}-indicator quin-2 was used to continuously monitor the $[Ca_o^{2+}]$ in these experiments, also enabling the calculation of the amount of Ca^{2+} taken up by the sarcoplasmic reticulum, Ca_i^{2+}. Fig. 1 shows $[Ca_o^{2+}]$-progress curves for a typical euthyroid sample started at various levels of $[Ca_o^{2+}]$. For comparison, two uptake curves of the same amount of euthyroid

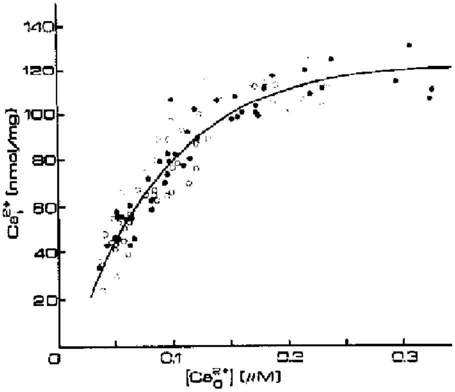

Fig. 2. Determinations of Ca^{2+}-filling level, Ca_i^{2+}, at various $[Ca_o^{2+}]$ were performed for seven euthyroid (●) and nine hypothyroid (○) preparations. The line through the data points was drawn by eye. The position of the line was independent of the amount of sarcoplasmic reticulum used (0.025–0.2 mg/ml) and also of the concentration of quin-2 (20–40 μM) and the temperature (25–37 ° C).

and hypothyroid sarcoplasmic reticulum, started at exactly the same [Ca^{2+}_o], are also given. The initial Ca^{2+}-uptake rate following the addition of ATP was higher in euthyroid material, in agreement with the data in Table I, but the amount of Ca^{2+} sequestered and the level of [Ca^{2+}_o] at which net uptake ceased were identical. This level was held for several minutes, followed by a very gradual efflux of the sequestered Ca^{2+} (not shown). Addition of an ATP-regenerating system did not alter the attained [Ca^{2+}_o], but only extended the time that it remained constant.

Analysis of such data yielded the relationship between the steady-state Ca^{2+}-filling level (Ca^{2+}_i) and the [Ca^{2+}_o], depicted in Fig. 2. The results were identical for both groups with a half maximal Ca^{2+}_i around 0.06 µM, levelling off to a value of 120 nmol/mg at 0.3 µM, which confirms the maximal filling level obtained with the Millipore filtration technique (Table I).

Steady-state (Ca^{2+} + Mg^{2+})-ATPase activity and Ca^{2+} fluxes

Determination of the total and background rate of ATP-hydrolysis under the conditions of Fig. 2 yielded the (Ca^{2+} + Mg^{2+})-ATPase activity associated with the maintenance of a Ca^{2+} gradient. A substantial steady-state activity was indeed found, which increased with increasing [Ca^{2+}_o] as shown

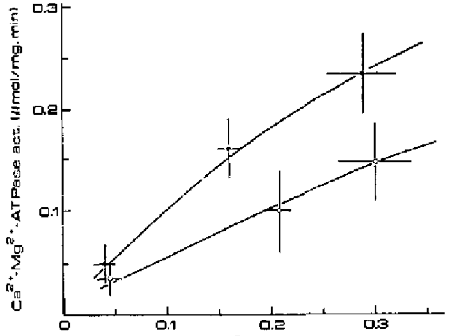

Fig. 3. The (Ca^{2+} + Mg^{2+})-ATPase activity at different Ca^{2+}-filling levels was determined for euthyroid (●) and hypothyroid (○) sarcoplasmic reticulum as described in Materials and Methods. The data points represent the means ± S.D. of 4–6 individual preparations. The Ca^{2+}-independent background ATPase activity (Mg^{2+}-ATPase) was 0.29 ± 0.13 and 0.16 ± 0.09 (± S.D.) µmol/mg per min for euthyroid and hypothyroid sarcoplasmic reticulum, respectively. In all determinations the (Ca^{2+} + Mg^{2+})-ATPase activity was constant for at least 3 min.

