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Adenosine triphosphatases during maturation of cultured human skeletal muscle cells and in adult human muscle

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Na^+/K^+-ATPase, Mg^{2+}-ATPase and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase are examined in cultured human skeletal muscle cells of different maturation grade and in human skeletal muscle. Na^+/K^+-ATPase is investigated by measuring ouabain binding and the activities of Na^+/K^+-ATPase and K^+-dependent 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase). SR Ca^{2+}-ATPase is examined by ELISA, Ca^{2+}-dependent phosphorylation and its activities on ATP and 3-*O*-methylfluorescein phosphate. Na^+/K^+-ATPase and SR Ca^{2+}-ATPase are localized by immunocytochemistry. The activities of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase show a good correlation with the other assayed parameters of these ion pumps. All ATPase parameters investigated increase with the maturation grade of the cultured muscle cells. The number of ouabain-binding sites and the activities of Na^+/K^+-ATPase and K^+-dependent 3-*O*-MFPase are significantly higher in cultured muscle cells than in muscle. The Mg^{2+}-ATPase activity, the content of SR Ca^{2+}-ATPase and the activities of SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-*O*-MFPase remain significantly lower in cultured cells than in muscle. The ouabain-binding constant and the molecular activities of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase are equal in muscle and cultured cells. During ageing of human muscle the activity as well as the concentration of SR Ca^{2+}-ATPase decrease. Thus the changes of the activities of the ATPases are caused by variations of the number of their molecules. Na^+/K^+-ATPase is localized in the periphery of fast- and slow-twitch muscle fibers and at the sarcomeric I-band. SR Ca^{2+}-ATPase is predominantly confined to the I-band, whereas fast-twitch fibers are much more immunoreactive than slow-twitch fibers. The presence of cross-striation for Na^+/K^+-ATPase and SR Ca^{2+}-ATPase in highly matured cultured muscle cells indicate the development and subcellular organization of a transverse tubular system and SR, respectively, which resembles the in vivo situation.

Introduction

Depolarization of the sarcolemma induces a Ca^{2+} release from the sarcoplasmic reticulum into the cytoplasm elevating the cytosolic Ca^{2+} concentration, which on its turn causes muscle contraction [1]. Each action potential is associated with an influx of Na^+ and an efflux of K^+. During the contraction muscles have a net potassium loss provoking an increase of the K^+ concentration in the interstitial fluid and blood plasma [2,3]. After excitation of skeletal muscle, ATP-dependent ion pumps restore the disturbed ion homeostasis.

Na^+/K^+-ATPase (EC 3.6.1.37) of the sarcolemma reestablishes the Na^+,K^+ gradient by K^+ uptake from the plasma into the muscle and Na^+ release [4]. An apparent relation exists between the Na^+/K^+-ATPase concentration and muscle performance [5]. The concentration of Na^+/K^+-ATPase in small muscle biopsies (1–5 mg) can be measured by vanadate-facilitated [^{3}H]ouabain binding [6]. It is nearly impossible to detect Na^+/K^+-ATPase activity in muscle homogenates, because of vesicle formation during homogenization, inhomogeneity of suspensions and the presence of other active ATPases. The K^+-dependent hydrolytic cleavage of the artificial substrate 3-*O*-methylfluorescein phos-

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Abbreviations: BSA, bovine serum albumin; CK-MM, creatine kinase muscle-specific isoenzyme MM; ELISA, enzyme-linked immunosorbent assay; Na^+/K^+-ATPase, $Na^+ + K^+$-dependent ATPase (EC 3.6.1.37); PBS, phosphate-buffered saline; SR Ca^{2+}-ATPase, Ca^{2+}-dependent Mg^{2+}-ATPase from sarcoplasmic reticulum (EC 3.6.1.38); 3-*O*-MFP, 3-*O*-methylfluorescein phosphate; 3-*O*-MFPase, 3-*O*-methylfluorescein phosphatase.

phate and its inhibition by ouabain can be used as a measure for Na^+/K^+-ATPase activity in muscle homogenates [7].

The growth and differentiation of skeletal muscle cells of chicken and rodents in vivo and/or in vitro is associated with an increase in the number of Na^+/K^+-pumps [8–13]. In cultured chicken muscle cells the Na^+/K^+-ATPase activity, measured by ouabain-suppressible $^{86}Rb^+$ uptake, rises simultaneously with the concentration of Na^+/K^+-ATPase [9].

The Ca^{2+}-dependent Mg^{2+}-ATPase of the sarcoplasmic reticulum membrane (SR Ca^{2+}-ATPase, EC 3.6.1.38) is responsible for the active transport of Ca^{2+} ions from the cytosol into the lumen of sarcoplasmic reticulum. SR Ca^{2+}-ATPase can be quantified by enzyme-linked immunosorbent assays [14] or Ca^{2+}-dependent steady-state phosphorylation from [γ-^{32}P]ATP [15,16] and by measuring its activity on the substrates ATP and 3-*O*-MFP [16,17]. All parameters are about 5–7-times higher in fast- than in slow-twitch muscles of rabbits and rodents. During postnatal development of rabbits and mice the SR Ca^{2+}-ATPase content and the Ca^{2+} uptake rate of fast-twitch fibers increase markedly, whereas these parameters nearly change in slow-twitch muscle [14,18]. The SR Ca^{2+}-ATPase content increases in cultured rat and chicken muscle cells during differentiation and the SR Ca^{2+}-ATPase activity rises concomitantly [15,19,20].

To our knowledge the amounts and activities of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase and the activities of K^+- and Ca^{2+}-dependent phosphatase have not yet been investigated in developing human skeletal muscle cells. By combining different growth and differentiation media we can obtain human skeletal muscle cells with different maturation grades [21]. On the basis of the percentage CK-MM, human skeletal muscle cells cultured on growth and differentiation media containing Ultroser G and rat brain extract reach a significantly higher maturation grade after 7–20 days of differentiation than cells which proliferate on fetal calf serum and chicken embryo extract and differentiate on horse serum and chicken embryo extract [21]. The myotubes cultured on Ultroser G-containing media are much longer, their nuclei are often localized in rows on the periphery, and they show more frequently cross-striation. Many biochemical parameters as activities of creatine kinase, citrate synthase, cytochrome-*c* oxidase, AMP deaminase and phophorylase increase with maturation. We used in this study cultured cells obtained from sera- or Ultroser G-containing media and various differentiation periods to investigate the effect of maturation on Na^+/K^+-ATPase and SR Ca^{2+}-ATPase. For comparison different parameters of these ion pumps were also measured in adult human muscle. Immunostaining is carried out in order to evaluate the localization of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase in adult skeletal muscle and cultured muscle cells.

