Stimulation of hormone-sensitive lipase activity by contractions in rat skeletal muscle

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Because the enzymic regulation of muscle triglyceride breakdown is poorly understood we studied whether neutral lipase in skeletal muscle is activated by contractions. Incubated soleus muscles from 70 g rats were electrically stimulated for 60 min. Neutral lipase activity against triacylglycerol increased after 1 and 5 min of contractions [0.36 ± 0.02 (basal) versus 0.49 ± 0.05 (1 min) and 0.54 ± 0.05 (5 min) m-unit·mg of protein⁻¹, means $\pm$ S.E.M., $P < 0.05$]. After 10 min the neutral lipase activity (0.40 ± 0.05 m-unit·mg of protein⁻¹) had decreased to basal values ($P > 0.05$). The contraction-mediated increase in lipase activity was increased by $\approx 110\%$ when muscle was stimulated in the presence of okadaic acid. Conversely, treatment of muscle homogenate with alkaline phosphatase completely reversed the contraction-mediated lipase activation. Lipase activity did not change during contractions when analysed in the presence of anti-hormone-sensitive-lipase (HSL) antibody [0.17 ± 0.02 (basal) versus 0.21 ± 0.02 (5 min) m-unit·mg of protein⁻¹, $P > 0.05$]. Furthermore, immunoprecipitation with affinity-purified anti-HSL antibody reduced muscle-HSL protein concentration by $81 \pm 4\%$

and caused similar reductions in lipase activity against triacylglycerol and in the contraction-induced increase in this activity. Neither prior sympathectomy [0.33 ± 0.02 (basal) versus 0.53 ± 0.06 (5 min) m-unit·mg of protein⁻¹, $P < 0.05$] nor propranolol impaired the lipase response to contractions. Glycogen phosphorylase activity in the absence of AMP increased after 1 min [27.3 ± 3.1 versus $8.9 \pm 1.8\%$ (activity without AMP/total activity with AMP), $P < 0.05$] and returned to basal levels after 5 min. In conclusion, skeletal-muscle-immunoreactive HSL is transiently stimulated by contractions and the mechanism probably involves phosphorylation. The time course of HSL activation is similar to that of glycogen phosphorylase. Apparently, the two enzymes are regulated in parallel by contraction-induced as well as hormonal mechanisms, allowing simultaneous recruitment of all major extra- and intra-muscular energy stores.

Key words: exercise, glycogen phosphorylase, lipolysis, metabolism, triglyceride.

INTRODUCTION

Triacylglycerol is stored in lipid droplets in the cytoplasm of skeletal-muscle cells [1,2]. The energy content of this triglyceride depot is higher than the energy content of the muscle glycogen pool [2], and the triglycerides can be mobilized by catecholamines [3,4]. For more than a decade it was believed that the breakdown of triglyceride in muscle was regulated by lipoprotein lipase [1]. However, this view has proven untenable because in muscle cells the enzyme is produced as a secretory protein and resides inside vesicles [1,2]. Furthermore, the intracellular milieu in muscle would not be compatible with optimum functioning of lipoprotein lipase which requires a pH of 8.5 and the presence of apoprotein C-2 [1,2]. Muscle also contains neutral lipase with a pH optimum of 7, and this type of lipase is an alternative regulator of triglyceride breakdown. The hormone-sensitive lipase (HSL), which controls lipolysis in adipose tissue, is a neutral lipase [5]. We have recently demonstrated that HSL is in fact present in skeletal muscle and can be stimulated by adrenaline via β -adrenergic activation of cAMP-dependent protein kinase [6]. Interestingly, adrenaline activates HSL and glycogen phosphorylase in parallel in muscle [6].

Intramuscular triglyceride stores may also be mobilized by exercise [1,2,7,8]. *In vivo*, the exercise-induced decrease in muscle triglyceride concentration can be reduced by β -adrenergic block-

ade and may, accordingly, to some extent be attributed to sympathetic stimulation of HSL [9]. However, intramuscular triglyceride may also be hydrolysed in response to electrical stimulation of isolated muscle *in vitro* [10,11]. The mechanism for this is not known. So, we have now studied whether contractions stimulate HSL in muscle. If this is the case then HSL would, like glycogen phosphorylase [12,13], be under the dual control of hormonal and contraction-related mechanisms. An interesting implication for metabolic regulation would be that activation of mobilization of both extra- and intra-muscular energy stores of triglyceride and glycogen occurs simultaneously.

We electrically stimulated soleus muscle *in vitro* and carefully cleaned the muscle fibres by microdissection before HSL analysis. However, to further ensure that any increase in HSL activity did not result from electrical stimulation of sympathetic nerve fibres to fat cells hiding between muscle cells, contractions were in some experiments elicited in the presence of a β -adrenergic blocking agent or following surgical sympathectomy.

EXPERIMENTAL

Materials

Tri[³H]olein (TO) and [³H]oleoyl-2-oleylglycerol [14] were synthesized by Lennart Krabich (Department of Cell and

Abbreviations used: HSL, hormone-sensitive lipase; MOME, 1(3)-mono-[³H]oleoyl-2-oleylglycerol; TO, tri[³H]olein.