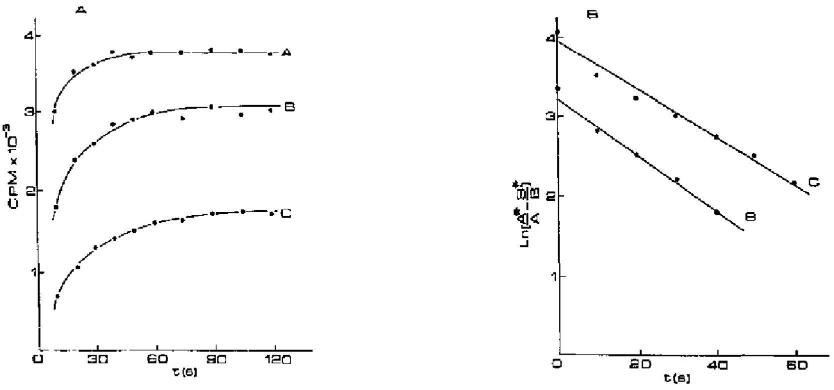

Fig. 4. Representative example of the determination of unidirectional Ca^{2+} flux, or Ca^{2+} cycling, when net uptake had ceased. The equilibration of a tracer pulse of $^{45}Ca^{2+}$ added at three different levels of [Ca^{2+}_o] (A, 0.20; B, 0.15 and C, 0.05 µM) is depicted in (A). The $^{45}Ca^{2+}$ taken up by the sarcoplasmic reticulum was determined by Millipore filtration and is given as cpm on the ordinate. The Ca^{2+}-filling level calculated from these data was similar to the value obtained from the fluorescence data, indicating complete equilibration of the $^{45}Ca^{2+}$ with the sarcoplasmic reticulum Ca^{2+} pool. The results in (A) were analysed as described in Materials and Methods yielding the straight lines depicted in (B). The Ca^{2+}-flux rate could be calculated from the slope of these lines. The rapid equilibration in curve A does not allow an accurate analysis.

TABLE II

UNIDIRECTIONAL Ca^{2+} FLUX (Ca^{2+} CYCLING) AT TWO STEADY-STATE LEVELS OF $[Ca_o^{2+}]$

Steady-state Ca^{2+} cycling was determined at two levels of $[Ca_o^{2+}]$, as described in the legend to Fig. 4. Data represent the means ± S.E. of (*n*) individual determinations.

	$[Ca_o^{2+}]$ (μM)	Ca_i^{2+} (nmol/mg)	Ca^{2+} flux (nmol/mg per min)
Euthyroid (4)	0.06 ± 0.01	42 ± 5	116 ± 23
Hypothyroid (3)	0.06 ± 0.01	37 ± 5	87 ± 6
Euthyroid (3)	0.12 ± 0.01	79 ± 5	307 ± 42
Hypothyroid (4)	0.13 ± 0.01	74 ± 7	212 ± 30

in Fig. 3. Sarcoplasmic reticulum of hypothyroid origin consumed some 30% less ATP than euthyroid material during the maintenance of the same gradient.

The rate of Ca^{2+}cycling at steady state was equally dependent on $[Ca_o^{2+}]$. This parameter was determined, as shown in Fig. 4, at two levels of $[Ca_o^{2+}]$. Although the average rate was again about 30% lower in the hypothyroid group, this difference was not statistically significant (Table II).