Materials and Methods

Materials

Ouabain, 3-*O*-methylfluorescein, 3-*O*-methylfluorescein phosphate and *p*-nitrophenyl phosphate were obtained from Sigma, St. Louis, MO, USA; thapsigargin from LC Services Corporation, Woburn, MA, USA; vanadate and creatine kinase NAC-activated monotest kit from Boehringer, Mannheim, Germany; [^{3}H]ouabain and [γ-^{32}P]ATP from Amersham International, Amersham, UK; Dulbecco's modified Eagle medium and Ultroser G from Gibco, Paisley, UK; fetal calf serum, horse serum and chicken embryo extract from Flow Laboratories, Irvine, UK.

Goat anti-rabbit renal Na^+/K^+-ATPase and rabbit anti-rat SR Ca^{2+}-ATPase were generous gifts from Dr. J.J.H.H.M de Pont (Department of Biochemistry, University of Nijmegen) and Dr. J.A.H. Timmermans (Department of Physiology, University of Nijmegen), respectively. The anti-rat SR Ca^{2+}-ATPase was raised against a mixture of slow- and fast-type SR Ca^{2+}-ATPase. Mouse anti-rabbit skeletal myosin fast and fluorescein-conjugated goat anti-rabbit IgG were from Sigma, St. Louis, MO, USA and fluorescein-conjugated donkey anti-goat IgG and tetramethylrhodamine-conjugated goat anti-mouse IgG from Nordic, Tilburg, Netherlands.

Muscle cell cultures

Muscle biopsies were obtained from individuals without any known muscular disorder, as approved by the Committee on Medical Ethics. Samples from m. quadriceps were obtained by open (100–250 mg) or percutaneous needle biopsy (25–50 mg) and from m. rectus abdominus and m. gluteus (200–400 mg) at cholecystectomy and orthopaedic surgery, respectively. The muscle samples were dissociated according to the dispersion technique of Yasin et al. [22]. The satellite cells were cultured on Dulbecco's modified Eagle medium with 20% fetal calf serum and 2% chicken embryo extract or with 4% Ultroser G and 10% rat brain extract [21]. After subculturing, when confluency has been reached, the cultures derived from fetal calf serum and Ultroser G differentiated on 10% horse serum and 2% chicken embryo extract or 0.4% Ultroser G and 10% rat brain extract, respectively [21]. The cells were cultured in a humidified CO_2 atmosphere at 37°C and all media were refreshed every third day. Cultures were harvested after 7–20 days of differentiation.

Preparation of homogenates

Homogenates of cultured muscle cells (1–5 mg protein/ml) or muscle (5% w/v) were prepared in 250

mM sucrose/2 mM EDTA/10 mM Tris-HCl (pH 7.4) at 0°C by 10 strokes in a glass homogenizer with a tight-fitting Teflon pestle (clearance is 50 μm) rotating at 1000 rev./min. To exclude sealed vesicles the homogenates were four times freeze-thawed and/or solubilized by detergents as indicated.

[^{3}H]Ouabain binding

[^{3}H]Ouabain binding capacity was determined using a method based on vanadate-facilitated binding [6]. Samples of muscle (4–11 mg) or intact cultured muscle cells (150–500 μg protein/dish) were labelled with 0.1 μM [^{3}H]ouabain. Unlabelled ouabain was added so as to give final concentrations from 0.1–5.0 μM. Correction for non-specific binding was determined by incubation in the presence of 1 mM unlabelled ouabain.

Na^+/K^+-ATPase, Mg^{2+}-ATPase and K^+-dependent 3-O-MFPase activity

Na^+/K^+-ATPase and Mg^{2+}-ATPase activity were determined in homogenates of muscle or cultured cells (100–250 μg protein) as ouabain-sensitive and -insensitive ATPase activity [23].

The K^+-dependent 3-*O*-MFPase activity was determined as described by Nørgaard et al. [7] with some modifications. Homogenates of muscle or cultured muscle cells (100–250 μg protein) were incubated for 10 min at 37°C in the presence of 10 mM KCl or 0.1 mM ouabain preceding the addition of 19.5 μM 3-*O*-MFP.

ELISA for SR Ca^{2+}-ATPase content

A mixture of slow- and fast-type SR Ca^{2+}-ATPase was purified from rat hind leg muscles as described by Timmermans et al. [24]. Wells of polystyrene microtiter plates were coated with 150 ng of this antigen in 150 μl coating buffer containing 100 mM Na_2CO_3/5 mM NaN_3 (pH 9.6) for 2 h at 37°C. Homogenates of muscle or cultured muscle cells were solubilized by mixing with an equal volume of a buffer containing 130 mM KCl/20 mM Hepes-KOH/0.4% Triton X-100 (pH 7.4) for 10 min at room temperature. After centrifugation at 100000 × *g* for 5 min in an airfuge (Beckman, Palo Alto, CA, USA) the supernatants of muscle and cultured cells were diluted 5 or 2 times, in 10 mM Tris-HCl/0.9% NaCl/0.05% Tween-20 (pH 7.5) (buffer B) containing 0.5% bovine serum albumin (BSA), respectively. In wells of a second microtiter plate standard SR Ca^{2+}-ATPase or samples (100 μl) were added to 100 μl rabbit anti-rat SR Ca^{2+}-ATPase (1 : 10000 in buffer B containing 0.5% BSA). After incubation overnight at 4°C, 150 μl of each well was transferred to a well of a polystyrene plate previously coated with purified SR Ca^{2+}-ATPase. The free antibody was allowed to complex with the coated antigen for 45 min (non-equilibrium condition [25]). After washing three times with buffer B, the antibodies bound to the SR Ca^{2+}-ATPase were incubated with 150 μl alkaline phosphatase-conjugated goat anti-rabbit IgG (1 : 2000 in buffer B containing 0.5% BSA) for 2 h at room temperature. After washing, 150 μl enzyme substrate (1 mg *p*-nitrophenyl phosphate per ml buffer containing 1 M diethanolamine/0.5 mM $MgCl_2$ (pH 9.8)) was added. The reaction was allowed to proceed for 20–30 min at room temperature and stopped by addition of 150 μl 5 M NaOH. The reaction product was measured spectrophotometrically at 405 nm using a Titertek Multiscan MCC/340 MK II (Labsystems Oy, Helsinki, Finland).

