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# Quantitative determination of $Ca^{2+}$-dependent $Mg^{2+}$-ATPase from sarcoplasmic reticulum in muscle biopsies

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The possibility of quantifying the total concentration of $Ca^{2+}$-dependent $Mg^{2+}$-ATPase of sarcoplasmic reticulum was investigated by measurement of the $Ca^{2+}$-dependent steady-state phosphorylation from [$\gamma$-$^{32}$P]ATP and the $Ca^{2+}$-dependent 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity in crude muscle homogenates. The $Ca^{2+}$-dependent phosphorylation at 0 °C (mean ± S.E.) was 40.0 ± 2.5 ($n = 6$) and 6.2 ± 0.7 ($n = 4$) nmol/g wet wt. in rat extensor digitorum longus (EDL) and soleus muscle, respectively ($P < 0.001$). The $Ca^{2+}$-dependent 3-*O*-MFPase activity at 37 °C was 1424 ± 238 ($n = 6$) and 335 ± 56 ($n = 4$) nmol/min per g wet wt. in rat EDL and soleus muscle, respectively ($P < 0.01$). The molecular activity calculated from these measurements amounted to 35 ± 5 $min^{-1}$ ($n = 6$) and 55 ± 10 $min^{-1}$ ($n = 4$) for EDL and soleus muscle respectively. These values were not different from the molecular activity calculated for purified $Ca^{2+}$-ATPase (36 $min^{-1}$). The $Ca^{2+}$-dependent $^{32}$P incorporation in soleus muscle decreased in the order mice > rats > guinea pigs. In EDL muscles from hypothyroid rats a 30% reduction of the $Ca^{2+}$-dependent phosphorylation was observed. The $Ca^{2+}$-dependent phosphorylation in vastus lateralis muscle from three human subjects amounted to 4.5 ± 0.8 nmol/g wet wt. It is concluded that measurement of the $Ca^{2+}$-dependent phosphorylation allows rapid and reproducible quantification of the concentration of $Ca^{2+}$-dependent $Mg^{2+}$-ATPase of sarcoplasmic reticulum. Since only 20–60 mg of tissue is required for the measurements, the method can also be used for biopsies obtained in clinical studies.

## INTRODUCTION

The $Ca^{2+}$-dependent $Mg^{2+}$-ATPase ($Ca^{2+}$-ATPase) of the sarcoplasmic reticulum (SR) membrane of skeletal muscle is the major transport system responsible for the removal of $Ca^{2+}$ ions from the sarcoplasm during relaxation. Relaxation rate and $Ca^{2+}$-ATPase concentration have been shown to vary with development (Close, 1964; Martonosi *et al.*, 1977), fibre type (Close, 1964; Wang *et al.*, 1979; Kim *et al.*, 1981; Dulhunty *et al.*, 1987) and thyroid status (Nicol & Bruce, 1981; Kim *et al.*, 1982; Simonides & van Hardeveld, 1985; Everts & van Hardeveld, 1987).

Isolation of SR vesicles requires relatively large muscle samples. Moreover, the recovery of the $Ca^{2+}$-ATPase after purification is at most 10–20% (Meissner *et al.*, 1973; Simonides & van Hardeveld, 1985) and may vary with fibre type (Kim *et al.*, 1981). The present study was undertaken to evaluate the possibility of quantifying the total concentration of $Ca^{2+}$-ATPase in crude muscle homogenates by measurement of the $Ca^{2+}$-dependent steady state phosphorylation from [$\gamma$-$^{32}$P]ATP (Martonosi *et al.*, 1977). Homogenates were prepared from samples weighing 20–60 mg obtained from the soleus (slow-twitch) and EDL (fast-twitch) muscle from rats, guinea pigs and mice, and from human VL (mixed type) muscle. The artificial substrate 3-*O*-methylfluorescein phosphate (3-*O*-MFP) (Hill *et al.*, 1968) was used to examine the $Ca^{2+}$-dependent phosphatase activity of the $Ca^{2+}$-ATPase.

Apart from the $Ca^{2+}$-ATPase, there are two other enzymes whose reaction pathway is characterized by the formation of a covalent aspartyl-phosphate. These include the Na,K-ATPase of the sarcolemma and the H,K-ATPase of the gastric mucosa (Pedersen & Carafoli, 1987; Jørgensen & Andersen, 1988). Therefore, we have also quantified the concentration of Na,K-ATPase by measurement of [$^3$H]ouabain binding capacity in some of the muscle preparations.