In order to assess to what extent the Ca^{2+} cycling and Ca^{2+}-pump activity were determined by passive diffusional leak of Ca^{2+}, we measured this flux at various filling levels (Fig. 5). The first-order rate constant was independent of the filling level and proved to be the same for both groups; see legend to Fig. 5. On the assumption for which Feher and Briggs have provided evidence [11], that the passive permeability is not perturbed by the active Ca^{2+} pump, i.e., in the presence of ATP, it is obvious that the measured steady-state (Ca^{2+} + Mg^{2+})-ATPase activities are too high to be the result of Ca^{2+}-uptake compensating passive efflux. For example, at 0.1 μM $[Ca_o^{2+}]$ the filling level of 70 nmol/mg would give a passive leak of 30 nmol/mg per min. Re-uptake would require 15 nmol ATP/mg per min in both groups, assuming a coupling ratio of 2 [19], whereas the actual activity was 100 and 60 nmol/mg per min for euthyroid and hypothyroid material, respectively (Fig. 3).

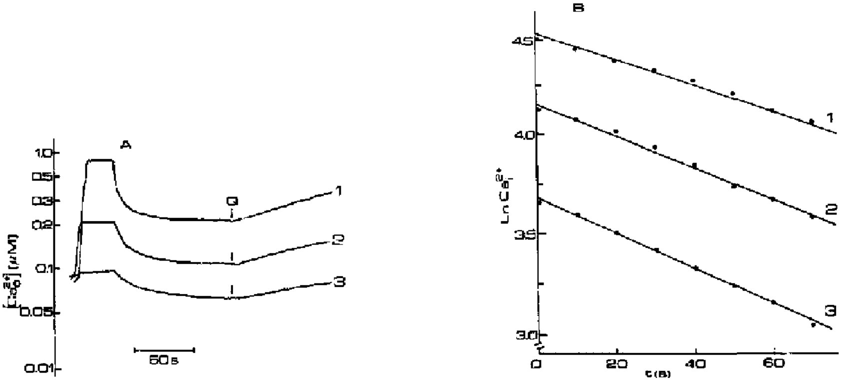

Fig. 5. Determination of passive efflux was accomplished by rapidly consuming the ATP present when a stable Ca^{2+}-filling level was reached (A). Ca^{2+} effluxed immediately following addition of quench reagent (glucose/hexokinase) at Q. The values for Ca_i^{2+} at 10-s intervals were calculated from the efflux curve and plotted logarithmically as a function of time in (B). The slope of the regression lines yielded the first-order efflux rate constant (K_e). The average value obtained for five euthyroid and eight hypothyroid sarcoplasmic reticulum preparations was 0.44 ± 0.05 and 0.40 ± 0.03 min^{-1} (± S.D.), respectively, confirming previous reports [11,12,29]. The value of K_e was independent of the level of Ca_i^{2+}, 55–125 nmol/mg, at which efflux was determined, suggesting the absence of an appreciable amount of high-affinity Ca^{2+}-binding sites in the sarcoplasmic reticulum. The observed efflux is not related to inversion of the Ca^{2+}-pump cycle, because the K_e was constant over a range of $[Ca_o^{2+}]$ of 0.04–0.25 μM in which pump reversal shows a 50% decrease in activity [33].

Contribution of leaky vesicles to steady-state (Ca^{2+} + Mg^{2+})-ATPase activity

It has been pointed out repeatedly that high steady-state ATPase rates may be explained by the presence of only a small fraction of leaky vesicles or unsealed fragments of sarcoplasmic reticulum, which will have a maximal (Ca^{2+} + Mg^{2+})-ATPase activity due to the absence of back inhibition by intravesicular Ca^{2+} [12,20]. To examine this possibility we determined the maximal (Ca^{2+} + Mg^{2+})-ATPase activity of euthyroid and hypothyroid preparations under the conditions used, by rendering them completely permeable to Ca^{2+} with the ionophore A23187. The results obtained at various [Ca_o^{2+}] are depicted in Fig. 6, showing a 4–5-fold higher activity, in both preparations, than measured in the absence of ionophore. Therefore, if the steady state (Ca^{2+} + Mg^{2+})-ATPase activities in Fig. 3 are to be explained by the presence of leaky material, this would require a fraction of 20% of the sarcoplasmic reticulum preparations used in this study. An estimate of the actual fraction of leaky or unsealed material was obtained by separating vesicles capable of Ca^{2+} sequestration from other material, using the increased density of vesicles loaded with Ca^{2+} in the presence of oxalate. The analysis of three control preparations indicated the presence of leaky membranes and non sarcoplasmic-reticulum material to a maximum of 10% (Table III).