Phosphorylation of SR Ca^{2+}-ATPase

The procedure was performed as described by Everts et al. [16] with some modifications. Homogenate of muscle or cultured muscle cells (50–250 μg protein) reacted with 40 μM ATP/10 μM [γ-^{32}P]ATP (pH 7.4) in the presence or absence of 0.55 mM $CaCl_2$. The reaction was quenched by 5% (w/v) trichloroacetic acid/0.1 M phosphoric acid. The denatured phosphoprotein was filtered on a 0.45 μm pore width membrane filter (Schleicher and Schuell, Dassel, Germany) and three times rapidly washed. The Ca^{2+}-dependent ^{32}P uptake, i.e. the concentration of phosphorylated intermediates of SR Ca^{2+}-ATPase, was calculated as the difference between the values obtained with or without $CaCl_2$.

SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-O-MFPase activity

SR Ca^{2+}-ATPase activity was determined as Ca^{2+}-dependent ATPase activity. Homogenates of muscle or cultured cells (50–200 μg protein) were incubated in 0.4 ml buffer containing 100 mM imidazole/100 mM KCl/5 mM $MgCl_2$/0.5 mM EGTA/5 mM ATP (pH 7.4) in the absence or presence of 0.55 mM $CaCl_2$ at 37°C. After 2 h the reaction was stopped and analyzed for inorganic phosphate as described for the Na^+/K^+-ATPase activity [23].

The Ca^{2+}-dependent 3-*O*-MFPase activity was assayed according to Everts et al. [16].

Other biochemical assays

For the determination of the percentage creatine kinase MM (CK-MM), as measure of the maturation grade of cultured muscle cells this isoenzyme was separated from other CK isoenzymes by anion exchange chromatography. This procedure and the assay of CK activity were described by Jacobs et al. [26]. The protein content was assayed according to Lowry et al. [27] with BSA as standard.

Immunocytochemistry

Muscle biopies were cryofixed in isopentane cooled with liquid nitrogen. Cryosections (4–8 μm) were air-

dried and frozen at −20°C. Cell cultures were washed three times with phosphate-buffered saline (PBS), air-dried and stored at −20°C. After rehydration in PBS, sections and cultures were blocked for 20 min with 20% normal goat serum in PBS, exept when goat anti-rabbit Na^+/K^+-ATPase anti serum was used, in which case blocking was done with 1% BSA in PBS. Primary antibodies were applied for 90 min, followed by washing three times with PBS. Incubation with fluorochrome-conjugated secondary antibodies was for 90 min, followed by washing and mounting. Localization of sarcomeric bands (e.g., A-and I-band), was done by phase-contrast microscopy and immunolocalization of desmin and myosin. Immunofluorescence was studied on a Zeiss Axioskop photomicroscope (Carl Zeiss, Oberkochen, Germany).

Statistics

Data represent means ± S.D. Statistical analysis was performed by means of the unpaired Student's *t*-test and significancy was set at $P < 0.01$.

Results

Na^+/K^+-ATPase and K^+-dependent 3-O-MFPase activity and ouabain binding

In order to expose latent Na^+/K^+-ATPase activity and to ensure full access of substrates, homogenates of cultured muscle cells were pretreated with different concentrations of detergents. Neither the mixture of Triton X-100/digitonin (1 : 1, w/w) nor SDS increase significantly the Na^+/K^+-ATPase activity, whether the homogenates are freeze-thawed or not (Fig. 1). The activity of Na^+/K^+-ATPase is maximal in freeze-thawed homogenates, in the absence of detergents. The Na^+/K^+-ATPase activity increases in cultured muscle cells when their maturation grade increases (Fig. 2a, Table I).

The Na^+/K^+-ATPase activity is not detectable in homogenates of muscle biopsies, when measured spectrophotometrically due to the excess of Mg^{2+}-ATPase activity. Therefore as a measure for the Na^+/K^+-ATPase activity only the K^+-dependent 3-*O*-MFPase activity could be determined in muscle. This activity increases in cultured muscle cells also with the maturation grade (Fig. 2b) and is always significantly higher than in muscle homogenates (Table I). Cells cultured on Ultroser G reach a higher maturation grade and higher Na^+/K^+-ATPase and K^+-dependent 3-*O*-MFPase activities than serum-based cultures. Ageing (14–58 year) had no effect on the 3-*O*-MFPase activity in human muscle (data not shown).

The maximal [^{3}H]ouabain binding capacity, i.e., the number of Na^+/K^+-pumps of cultured muscle cells increases with the maturation grade too (Fig. 2c) and is 2- to 3-fold higher than in muscle biopsies (Table I). The number of [^{3}H]ouabain binding sites of human skeletal muscle (360 ± 70 pmol/g wet wt.; $n = 5$) is comparable to previously published data [16,28–30]. The dissociation constant and the molecular activities do not differ in muscle and cultured cells of different maturation grade (Table I). These observations indicate that the differences of the Na^+/K^+-ATPase activity are due to variations of the number of Na^+/K^+-ATPase molecules.

Mg^{2+}-ATPase activity

The Mg^{2+}-ATPase activity also increases, when the maturation grade of the cultured muscle cells increases (results not shown). The activities of Mg^{2+}-ATPase in muscle cells cultured on serum or Ultroser G are 14.2 ± 2.6 ($n = 20$) and 22.0 ± 2.9 mU/mg protein ($n = 51$; $P < 0.01$), respectively. However, they remain lower than the activity in homogenates of muscle (41.9 ± 8.4 mU/mg protein, $n = 7$; $P < 0.01$).

SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-O-MFPase activity and SR Ca^{2+}-ATPase content

In experiments in which the Ca^{2+} dependence of the SR Ca^{2+}-ATPase is tested, Ca^{2+}-(0.5 mM)EGTA

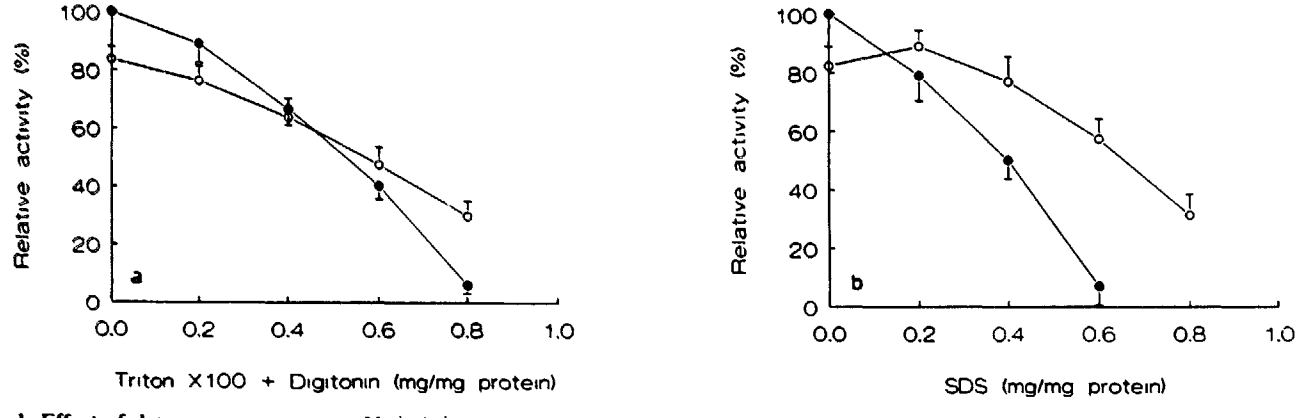

Fig. 1. Effect of detergent treatment on Na^+/K^+-ATPase activity. Fresh (○) or freeze-thawed (●) homogenates of cultured muscle cells were incubated with a mixture of Triton X-100 and digitonin (1 : 1, w/w) (a) or SDS (b).