The results show that the concentration of $Ca^{2+}$-ATPase in rat EDL muscle is 6–8-fold higher than in rat soleus and human VL muscle. Moreover, the concentration of $Ca^{2+}$-ATPase in rat soleus and EDL muscle is 25–125 times higher than the concentration of Na,K-ATPase in the same muscle preparations.

It is concluded that measurement of the $Ca^{2+}$-dependent phosphorylation in crude homogenates allows a rapid and reproducible quantification of the concentration of $Ca^{2+}$-ATPase. Because of the small sample size, the method can also be applied in clinical studies.

## MATERIALS AND METHODS

### Animals

Soleus and EDL muscles were prepared from adult female fed animals; Wistar rats (200 g), mice (25 g) and guinea pigs (800–1000 g). Hypothyroidism was induced by maintaining 10-week-old rats on an iodine-deficient diet (0.5 $\mu$mol of I/kg) (Altromin, Germany) for 5–6 weeks with distilled water during the first 2 weeks and drinking water containing 144 mM-$KClO_4$ for the fol-

Abbreviations used: $Ca^{2+}$-ATPase, $Ca^{2+}$-dependent $Mg^{2+}$-ATPase from sarcoplasmic reticulum (EC 3.6.1.38); $C_{12}E_8$, octaethylene glycol mono-n-dodecyl ether; EDL, extensor digitorum longus; Na,K-ATPase, $Na^+ + K^+$-dependent ATPase (EC 3.6.1.37), 3-*O*-MFP: 3-*O*-methylfluorescein phosphate; SR, sarcoplasmic reticulum; VL, vastus lateralis.

lowing 3–4 weeks (van Hardeveld & Clausen, 1984; Everts & Clausen, 1986).

### Human muscle samples

These were obtained from three patients, 41, 68 and 72 years old, respectively, who had given their informed consent. The samples were taken from the vastus lateralis (VL) muscle during surgery to the hip region. It was ensured that the patients had no history of muscular disease and were clinically euthyroid.

### Preparation of homogenates

Immediately after dissection, all muscle samples were weighed and stored in liquid $N_2$ for 15–60 min. As a standard procedure the muscle samples (20–60 mg) were homogenized at 0 °C for two periods of 30 s at full speed on an Ultra-Turrax tissue homogenizer (Janke and Kunkel) in 40 vol. of buffer containing 5 mM-Hepes and 300 mM-sucrose (pH 7.4). The knife was rinsed with 40 vol. of buffer. The suspension was further homogenized at 0 °C by 10 strokes in a glass homogenizer with a tight-fitting Teflon pestle rotating at 1000 rev./min. The final concentration of the homogenates was 10 mg of muscle tissue/ml. All homogenates were divided into portions of 1 ml and were either used immediately for experiments or stored at −60 °C until use.

For the preparation of material for control experiments 10 EDL or soleus muscles were pooled and homogenates were prepared at a concentration of 100 mg of tissue/ml. For the development of the 3-*O*-MFPase assay, SR $Ca^{2+}$-ATPase was purified from rabbit hind limb muscle (Andersen *et al.*, 1982), a procedure yielding enzyme with at least 90% purity as judged by SDS/polyacrylamide-gel electrophoresis.

### Determination of phosphoprotein concentration with [$\gamma$-$^{32}$P]ATP

This was essentially performed as described by Martonosi *et al.* (1977) with some modifications. In the routine assay procedure 200 $\mu$l aliquots of the homogenate (10 mg/ml) were reacted for 30 s at 0 °C in a medium (final volume 3.0 ml) containing in mM: imidazole, 100; KCl, 100; $MgCl_2$, 5; EGTA, 0.5; ATP, 0.05; [$\gamma$-$^{32}$P]ATP (0.3 $\mu$Ci/ml) was added as a tracer (pH 7.4). In a parallel assay $CaCl_2$ (0.55 mM) was also added to the medium. Each assay was carried out in duplicate. In control experiments 100 $\mu$l aliquots of homogenates containing 100 mg/ml were used for each incubation.