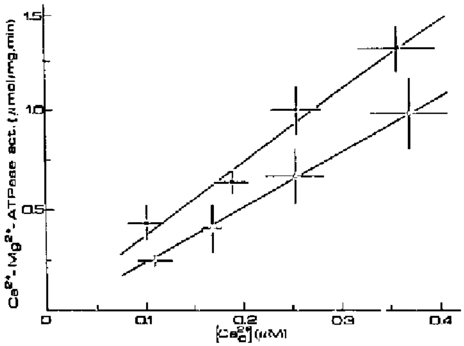

Fig. 6. (Ca^{2+} + Mg^{2+})-ATPase activity of euthyroid (●) and hypothyroid (○) sarcoplasmic reticulum, rendered permeable to Ca^{2+} by addition of 1 μM A23187, at different levels of [Ca_o^{2+}]. Conditions were otherwise identical to those described for Fig. 3, but the ATPase activity was determined using a coupled enzyme assay (pyruvate kinase/lactate dehydrogenase) by measuring NADH absorbance. Identical results were obtained by direct measurement of P_i liberation. Data points are the means ± S.D. of 6–8 individual preparations.

TABLE III

DETERMINATION OF THE FRACTION OF SEALED MEMBRANE VESICLES

The oxalate supported Ca^{2+} loading (plateau) was determined with the Ca^{2+}-stat method using 1 mg sarcoplasmic reticulum protein as described in Materials and Methods, in a 30 min incubation. Ca^{2+}-loaded, i.e., intact vesicles were separated from leaky vesicles and other contaminating material by sedimentation of the former through a sucrose layer. The protein content of the pellet and the non-sedimentable fraction (other material) was determined. Control experiments without ATP invariably yielded no sedimentable material. Data represent the means ± S.E. of three individual control preparations (euthyroid).

Ca^{2+} loading (μmol/mg)	Ca^{2+}-loaded vesicles (mg)	Other material (mg)
11.5 ± 0.7	0.92 ± 0.02	0.10 ± 0.02

Discussion

The results of this study can be summarized as follows:

(1) Sarcoplasmic reticulum consumes about 5-times more ATP than expected on the basis of passive Ca^{2+}-permeability of the membrane, while maintaining a gradient at low external [Ca^{2+}].

(2) The lower Ca^{2+}-pump activity of hypothyroid sarcoplasmic reticulum does not affect the attainable Ca^{2+} gradient, yet it reduces significantly the (Ca^{2+} + Mg^{2+})-ATPase activity associated with its maintenance.

Before discussing the implications for the interpretation of the resting metabolic rate of skeletal muscle in euthyroidism and hypothyroidism, we will first consider some general aspects of the present data.