buffers were used and the free Ca^{2+} concentration is determined in the presence of homogenate with the Ca^{2+}-indicator fura-2 [31]. At a free Ca^{2+} concentration of about 5 μM the SR Ca^{2+}-ATPase is maximally activated (Fig. 3a). The K_m values of 0.43 and 0.55 μM in homogenates of cultured cells and muscle, respectively, fall in the range of published data [17,32,33]. The ion pump is inhibited by a large excess of Ca^{2+} and of vanadate (Figs. 3a,b), but less than 10% of the Ca^{2+}-dependent ATPase activity is inhibited by 5 μM vanadate. Michalak et al. showed that this vanadate concentration completely inhibits sarcolemmal Ca^{2+}-ATPase [34]. Besides 1 μM thapsigargin, a specific inhibitor of SR Ca^{2+}-ATPase [35], inhibits 92 ± 6% (n = 4) and 89 ± 5% (n = 3) of the Ca^{2+}-dependent ATPase activity in adult muscle and cultured cells, respectively. The measured Ca^{2+}-ATPase activity represents thus quite well SR Ca^{2+}-ATPase activity. The concentration of 5 μM free Ca^{2+} was therefore used in the assays of SR Ca^{2+}-ATPase with ATP or 3-*O*-MFP as substrate and by phosphorylation.

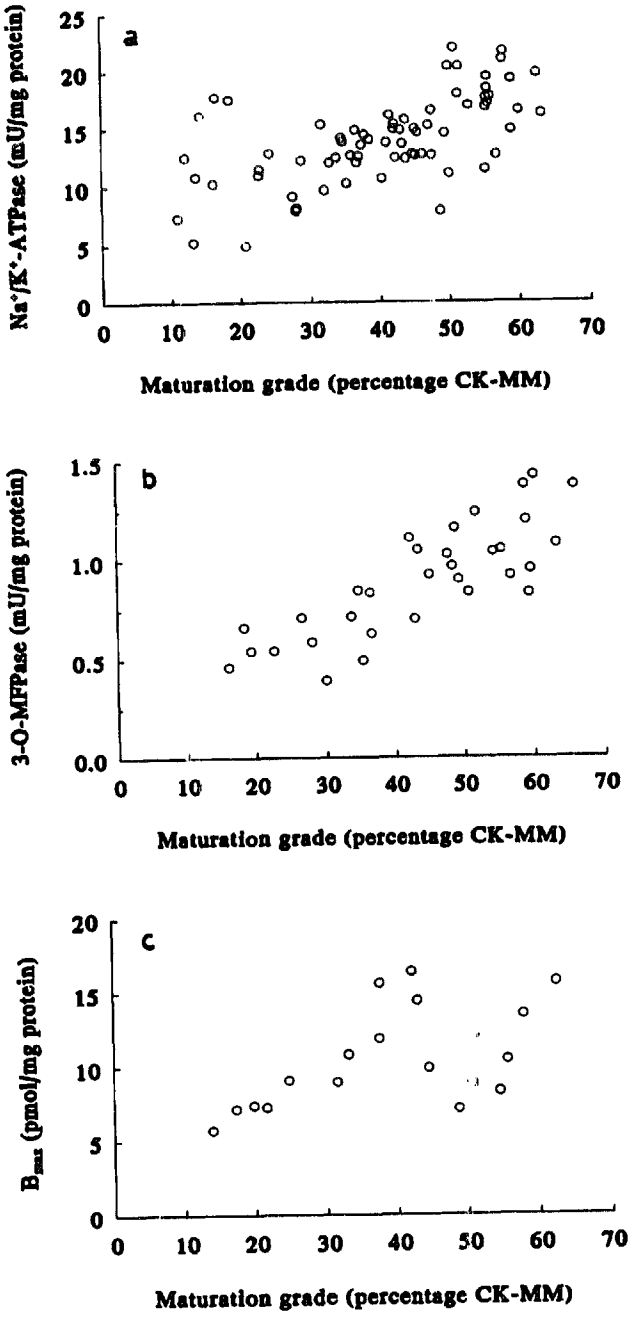

Fig. 2. The relation between the maturation grade of cultured muscle cells expressed in percentage CK-MM and the Na^+/K^+-ATPase activity (a), the K^+-dependent 3-*O*-MFPase activity (b) and the maximum number of ouabain-binding sites (c).

The activities of SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-*O*-MFPase increase in muscle cells with maturation (Figs. 4a,b), but remain significantly lower than in muscle (Table II). The highly matured cells derived from Ultroser G-containing media reach a higher SR Ca^{2+}-ATPase activity with both ATP and 3-*O*-MFP as substrates.

The number of SR Ca^{2+}-pumps was quantified as well as by Ca^{2+}-dependent phosphoprotein formation as by an ELISA. The former assay assesses the total concentration of SR Ca^{2+}-ATPase under steady state conditions, where all enzyme molecules are in a phosphorylated state since dephosphorylation of the phosphoenzyme is the rate-limiting step in fast- as well as in slow-twitch muscle [16]. Additionally thapsigargin (1 μM) inhibits 94 ± 4% (n = 4) of the Ca^{2+}-dependent phosphorylation. The ELISA also measures total SR Ca^{2+}-ATPase since an antiserum raised against a mixture of fast- and slow-type SR Ca^{2+}-ATPase is used. Both parameters increase in cultured cells when their maturation grade increases (Figs. 4c,d) and remain lower than in muscle (Table II). The concentration of SR Ca^{2+}-ATPase of adult human skeletal muscle (6.7 ± 1.3 nmol/g wet wt.; n = 20) agrees with previously published data [16,30]. The activity and the content of SR Ca^{2+}-ATPase show a slight, but significant age-related decrease in human muscle (data not shown). The molecular activities of SR Ca^{2+}-ATPase calculated for ATP and 3-*O*-MFP do not differ in muscle from those in cultured cells and are independent of the maturation grade (Table II). These observations imply that the changes of the SR Ca^{2+}-ATPase activity are related to the variations of the SR Ca^{2+}-ATPase content.