The reaction was started by addition of the homogenate. The reaction was quenched with 3 ml of a solution containing in mM: trichloroacetic acid, 600; $Na_4P_2O_7$, 10; $KH_2PO_4$, 10, and the suspension was centrifuged (40 min, 4000 ***g***, 4 °C). The pellet was resuspended in a solution containing in mM: trichloroacetic acid, 60; $Na_4P_2O_7$, 10; $KH_2PO_4$, 10, and the centrifugation (20 min, 4000 ***g***, 4 °C) was repeated. Thereafter, the pellet was resuspended once more in the same medium and centrifuged. After dissolving the pellet in 0.5 ml of 1 M-NaOH at 55 °C, 0.6 ml of water was added. An aliquot (1 ml) of this suspension was neutralized with 1 ml of 0.25 M-$H_2SO_4$ and 10 ml of Triton/toluene-based scintillation fluid was added to the mixture for counting of radioactivity. From the incubation medium, 0.2 ml aliquots (obtained from the supernatant after the first centrifugation) were used for counting after addition of 0.5 ml of 1 M-NaOH, 0.3 ml of water, 1 ml of 0.25 M-$H_2SO_4$ and 10 ml of the same scintillation fluid. Counting efficiency was the same in the samples from the pellet and the incubation medium and amounted to 98%.

The $^{32}$P activity in the pellet was expressed as the relative uptake, i.e. $^{32}$P/ml of incubation medium divided by the muscle wet weight. The relative $^{32}$P uptake was multiplied by the ATP concentration of the medium to obtain absolute values for $^{32}$P uptake (in nmol/g wet wt.). The $Ca^{2+}$-dependent $^{32}$P uptake (or $Ca^{2+}$-ATPase concentration) was calculated as the difference between the values obtained in the presence and absence of $CaCl_2$.

Part of the experiments were repeated with [U-$^{14}$C]ATP (0.3 $\mu$Ci/ml) instead of [$\gamma$-$^{32}$P]ATP.

In one series of experiments, the phosphorylation reaction was, after acid precipitation, followed by incubation at 0 °C in 0.1 M-phosphate buffer (pH 5.5) in the absence or presence of 1 M-hydroxylamine (Martonosi *et al.*, 1977).

### Determination of hydrolytic activity with 3-*O*-MFP

The incubation medium contained in mM: Tris, 80; $MgCl_2$, 4; EGTA, 0.5; KCl, 100; 3-*O*-MFP, 0.05 (pH 6.98), and was kept at 37 °C in a thermostated cuvette under continuous stirring. After recording of the spontaneous hydrolytic activity for 2–5 min, 25–50 $\mu$l of the homogenate (10 mg/ml) was added. The reaction was started 2 min later by the addition of $CaCl_2$ (0.45 mM) and was allowed to proceed for 4–6 min. Final volume of the reaction medium was 2650 $\mu$l.

The $K_m$ of $Ca^{2+}$-ATPase for 3-*O*-MFP was determined by using purified $Ca^{2+}$-ATPase and a 3-*O*-MFP concentration varying between 5 and 80 $\mu$M.

The fluorimetric assay was performed using a Perkin–Elmer MPF-44A spectrofluorimeter. Excitation wavelength was 475 nm, emission wavelength 515 nm and slitwidths 5 nm. For calibration, the fluorescence was measured with 2500 $\mu$l of the incubation medium and 3-*O*-methylfluorescein was added cumulatively in steps of 80 nM.

The $Ca^{2+}$-dependent phosphatase activity was calculated as the slope of the line recording the fluorescence in the presence of $CaCl_2$ corrected for $Ca^{2+}$-independent hydrolytic activity, and was expressed as nmol/min per g muscle wet wt. for homogenates and as nmol/min per mg of protein for purified $Ca^{2+}$-ATPase.

### Determination of Na,K-ATPase concentration

This was quantified by measuring [$^3$H]ouabain binding capacity in the presence of vanadate as described by Nørgaard *et al.* (1983). This procedure has been shown to allow the quantification of all functional $Na^+ + K^+$ pumps in skeletal muscle (Clausen *et al.*, 1987).

### Protein determination

The protein content of the crude homogenates was measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

### Chemicals

All chemicals were of analytical grade. Bovine serum albumin, 3-*O*-methylfluorescein, 3-*O*-MFP and ouabain were from Sigma. $C_{12}E_8$ was from Nirro Chemicals, Tokyo, Japan. [$^3$H]Ouabain (20.9 Ci/mmol) was from New England Nuclear. The purity of [$^3$H]ouabain was determined by the Na,K-ATPase extraction method (Hansen & Skou, 1973) and amounted to 95%. [U-

**Table 1. Effects of $Ca^{2+}$ and ATP concentration and incubation time on $^{32}P$ uptake in homogenates from EDL muscle from 12–14-week-old rats**