Steady-state Ca^{2+}filling and (Ca^{2+} + Mg^{2+})-ATPase activity

The conditions used in the assay of the steady-state Ca^{2+}-filling level at low [Ca_o^{2+}] were such, that less than 20% of the ATP present was consumed during the measurement. This means that the ratio of [ATP]/[ADP]·[P_i], the phosphorylation potential, decreased continuously, but never dropped below $2 \cdot 10^4$ M^{-1}; for the upper curve (euthyroid) in the example of Fig. 1 the ratio was $4 \cdot 10^5$ at $t = 1$ min and $3 \cdot 10^4$ at $t = 4$ min. We chose these conditions because Trevorrow and Haynes have shown that the Ca^{2+} gradient attained by sarcoplasmic reticulum in vitro at 0.05 μM [Ca^{2+}_o] is constant and independent of the phosphorylation potential down to a value of this parameter of $1 \cdot 10^4$ M^{-1} [21]. Below this value the gradient decreased proportionately, indicating thermodynamic equilibrium of the Ca^{2+}-transport process. The stability of the Ca^{2+}-filling level, at phosphorylation potentials $> 2 \cdot 10^4$ M^{-1}, as observed by us, confirms these results. We consider the applied conditions physiologically relevant, since recent determinations of the phosphorylation potential in resting skeletal muscle invariably show values above $2 \cdot 10^4$ M^{-1}, e.g., $2.7 \cdot 10^4$ [22], $2.2 \cdot 10^4$ and $4.9 \cdot 10^4$ [23] and $7.7 \cdot 10^4$ (Ref. 24, data recalculated with the accepted K_{cpk} of $1.66 \cdot 10^9$ M^{-1} [25]).

The limits of the Ca^{2+} gradient at low [Ca^{2+}_o] and phosphorylation potentials above $2 \cdot 10^4$ M^{-1} or at increasing [Ca^{2+}_o] are apparently not determined by a mere balance between passive efflux and active uptake of Ca^{2+} as previously suggested [9,21], in which case the lower Ca^{2+}-pump activity, but unaltered passive permeability in hypothyroidism should have resulted in a shift of the Ca^{2+}_i-[Ca^{2+}_o] relationship (Fig. 2). The absence of such an effect is in line with results obtained by Haynes and Mandveno [26], who observed the same Ca^{2+}_i-[Ca^{2+}_o] relationship as reported here and showed that reducing the Ca^{2+}-pump activity by 90% by raising the pH from 7.0 to 8.0, left the relationship unaltered. Since the passive efflux of Ca^{2+} shows no such pH dependence, this precludes the balance of active uptake and passive efflux as a determinant of the Ca^{2+} gradient.

Ca^{2+}-exchange studies on the other hand, have led to the conclusion that at steady state, Ca^{2+} efflux is mediated by the Ca^{2+} pump itself. Such tight coupling of Ca^{2+}-influx and -efflux was suggested by several groups who observed for the same maximal filling at high [Ca^{2+}_o], widely different Ca^{2+}-exchange rates proportional to the Ca^{2+}-pump activity [10,12,27,29,34,35]. Two basically different, but not mutually exclusive mechanisms have been proposed, in which Ca^{2+} effluxes through reversal of the pump cycle with resynthesis of ATP [11], or Ca^{2+} effluxes as part of the normal forward reaction cycle, with the return of the enzyme's Ca^{2+} translocator to the exterior position, i.e., coupled to ATP-hydrolysis [10,12]. A carefull study by Gerdes and Møller provided evidence for the existence of the latter efflux route, at least at 100 μM [Ca^{2+}_o] and filling levels of the sarcoplasmic reticulum > 30% of the maximal value [12]. Furthermore, net ATP-consumption by the Ca^{2+} pump at low [Ca^{2+}_o], 0.3–3.5 μM and steady-state filling levels of 14–60 nmol/mg (heart sarcoplasmic reticulum) was also observed in Ref. 11.

Although for both proposed mechanisms, the filling level at which influx equals efflux is independent of the Ca^{2+}-pump activity of a preparation, which is in line with the present results, the scheme proposed by Gerdes and Møller also accounts for the observed high rate of net ATP-consumption at steady state. It is unlikely that the (Ca^{2+} + Mg^{2+})-ATPase activities in the present study are due to a fraction of leaky or unsealed sarcoplasmic reticulum fragments. The equal stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity after making all material permeable to Ca^{2+}, indicated that preparations from both groups contained similar fractions of unsealed material. This experiment furthermore indicated that 20% of the material should be leaky if this were to explain the observed steady-state (Ca^{2+} + Mg^{2+})-ATPase activities. The obtained estimate of the actual fraction that was unable to sequester Ca^{2+} of 10%, comprises at least 4% plasmamembrane and 1% mitochondrial contamination, as indicated by marker-enzyme analysis [30]. The presence of 5% unsealed sarcoplasmic reticulum fragments in the preparations is therefore a more realistic estimate, suggesting that the greater part of the observed steady-state (Ca^{2+} + Mg^{2+})-ATPase activity is associated with the maintenance of the Ca^{2+} gradient of intact vesicles, rather than with leaky material.