Immunocytochemistry

Goat anti-rabbit Na^+/K^+-ATPase and rabbit anti-rat SR Ca^{2+}-ATPase were used to localize Na^+/K^+-ATPase and SR Ca^{2+}-ATPase, respectively. The specificity of the anti-sera was established by Western blotting (results not shown). When muscle sections are stained for Na^+/K^+-ATPase, both fast- and slow-twitch muscle fibers are strongly positive (Figs. 5a,b). Endothelial and smooth muscle cells of blood vessels are immunoreactive as well. In longitudinal sections cross-striation is visible at the I-band (Fig. 5c). Cultured myotubes are also immunoreactive, but cross-striation

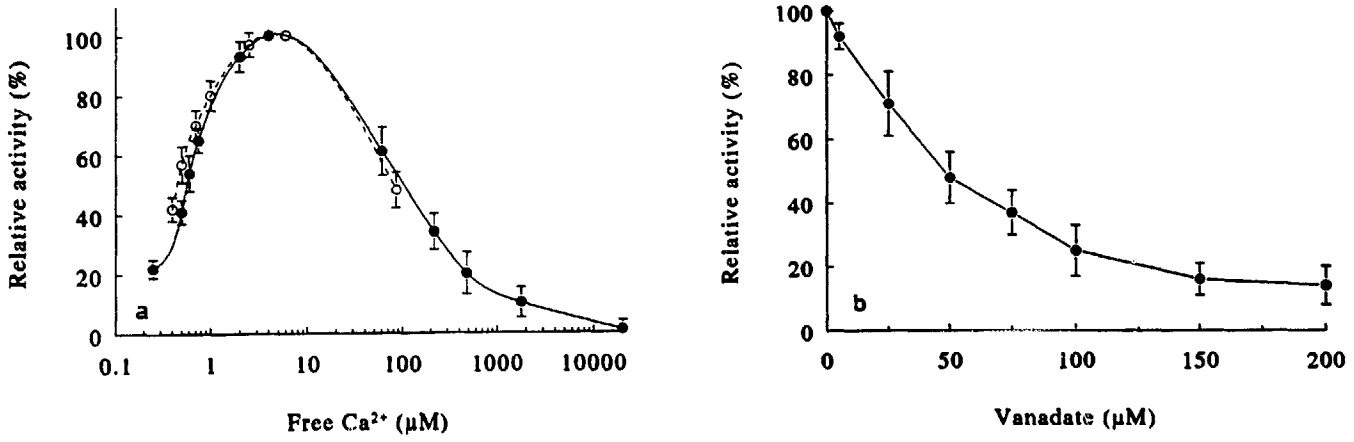

Fig. 3. Effects of the free Ca^{2+}-concentration (a) and the vanadate concentration (b) on the SR Ca^{2+}-ATPase activities of muscle (●———●) and cultured muscle cells (○ - - - ○).

at the I-band is only observed in highly matured cells (Fig. 5d).

When muscle sections are incubated with the antiserum against SR Ca^{2+}-ATPase, fast-twitch fibers are strongly positive, whereas slow-twitch fibers stain weakly (Figs. 6a,b). Endothelial cells are negative. Cross-striation is detectable at the I-band and a faint staining is associated with the H-band (Fig. 6c). Cultured myotubes are strongly stained and highly matured cells display cross-striation (Fig. 6d).

Discussion

Ultroser G and rat brain extract markedly enhance the growth and differentiation of human and rat skeletal muscle cells in vitro compared to sera [21,36]. Upon

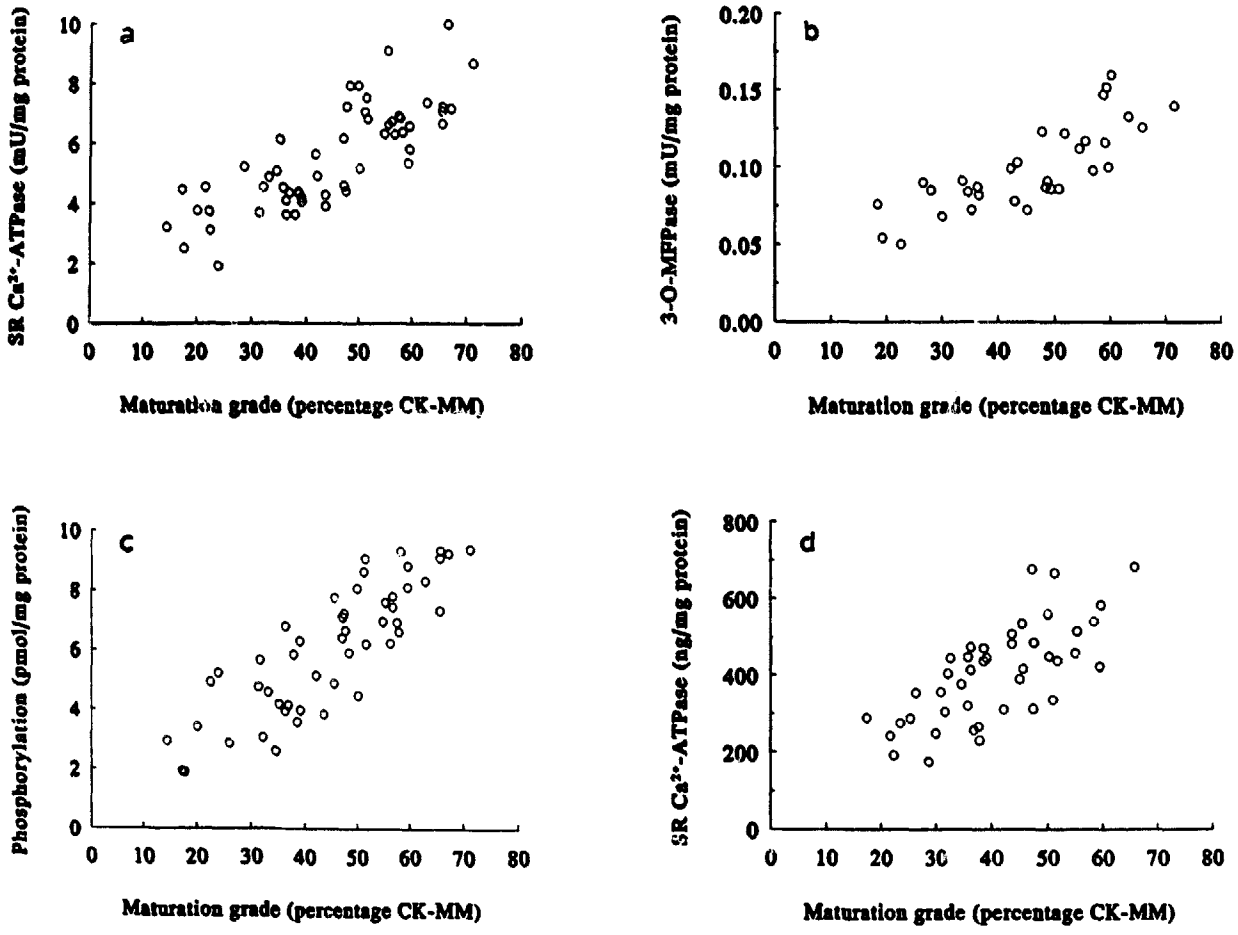

Fig. 4. The relation between the maturation grade of cultured muscle cells and the SR Ca^{2+}-ATPase activity (a), the Ca^{2+}-dependent 3-*O*-MFPase activity (b) and the SR Ca^{2+}-ATPase content as quantified by phosphorylation (c) or ELISA (d).