Homogenates were prepared from ten pooled EDL muscles from adult (12–14-week-old) rats and $^{32}P$ uptake was determined as described in the Materials and methods section except that the final concentration of the homogenate was 100 mg of tissue/ml and 100 $\mu$l aliquots were used for the assay. When the ATP concentration was varied (*a*), the $CaCl_2$ concentration was 0.55 mM and incubation time was 30 s. When the $Ca^{2+}$ concentration was varied (*b*), ATP concentration was 50 $\mu$M and incubation time was 30 s. Incubation time (*c*) was varied with an ATP concentration of 50 $\mu$M and a $CaCl_2$ concentration of 0.55 mM. The $Ca^{2+}$-dependent $^{32}P$ incorporation (third column) was calculated as the difference between the values obtained in the presence (first column) and absence (second column) of $CaCl_2$. The data represent means of duplicate determinations.

| Experimental conditions | $^{32}P$ uptake (nmol/g wet wt.) $+Ca^{2+}$ | $-Ca^{2+}$ | $Ca^{2+}$-dependent |
|---|---|---|---|
| (*a*) ATP concentration ($\mu$M) | | | |
| 10 | 38.1 | 3.9 | 34.2 |
| 25 | 47.9 | 5.0 | 42.9 |
| 50 | 57.8 | 6.7 | 51.0 |
| 100 | 57.4 | 8.2 | 49.2 |
| (*b*) $Ca^{2+}$ concentration ($\mu$M) (excess over 0.5 mM-EGTA) | | | |
| 0 | 45.7 | | 39.0 |
| 10 | 49.9 | | 43.2 |
| 25 | 52.7 | 6.7 | 46.0 |
| 50 | 53.7 | | 47.0 |
| 100 | 52.2 | | 45.5 |
| (*c*) Incubation time (s) | | | |
| 10 | 56.2 | 15.7 | 40.5 |
| 20 | 58.2 | 10.0 | 48.2 |
| 30 | 61.2 | 8.5 | 53.1 |
| 120 | 57.4 | 7.2 | 50.2 |

$^{14}C$]ATP (0.5 Ci/mmol) was obtained from Amersham and [$\gamma$-$^{32}P$]ATP (33.8 Ci/mmol) was from New England Nuclear. [U-$^{14}C$]ATP and [$\gamma$-$^{32}P$]ATP were purified by column chromatography (Nørby & Jensen, 1971).

### Statistics

All results are given as mean values ± S.E. with the number of observations in parentheses. The statistical significance of any difference was throughout ascertained using the two-tailed *t*-test for non-paired observations.

## RESULTS

### Phosphoprotein formation in EDL and soleus muscle

The optimum conditions (ATP, $CaCl_2$ concentration and incubation time) for measurement of $Ca^{2+}$-dependent $^{32}P$ incorporation in EDL muscle were established using a homogenate based on 10 pooled muscles (Table 1). The $^{32}P$ uptake in the presence of $CaCl_2$ is shown in the first column and that in the absence of $CaCl_2$ in the second. The $Ca^{2+}$-dependent $^{32}P$ uptake or $Ca^{2+}$-dependent $^{32}P$ incorporation was calculated as the difference between the values obtained in the presence and absence of $CaCl_2$ (third column).

**Table 2. Comparison of [$^{32}P$]ATP and [$^{14}C$]ATP uptake in homogenates of rat EDL and soleus muscle**

Homogenates (10 mg/ml) were prepared from individual EDL and soleus muscles from adult rats and $^{32}P$ uptake was determined as described in the Materials and methods section; 200 $\mu$l aliquots (2 mg of tissue) were used for each assay. In part of the experiments [U-$^{14}C$]ATP (0.3 $\mu$Ci/ml) was added as a tracer instead of [$\gamma$-$^{32}P$]ATP. Data represent means ± S.E. of duplicate determinations on six different homogenates of each muscle.

| Muscle (*n*) | $^{32}P$ uptake (nmol/g wet wt.) $-Ca^{2+}$ | $+Ca^{2+}$ | $^{14}C$ uptake (nmol/g wet wt.) $-Ca^{2+}$ | $+Ca^{2+}$ |
|---|---|---|---|---|
| EDL (6) | 7.0 ± 0.4 | 49.5 ± 0.7 | 3.6 ± 0.8 | 4.0 ± 0.8 |
| Soleus (6) | 5.6 ± 0.4 | 13.8 ± 0.5 | 3.0 ± 0.2 | 2.9 ± 0.3 |
| | $P < 0.05$ | $P < 0.001$ | $P > 0.40$ | $P > 0.30$ |

As can be seen from Table 1(*a*) the $Ca^{2+}$-dependent $^{32}P$ incorporation was saturated at an ATP concentration of 50 $\mu$M. Furthermore, the highest value of $Ca^{2+}$-dependent phosphoprotein formation was obtained at an excess $Ca^{2+}$ concentration of 50 $\mu$M (i.e. 0.5 mM-EGTA and 0.55 mM-$CaCl_2$) (Table 1*b*), and an incubation time of 30 s (Table 1*c*). Under the optimum conditions thus established, the $^{32}P$ uptake in the absence of $CaCl_2$ amounted to 13–16 % of the $Ca^{2+}$-dependent phosphorylation. The optimum conditions for measurement of $Ca^{2+}$-dependent phosphoprotein formation in soleus muscle were the same as for EDL muscle (not shown).