If we then compare the steady-state (Ca^{2+} + Mg^{2+})-ATPase activity (Fig. 3), corrected for 5% unsealed material (Fig. 6), with the Ca^{2+}-cycling rate of Table II, it shows that approx. 50% can be accounted for by Ca^{2+} cycling coupled to net ATP hydrolysis, using a coupling ratio of 2. The remainder would constitute exchange of Ca^{2+} through (partial) reversal of the Ca^{2+}-pump cycle. This corroborates the results of Feher and Briggs, who reported that 40% of their Ca^{2+}-cycling activity was possibly not related to pump reversal [11].

Implications for the in vivo situation: the effect of hypothyroidism

In summary, the interpretation of the results implies, that the maintenance of a Ca^{2+} gradient by the sarcoplasmic reticulum under conditions prevailing in resting skeletal muscle, i.e., [Ca$_o^{2+}$] of 0.05 μM and a phosphorylation potential $> 2 \cdot 10^4$ M^{-1}, is associated with a substantial energy turnover proportional to the Ca^{2+}-pump content of the membrane. On the other hand, the Ca^{2+} gradient is relatively insensitive to variations of the energy state of the cell (phosphorylation potential), which precludes fluctuations of the Ca^{2+} gradient that in turn would affect the important homeostasis of the low cytosolic [Ca^{2+}].

The results indicate a slight (−15%, Table I), but significant reduction of the Ca^{2+}-pump content of sarcoplasmic reticulum in hypothyroidism, which is in line with a similar but more pronounced effect on the Ca^{2+}-pump density observed for soleus muscle [4]. The somewhat larger difference in maximal (Ca^{2+} + Mg^{2+})-ATPase activity between the preparations used here (-25%, Table I), is accounted for by the lower catalytic turnover number of the Ca^{2+} pump in hypothyroidism, which is related to a higher energy of activation of the enzyme in this thyroid state [3].

However, as the Ca^{2+}-pump activity does not determine the attainable Ca^{2+} gradient, hypothyroidism has no effect on the Ca^{2+}-sequestering capacity of sarcoplasmic reticulum, which at 0.05 μM [Ca$_o^{2+}$] gives a Ca^{2+}-filling level of 30% of its maximum. This value corroborates earlier estimates of the fractional filling level of sarcoplasmic reticulum in vivo [31]. Nevertheless, the lower Ca^{2+}-pump activity in hypothyroidism gives rise to a proportionately lower steady-state (Ca^{2+} + Mg^{2+})-ATPase activity coupled to the (futile) cycling of Ca^{2+}. The consequences of this for the in vivo situation were assessed using the previously determined sarcoplasmic reticulum content of gastrocnemius-plantaris muscle of 6.8 and 4.7 mg/g wet weight in euthyroidism and hypothyroidism, respectively [3]. The ATP consumption related to the maintenance of a Ca^{2+} gradient at 0.05 μM [Ca$_o^{2+}$] and 37°C was calculated, making corrections for the temperature [3] and the contribution of 5% leaky vesicles to the ATPase data in Fig. 3. We arrive at an activity of 0.88 and 0.44 μmol ATP/g per min for euthyroid and hypothyroid muscle, respectively. In both cases this is 20% of the resting metabolic rate, as determined for predominantly fast muscle in hindlimb perfusion experiments [2].

These results, in conclusion, suggest that nearly one third of the decrease in basal metabolic rate of skeletal muscle in hypothyroidism is accounted for by the decreased sarcoplasmic reticulum content of the muscle and the lower Ca^{2+}-pump activity of this organel.

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