fusion of myoblasts to form multinucleated myotubes there is a coordinated expression of metabolic enzymes, muscle-specific isoenzymes and contractile proteins. The sarcolemma and basement membrane likewise become structurally and functionally more differentiated [37]. In this study we observed also major effects of Ultroser G and rat brain extract on the concentration and activities of both Na^+/K^+-ATPase and SR Ca^{2+}-ATPase.

A tight control of the Na^+/K^+ transport is essential for the maintenance of optimal muscle function [4]. In skeletal muscle, short-term activation of the Na^+/K^+-pump is induced by excitation, catecholamines and insulin. Long-term regulation of muscle Na^+/K^+-ATPase is determined by differentiation, age, muscle activity and hormonal status [4,38,39]. In our cultured human skeletal muscle cells the activities of Na^+/K^+-ATPase and K^+-dependent 3-*O*-MFPase increase during maturation concomitantly with the concentration of Na^+/K^+-pumps. The molecular activities and dissociation constant do not change. This agrees with the indications that the changes in the Na^+/K^+-ATPase activity reflect changes in the number of [^{3}H]ouabain-binding sites during differentiation and life both in vitro as in vivo. A marked increase of ouabain-sensitive $^{86}Rb^+$ uptake and of the number of Na^+/K^+-ATPase molecules, quantified by ouabain binding or with antibodies, is observed upon the fusion of cultured chicken myoblasts into myotubes and differentiation [9,12,13]. In cultured rat muscle cells the concentration of Na^+/K^+-ATPase also increased upon fusion [11]. The number of ouabain-binding sites (per mg protein) is comparable in cultured chick embryo myotubes [9], cultured rat neonatal muscle cells [11] and our cultured human muscle cells. The ouabain-binding capacity is of the same order in muscle of rat, mouse, guinea pig [8,10], chicken [9] and human (Table I), but species-variations are present in the changes of this capacity or

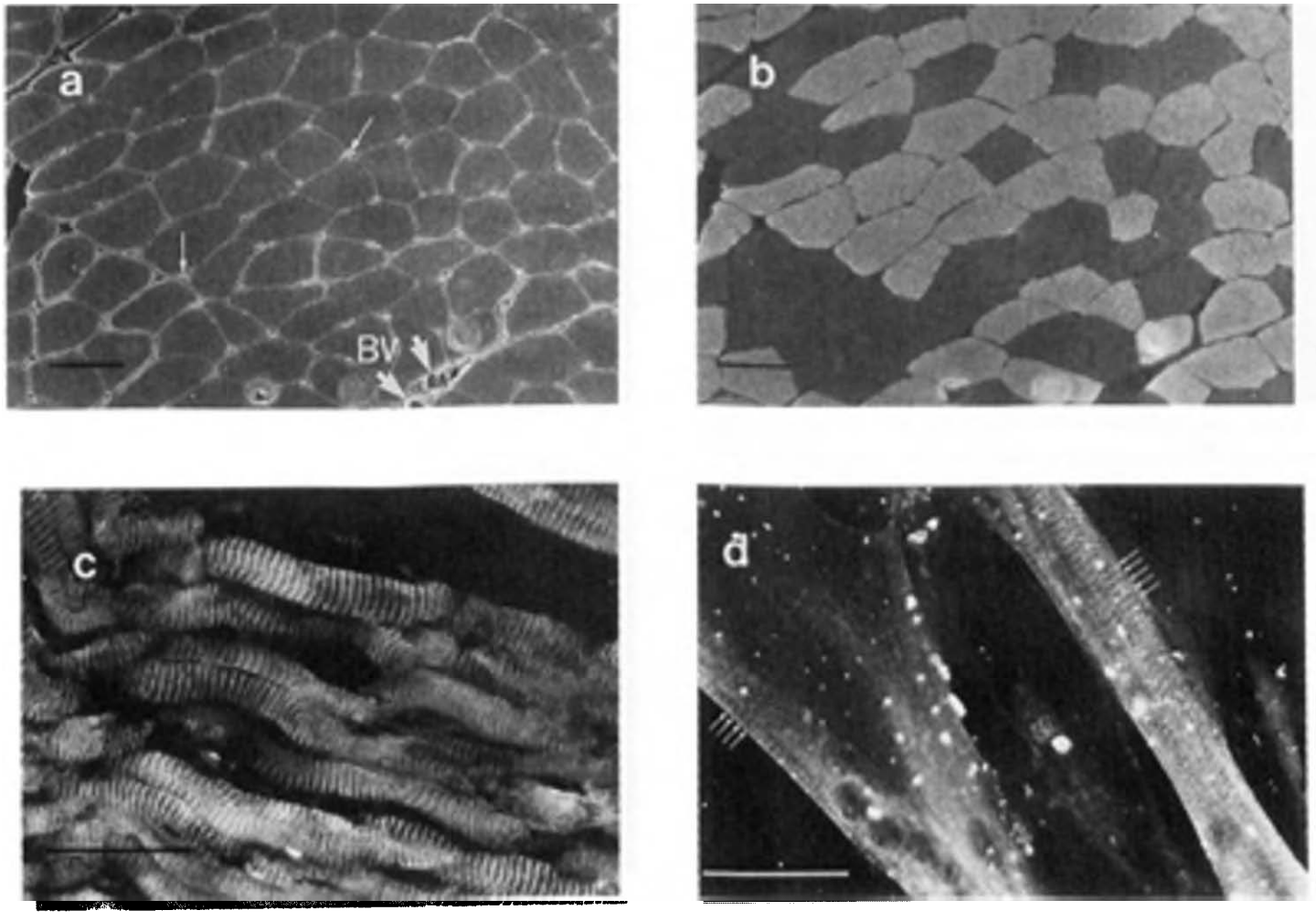

Fig. 5. Immunolocalization of Na^+/K^+-ATPase in human skeletal muscle fibers (a–c) and cultured myotubes (d). Antibodies to Na^+/K^+-ATPase are visualized using fluorescein-conjugated secondary antibodies. Fast-twitch muscle fibers are localized in the same section using anti-myosin fast antibodies which are visualized by tetramethylrhodamine-conjugated secondary antibodies. Bars represent 40 μm. (a,b) Transverse cryosection. Na^+/K^+-ATPase (a); myosin fast (b). Fast-twitch as well as slow-twitch muscle fibers are positive for Na^+/K^+-ATPase. Blood vessels (BV) and capillaries (arrows) also react positively. (c) Longitudinal cryosection. Na^+/K^+-ATPase staining is confined to the sarcomeric I-band. (d) Muscle cell culture. Myotubes sometimes display cross-striation (arrows), corresponding to the sarcomeric I-band.