Comparison of the phosphoprotein formation in rat EDL and soleus muscle is shown in Table 2. In the absence of $Ca^{2+}$ a slight but significant difference in $^{32}P$ uptake was observed between EDL and soleus (first column). In the presence of $Ca^{2+}$, the difference was much larger (3.6-fold) (second column). The calculated $Ca^{2+}$-dependent $^{32}P$ incorporation amounted to 42.5 ± 0.8 nmol/g wet wt. in EDL ($n = 6$) and to 8.2 ± 0.6 nmol/g wet wt. in soleus muscle ($n = 6$) ($P < 0.001$). To examine whether $^{32}P$ uptake in the absence of $CaCl_2$ could be accounted for by unspecific trapping of isotope in the pellet, parallel experiments were performed with [U-$^{14}C$]ATP. No significant difference between EDL and soleus was observed with respect to [$^{14}C$]ATP uptake, either in the absence (third column) or in the presence of $Ca^{2+}$ (fourth column). It can be seen that around 50 % of the $^{32}P$ uptake in the absence of $CaCl_2$ in both muscles could be accounted for by unspecific trapping of [$^{32}P$]ATP in the pellet. In addition, 99 % of the $Ca^{2+}$-dependent $^{32}P$ incorporation in EDL muscle could be removed by addition of hydroxylamine after acid precipitation. This indicated that $^{32}P$ is bound to an aspartic acid residue (Nagano *et al.*, 1965).

### 3-*O*-Methylfluorescein phosphatase activity

Fig. 1 shows a representative determination of the $Ca^{2+}$-dependent 3-*O*-MFPase activity of purified $Ca^{2+}$-ATPase. The addition of $CaCl_2$ clearly produced an

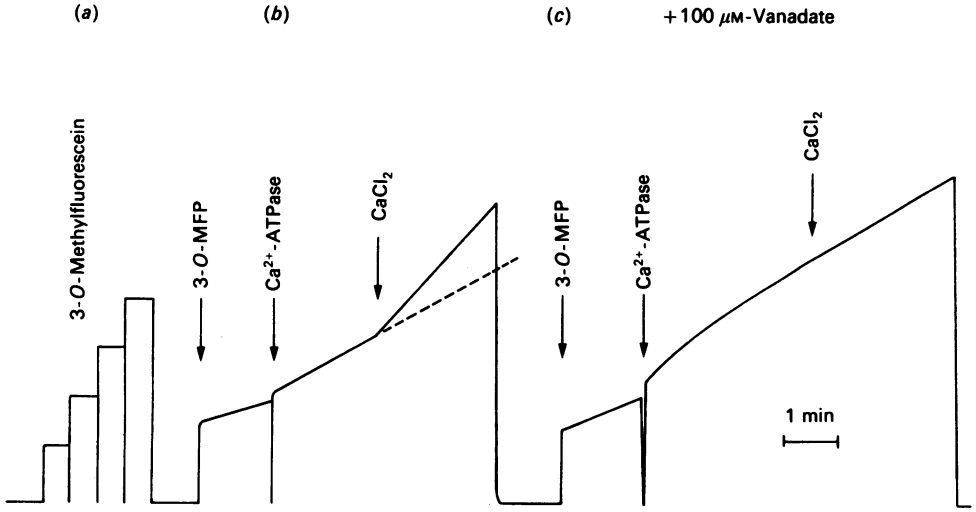


**Fig. 1. Representative recordings showing the $Ca^{2+}$-dependent 3-*O*-MFPase activity of purified $Ca^{2+}$-ATPase in the absence (*b*) and presence (*c*) of vanadate (100 μM)**

Purified $Ca^{2+}$-ATPase (approx. 0.3 μg/ml of medium) was assayed for 3-*O*-MFPase activity as described in the Materials and methods section. Arrows in (*b*) and (*c*) indicate the addition of 3-*O*-MFP, the $Ca^{2+}$-ATPase or $CaCl_2$; (*a*) shows calibration by stepwise addition of 80 nM-3-*O*-methylfluorescein.

increase in fluorescence (Fig. 1*b*) which was prevented by vanadate (100 μM) (Fig. 1*c*).

To determine the $K_m$ of $Ca^{2+}$-ATPase for 3-*O*-MFP, the hydrolytic activity of purified $Ca^{2+}$-ATPase was measured over a concentration range of 5–80 μM-3-*O*-MFP. When the data were expressed as $1/v$ against 1/[S] (Fig. 2), a straight line was obtained ($r = 0.969$, $P < 0.001$), indicating that only one component is involved in the phosphatase reaction. The concentration of 3-*O*-MFP giving half-maximal reaction velocity was 25 μM. The 3-*O*-MFPase activity with 50 μM-3-*O*-MFP (the concentration used for the determinations on muscle homogenates) amounted to 184 nmol/min per mg of protein. The molecular activity calculated from the 3-*O*-MFPase activity and the number of phosphorylation sites (5.1 nmol/mg of protein) of the purified $Ca^{2+}$-ATPase was 36.1 $min^{-1}$ (mol of 3-*O*-MFP split/mol of phosphorylation sites per min).

Table 3 shows a comparison of $Ca^{2+}$-dependent phosphorylation and 3-*O*-MFPase activity in rat EDL and soleus muscle. The $Ca^{2+}$-dependent $^{32}P$ incorporation was 6.5-fold larger in EDL than in soleus (first column),

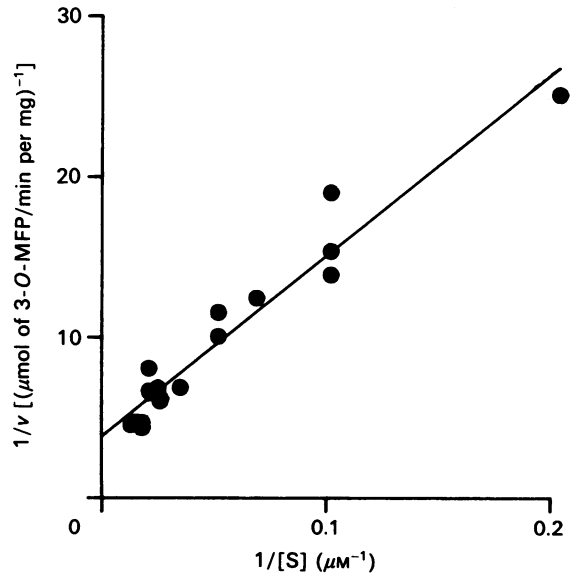


**Fig. 2. Double-reciprocal plot of the hydrolytic activity of purified $Ca^{2+}$-ATPase against 3-*O*-MFP concentration**

The $Ca^{2+}$-dependent 3-*O*-MFPase activity was determined as described in the Materials and methods section with 5–80 μM-3-*O*-MFP as substrate. The results were obtained in two separate experiments where measurements of 3-*O*-MFPase activity were carried out in duplicate. Linear regression analysis of $1/v$ against 1/[S] showed a straight line with a correlation coefficient $r = 0.969$ ($P < 0.001$).

**Table 3. $Ca^{2+}$-dependent [$^{32}$P]ATP incorporation, $Ca^{2+}$-dependent 3-*O*-MFPase activity and the calculated molecular activity in rat EDL and soleus muscle**

Homogenates (10 mg/ml) were prepared from individual EDL and soleus muscles from adult rats and $Ca^{2+}$-dependent $^{32}P$ incorporation and 3-*O*-MFPase activity were determined as described in the Materials and methods section; 200 μl aliquots were used for the determination of the $Ca^{2+}$-dependent $^{32}P$ incorporation and 25–50 μl aliquots for the 3-*O*-MFPase assay. In the 3-*O*-MFPase assay 50 μM-3-*O*-MFP was present as a substrate. Data represent means ± S.E. of duplicate determinations on six EDL and four soleus muscle homogenates.

| Muscle (*n*) | $Ca^{2+}$-dependent $^{32}P$ incorporation (nmol/g wet wt.) | $Ca^{2+}$-dependent 3-*O*-MFPase activity (nmol/min per g wet wt.) | Molecular activity ($min^{-1}$) |
|---|---|---|---|
| EDL (6) | 40.0 ± 2.5 | 1424 ± 238 | 34.7 ± 4.7 |
| Soleus (4) | 6.2 ± 0.7 | 335 ± 56 | 55.4 ± 9.8 |
| | $P < 0.001$ | $P < 0.01$ | $P > 0.05$ |