Na^+/K^+-ATPase activity at different ages. In chicken skeletal muscle the content and activity of the Na^+/K^+-pump increased in ovo up to 9 days after hatching, but decreased again by 50% [9,40]. Rat and mouse muscles showed an increase of ouabain-binding capacity after birth up to 4 wk, followed by a decrease of the same order to a constant level at 8–12 wk [8,10]. Guinea pig muscle showed a maximal value at birth [10]. Our observation on the constant 3-*O*-MFPase activity in human muscle during ageing is in agreement with the absence of age-dependent changes of the ouabain-binding capacity between 0 and 86 year [28,41,42]. No data are available on parameters of the Na^+/K^+-pump in fetal or neonatal mammalian muscle. The different maturation grade of muscle in various species at birth [43] may cause a variable balance of synthesis of Na^+/K^+-ATPase and other proteins and in this way a species-related age-pattern. Guinea pig and human muscle may also show the biphasic age-pattern of increase and decrease as chicken and rodents, but with the increase in the prenatal or neonatal period. The higher value of the Na^+/K^+-pump parameters in cultured human muscle cells than in adult muscle and their increase at maturation may reflect a preferential synthesis of the transverse tubular system proteins as Na^+/K^+-ATPase in the first phase of muscle growth.

SR Ca^{2+}-ATPase constitutes about 90% of the total protein content in SR membrane [44]. The SR Ca^{2+}-ATPase content is regulated by differentiation, ageing, muscle activity and hormonal and neural control [45–47]. The activities of SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-*O*-MFPase and the concentration of SR Ca^{2+}-ATPase increase concomitantly with the maturation of the cultured human muscle cells. The molecular activities do not change. The $^{45}Ca^{2+}$ uptake and the amount of SR Ca^{2+}-ATPase increase 3–5 times in cultured chicken muscle cells upon fusion and differen-

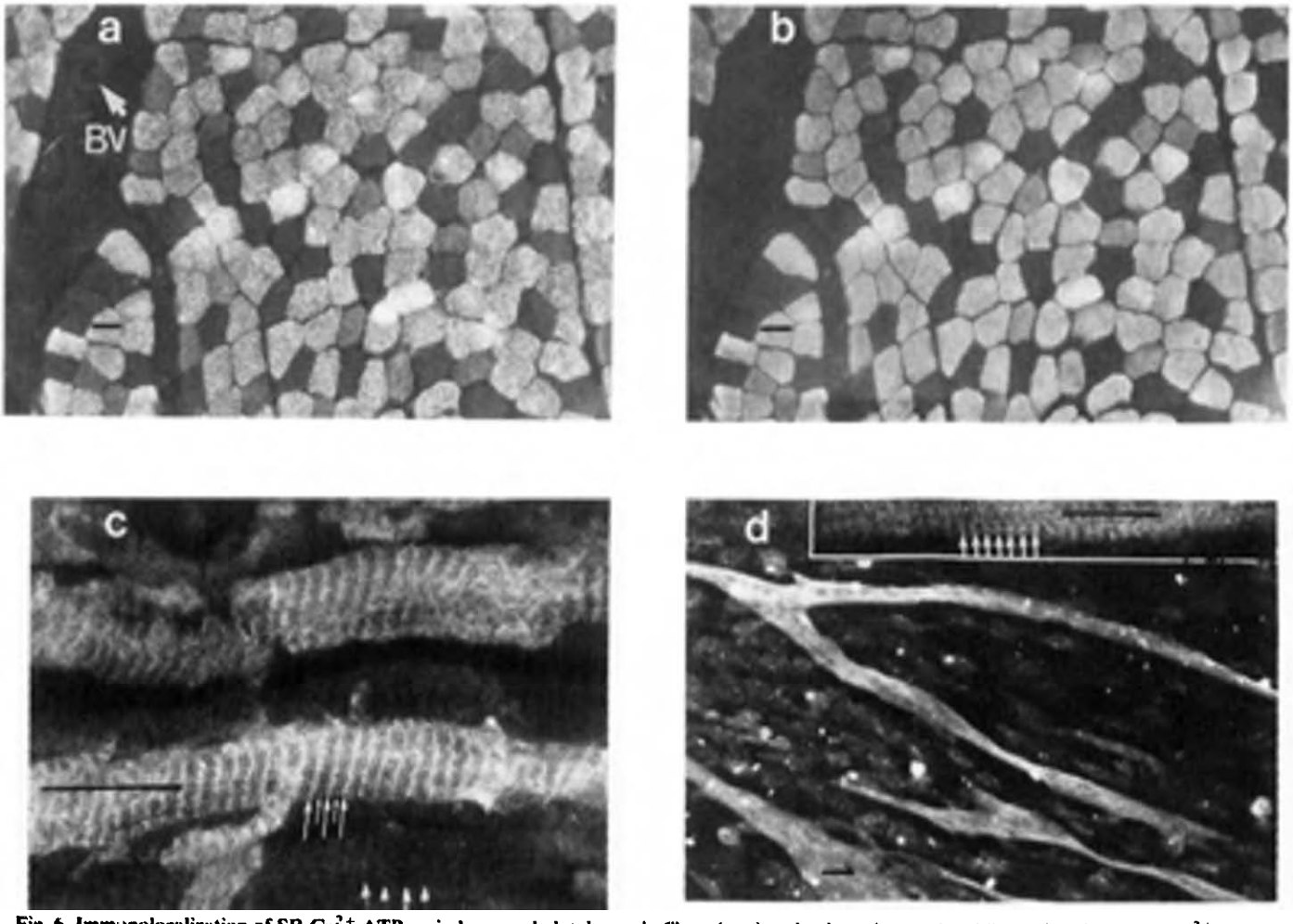

Fig. 6. Immunolocalization of SR Ca^{2+}-ATPase in human skeletal muscle fibers (a–c) and cultured myotubes (d). Antibodies to SR Ca^{2+}-ATPase are visualized using fluorescein-conjugated secondary antibodies. Fast-twitch muscle fibers are localized in the same section according to the procedure in Fig. 5. Bars represent 20 μm. (a,b) Transverse cryosection. Muscle cells strongly positive for SR Ca^{2+}-ATPase (a) correspond to fast-twitch cells (b). Capillary endothelium and blood vessels (BV) are negative. (c) Longitudinal cryosection. SR Ca^{2+}-ATPase is predominantly localized in the sarcomeric I-band (large arrows). A faint band corresponding to the H-band can be observed (small arrows). Slow-twitch muscle cells display a weak staining, confined mainly to the I-band (triangles). (d) Muscle cell culture. Myotubes are positive for SR Ca^{2+}-ATPase and display cross-striation at some places (inset, arrows).