**Table 4. $Ca^{2+}$-dependent $^{32}P$ incorporation in EDL and soleus muscle of mice, guinea pigs and rats**

From each species muscle homogenates with a concentration of 10 mg of tissue/ml were prepared and $Ca^{2+}$-dependent $^{32}P$ incorporation was determined as described in the Materials and methods section; 200 $\mu$l aliquots of the homogenates were used in each assay. Values represent means ± S.E. with the number of animals in parentheses. *$P < 0.005$ as compared with rat EDL, **$P < 0.001$ as compared with rat soleus.

| | $Ca^{2+}$-dependent $^{32}P$ incorporation (nmol/g wet wt.) | |
|---|---|---|
| Species | EDL ($n$) | Soleus ($n$) |
| Mouse | 38.2 ± 1.9 (4) | 9.0 ± 0.2** (4) |
| Rat | 38.7 ± 1.0 (6) | 6.4 ± 0.3 (5) |
| Guinea pig | 30.9 ± 1.7* (4) | 2.9 ± 0.4** (4) |

whereas 3-*O*-MFPase activity was 4.3-fold higher (second column). The calculated molecular activity was not significantly different in the two muscles (third column) and was in the same range as that determined for purified $Ca^{2+}$-ATPase (36.1 $min^{-1}$). Treatment of the homogenates with detergent ($C_{12}E_8$) did not increase the $Ca^{2+}$-dependent phosphorylation or the $Ca^{2+}$-dependent 3-*O*-MFPase activity.

### Comparison of phosphoprotein formation in muscle of mice, rats, guinea pigs and man

A comparison of the $Ca^{2+}$-dependent $^{32}P$ incorporation in the EDL and soleus muscle of three animal species is shown in Table 4. The phosphoprotein formation in EDL muscle was the same in mice and rats, but around 20% lower in guinea pigs (Table 4, first column). In soleus muscle a significant decrease in $^{32}P$ incorporation was seen in the order mice > rats > guinea pigs (Table 4, second column). The protein content of the homogenates did not vary with fibre type nor with species (all muscles contained around 200 mg of protein/g wet wt.).

The $Ca^{2+}$-dependent phosphorylation in samples (20–60 mg) of human VL muscle was 4.5 ± 0.8 nmol/g wet wt. ($n = 3$). The protein content of these homogenates was around 150 mg/g wet wt. Expressed per mg of protein, the phosphoprotein formation in the human VL muscle amounted to around 30 pmol/mg of protein.

### Effect of hypothyroidism

Hypothyroidism induced a 24% decrease in phosphoprotein formation in rat EDL muscle [control 39.2 ± 0.1 nmol/g wet wt. ($n = 4$) versus hypothyroid 29.9 ± 0.7 nmol/g wet wt. ($n = 5$) ($P < 0.001$)]. This effect could not be ascribed to a decrease in the total protein content of the homogenates. When expressed per mg of protein, the decrease in $Ca^{2+}$-dependent $^{32}P$ incorporation after thyroid hormone depletion amounted to 30% ($P < 0.001$). Our results are in good agreement with those obtained with isolated SR vesicles of rat gastrocnemius (fast-twitch) muscle (Simonides & van Hardeveld, 1985). In that study it was calculated that hypothyroidism resulted in a 30% reduction of the entire SR membrane system.

### Comparison of $Ca^{2+}$-ATPase with Na,K-ATPase concentration

The Na,K-ATPase concentration as quantified by [$^3$H]ouabain binding was 315 ± 7 pmol/g wet wt. ($n = 6$) and 251 ± 6 pmol/g wet wt. ($n = 6$) in rat EDL and soleus muscle, respectively. The total concentration of $Ca^{2+}$-ATPase (Table 3) was 125- and 25-fold larger than that of Na,K-ATPase in rat EDL and soleus muscle, respectively. The [$^3$H]ouabain binding site concentration in human VL muscle was previously described to amount to 278 ± 15 pmol/g wet wt. ($n = 20$) (Nørgaard *et al.*, 1984). This implies that in human VL muscle the $Ca^{2+}$-ATPase concentration is at least 15-fold larger than the Na,K-ATPase concentration.

## DISCUSSION

The results of the present study show that the $Ca^{2+}$-ATPase concentration can be quantified in crude muscle homogenates by measurement of $Ca^{2+}$-dependent $^{32}P$ incorporation as well as 3-*O*-MFPase activity.