TABLE I

Na^+/K^+-ATPase in human muscle and cultured muscle cells

Values are means ± S.D. of the number of experiments given between parentheses. Units of activity represent μmol inorganic phosphate or 3-*O*-methylfluorescein formed per min at 37°C. The maturation grade (percentage CK-MM) of the muscle cells cultured on serum or Ultroser G are 22.4 ± 7.7 and 47.5 ± 8.2, respectively. Parameters are significantly different from highly matured muscle cells cultured on Ultroser G with: * $P < 0.01$ (Student's unpaired *t*-test). n.d., not detectable.

Parameters	Muscle	Muscle cells cultured on	
		serum	Ultroser G
Na^+/K^+-ATPase (mU/mg protein)	n.d.	11.1 ± 3.6 * (20)	15.2 ± 3.1 (51)
K^+-dependent 3-*O*-MFPase (μU/mg protein)	382 ± 73 * (14)	547 ± 105 * (8)	1007 ± 214 (24)
B_{max} (ouabain binding) (pmol/mg protein)	4.1 ± 0.8 * (5)	8.0 ± 1.7 * (7)	12.0 ± 3.1 (12)
K_d (ouabain binding) (nM)	257 ± 82 (5)	249 ± 57 (7)	240 ± 41 (12)
Molecular activity (ATP) (min^{-1})	-	1399 ± 280 (5)	1428 ± 302 (8)
Molecular activity (3-*O*-MFP) (min^{-1})	85 ± 15 (3)	77 ± 14 (3)	87 ± 17 (5)

tiation [15,20,44]. Recently Charuk et al. demonstrated that the biogenesis of SR Ca^{2+}-ATPase in cultured chicken muscle cells depends on their contractile activity [45]. In cultured rat muscle cells a striking increase of the SR Ca^{2+}-ATPase concentration is seen during fusion [19,48]. Fusion, however, is not essential for this increase. In rodent muscle the initial Ca^{2+} uptake rate and/or SR Ca^{2+}-ATPase content rise about 3-fold from birth to adult age [14,18]. In developing chicken muscle the Ca^{2+} uptake and the SR Ca^{2+}-ATPase content increase 30–40 times from 12 days in ovo to 30 days in vivo [15,20]. The concentration of SR Ca^{2+}-ATPase in cultured chicken muscle cells is only 10% of that found in adult chicken muscle [15]. In our study we found the same differences for human between the cultured cells and adult muscle. The decrease of the activity and the content of SR Ca^{2+}-ATPase in human skeletal muscle upon ageing is related to a selective atrophy of type II fibres [49].

TABLE II

Sarcoplasmic reticulum Ca^{2+}-ATPase in human muscle and cultured muscle cells

Values are means ± S.D. of the number of experiments given between parentheses. The maturation grade (percentage CK-MM) of the muscle cells cultured on serum or Ultroser G are 25.9 ± 7.8 and 50.7 ± 10.6, respectively. Parameters are significantly different from highly matured muscle cells cultured on Ultroser G with: * $P < 0.01$ (Student's unpaired *t*-test).

Parameters	Muscle	Muscle cells cultured on	
		serum	Ultroser G
SR Ca^{2+}-ATPase (mU/mg protein)	42.8 ± 6.4 * (23)	3.70 ± 0.86 * (14)	6.06 ± 1.49 (42)
Ca^{2+}-dependent 3-*O*-MFPase (μU/mg protein)	1134 ± 227 * (14)	73 ± 14 * (9)	112 ± 29 (23)
Phosphorylation (pmol/mg protein)	56.0 ± 6.7 * (22)	3.89 ± 1.48 * (14)	6.85 ± 1.76 (36)
SR Ca^{2+}-ATPase content (ng/mg protein)	6167 ± 2166 * (13)	304 ± 83 * (17)	472 ± 105 (26)
Molecular activity (ATP) (min^{-1})	789 ± 114 (21)	939 ± 288 (12)	903 ± 170 (35)
Molecular activity (3-*O*-MFP) (min^{-1})	18 ± 3 (8)	17 ± 4 (5)	17 ± 4 (18)

The similar staining for Na^+/K^+-ATPase in human fast- and slow-twitch muscle fibers indicates that there are no marked differences of ion pump density between both fiber types. Fambrough et al., however, observed variation in fluorescence intensity between chicken fast- and slow-twitch muscle fibers [13]. The Na^+/K^+-ATPase distribution in human muscle fibers is consistent with its sarcolemmal localization, including the T-tubules. The Na^+/K^+-ATPase cross-striation in highly matured cultured human muscle cells indicates the development of a T-tubule system in vitro. These observations are comparable with those in cultured chicken muscle cells [12].

Human skeletal fast-twitch muscle fibers have a higher content of SR Ca^{2+}-ATPase than slow-twitch fibers. Jorgensen et al. observed the same fiber type-dependent distribution in rat skeletal muscle in vivo [50]. Biochemical studies in rabbit, rat, mouse and guinea pig support these results [14,16,18]. The presence of SR Ca^{2+}-ATPase cross-striation at the H- and I-band in adult muscle indicates that the ion pumps are rather uniformly distributed throughout the longitudinal regions of the SR. The cross-striation was undetectable in cultured rat skeletal muscle cells [51], in contrast to in our highly matured cultured human muscle cells and cultured mouse muscle cells [52]. The latter phenomenon indicates a good development of SR in our cell system.

In conclusion, the activities and the concentrations of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase increase coordinately in cultured human muscle cells and correlate to their maturation grade. The Na^+/K^+-ATPase parameters are always higher in cultured muscle cells than in adult human muscle, but the SR Ca^{2+}-ATPase parameters remain lower. The molecular activity of both ion pumps does not change in cultured cells as well as adult muscle. Cultured human muscle cells may well be applied in studies of ion transport during myogenesis and muscle diseases.

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