The quantitative determination of the $Ca^{2+}$-ATPase concentration by phosphorylation in the presence of a relatively high concentration of $Ca^{2+}$ rests on the assumption that in steady state all enzyme molecules are in a phosphorylated state. For the SR $Ca^{2+}$-ATPase of fast-twitch muscle, it is well established that the rate-limiting reaction step is a conformational change of the phosphoenzyme ($E_1P$–$E_2P$ transition) (Martonosi & Beeler, 1983). For slow-twitch muscle, there is also evidence that decomposition of the phosphoenzyme is considerably slower than its formation, so that the phosphorylated form of the protein accumulates during ATP hydrolysis (Wang *et al.*, 1979). Therefore, there is a firm basis for using the steady-state phosphorylation level as a measurement of the concentration of $Ca^{2+}$-ATPase.

It is less certain that the 3-*O*-MFPase activity directly reflects the concentration of $Ca^{2+}$-ATPase in various muscle types, since the reaction cycle is dependent on the muscle type. The low molecular activity of the purified $Ca^{2+}$-ATPase with 3-*O*-MFP (36.1 $min^{-1}$) is not unexpected in view of the fact that a similar low molecular activity (10–60 $min^{-1}$) was found with another artificial substrate (*p*-nitrophenyl phosphate) (Inesi, 1971). The molecular activities calculated for EDL and soleus homogenates were not different from that calculated for purified $Ca^{2+}$-ATPase. This indirectly suggests that the phosphorylation measurements mainly include phosphorylation of the $Ca^{2+}$-ATPase. If other proteins were included for a substantial part, molecular activities lower than that of the $Ca^{2+}$-ATPase would be expected.

The $Ca^{2+}$-dependent $^{32}P$ incorporation in rat EDL muscle was around 6-fold larger than in soleus muscle, which is in good agreement with the relative difference reported by Dulhunty *et al.* (1987) based on immunofluorescence and electron microscopy with monoclonal antibodies. Using an immunochemical method, Gundersen *et al.* (1988) have reported values of around 3 mg of $Ca^{2+}$-ATPase/g total protein for adult rat soleus and around 20 mg of $Ca^{2+}$-ATPase/g total protein for EDL muscle. Assuming a molecular mass of the $Ca^{2+}$-ATPase of around 100 kDa (Martonosi & Beeler, 1983) and a total muscle protein content of 200 mg/g wet wt. for both muscles (see the description of Table 4 in the Results section), $Ca^{2+}$-ATPase concentrations of 6 and

40 nmol/g wet wt. for soleus and EDL muscle can be calculated, values which are in good agreement with our data.

The $Ca^{2+}$-dependent $^{32}P$ incorporation in mouse EDL muscle was of the same order of magnitude as previously reported by Martonosi *et al.* (1977) (30–60 nmol/g wet wt.). Our results show that also in mice and in guinea-pigs the $Ca^{2+}$-ATPase concentration is considerably larger in fast- as compared with slow-twitch muscle. It is, however, interesting to note that the $Ca^{2+}$-ATPase concentration in EDL and soleus muscle seems to decrease with increasing size of the animal roughly in the same way as the shortening velocity (Close, 1972). This observation has in turn been related to the fact that limb movements are much more rapid in small as compared with larger mammals. Extrapolation to human muscle would predict that the $Ca^{2+}$-ATPase concentration in both fast- and slow-twitch human fibres will be lower than in the respective muscles of guinea pigs. This might provide an explanation for the relatively low $Ca^{2+}$-ATPase concentration found in the human VL (mixed fibre-type) muscle.

The concentration of $Ca^{2+}$-ATPase in rat muscle was 25–125-fold greater than that of Na,K-ATPase. Even if it is taken into account that the surface area of the sarcolemma is only one-tenth that of the SR (Eisenberg, 1983), the difference between $Ca^{2+}$-ATPase and Na,K-ATPase concentration is still considerable. This implies that, even if our measurements of phosphoprotein formation in the crude homogenates include the Na,K-ATPase, it would be of minor importance for the values of the $Ca^{2+}$-ATPase concentration. The question may arise whether the difference in $Ca^{2+}$-ATPase and Na,K-ATPase concentration has significance for their contribution to the energy metabolism. In resting skeletal muscle, $Na^+ + K^+$ transport and $Ca^{2+}$ transport each account for less than 10 % of the total energy expenditure (Biron *et al.*, 1979; Hasselbach & Oetliker, 1983). However, the contribution of the $Ca^{2+}$-ATPase to the extra energy consumption during work may amount to 30–50 % (Homsher *et al.*, 1972; Rall, 1982), whereas the major remaining part is accounted for by the crossbridge cycling and only a minor part by the Na,K-ATPase (Kushmerick, 1983). This suggests that variations in the $Ca^{2+}$-ATPase concentration are of much more importance for the energetic efficiency of muscular work than changes in the Na,K-ATPase concentration.

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