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Molecular Biology, Lund University, Lund, Sweden). An antibody was raised in hens against recombinant rat adipocyte 84-kDa HSL protein [15]. The antibody does not cross-react with lipoprotein lipase, hepatic lipase or monoacylglycerol lipase. Essentially fatty-acid-free BSA, pepstatin A, antipain, leupeptin and D,L-propranolol were from Sigma, and okadaic acid and calf intestine alkaline phosphatase were from Boehringer Mannheim.

Animals

Male Wistar rats (25 days old) weighing 65–75 g were obtained from the laboratory animal-breeding facility at the Panum Institute, Copenhagen, Denmark. Rats were kept in a constant light/dark cycle (12 h each) and received standard rat chow and water *ad libitum*. They were anaesthetized by intraperitoneal injection of sodium pentobarbital (5 mg per 100 g of body weight).

Soleus muscle incubation and stimulation

Hindquarters were perfused as described previously [12,16] for 3–4 min ($25 \text{ ml} \cdot \text{min}^{-1}$) to wash out blood. Then soleus muscles were removed with intact tendons and incubated in separate test tubes for 1 h. Perfusion and incubation media (15 ml/tube) were Krebs–Ringer bicarbonate buffer containing 8 mM glucose, 1 mM pyruvate and 0.2% BSA at pH 7.4. The incubation medium had a 95% O_2 /5% CO_2 mixture bubbled through it continuously and the temperature was maintained at 29 °C. After 1 h preincubation muscles were transferred to fresh incubation medium and fixed in the vertical position and at resting length by small clips attached to the tendons. The upper tendon was connected to a force transducer. Subsequently, muscles either remained resting or were stimulated electrically through electrodes in both ends to perform repeated ($1 \cdot \text{s}^{-1}$) maximal tetanic contractions (200-ms trains of 100 Hz, impulse duration 0.2 ms, 25 V) for 1, 5, 10 or 60 min while tension was recorded. In some experiments, 10^{-7} M propranolol was present during perfusion, incubation and 5 min of electrical stimulation. In other experiments muscles were incubated in the presence of 150 nM okadaic acid for 35 min and stimulated electrically during the last 5 min. At the end of incubation muscles were freeze-clamped with aluminium tongs cooled in liquid nitrogen and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to ensure further that findings reflected the biology of muscle cells, muscle fibres were in most experiments before analysis and after freeze-drying isolated from other tissue components by microdissection using a stereomicroscope. Generally, muscles were homogenized (Polytron PT 3100, maximum speed) on ice in 10 vols of 0.25 M sucrose, 1 mM dithioerythritol, 40 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 0.31 μM okadaic acid, 20 $\mu\text{g} \cdot \text{ml}^{-1}$ leupeptin, 10 $\mu\text{g} \cdot \text{ml}^{-1}$ antipain and 1 $\mu\text{g} \cdot \text{ml}^{-1}$ pepstatin, pH 7.0. The crude homogenate was centrifuged at 15800 *g* in an Eppendorf microfuge tube at 4 °C for 45 s. The supernatant was recovered and stored at -80 °C until analysis within 1 week. In experiments evaluating the effect of phosphatase treatment on muscle homogenate the homogenization buffer contained 20 mM Pipes (pH 7.0) instead of sodium pyrophosphate and glycerophosphate.

Adipose-tissue incubation and stimulation

Epididymal fat pads were incubated, stimulated electrically for 5 min and homogenized as described above for muscles.

Sympathectomy

In a series of experiments unilateral hindleg sympathectomy was carried out 7 days before soleus muscle incubation as described previously [17,18]. An abdominal midline incision was made and at least 1.2 cm of the left sympathetic chain (3–4 ganglia) was removed at the kidney level using a Zeiss microscope (Opmic 1). In our laboratory this procedure has previously been shown to reduce noradrenaline concentrations in soleus, gastrocnemius and vastus muscles in sympathectomized legs by more than 90% [17,18]. In the present study the denervation was confirmed by the finding of lower noradrenaline concentrations in gastrocnemius muscles from sympathectomized compared with contralateral legs ($P < 0.05$, results not shown).

Assays

In the basal state the catalytic activity of adipose-tissue HSL towards diglyceride substrates is about 10-fold higher than the activity towards triglyceride substrates [5]. HSL assays are based on measurements of release of [^3H]oleic acid from 1(3)-mono-[^3H]oleoyl-2-oleylglycerol (MOME), a diacylglycerol analogue not hydrolysable at position 2 [14], and from TO. Upon phosphorylation by protein kinase A the activity of adipose tissue HSL towards TO increases markedly, whereas the MOME activity does not change significantly [5,19]. Accordingly, MOME activity is a measure of the total enzyme concentration while TO activity is a measure of the activated form of HSL and the assay of choice for monitoring changes in the activation state of HSL. The TO and MOME substrates were emulsified with phospholipids by sonication [15]. BSA was used as fatty acid acceptor. For measurements of TO activity 14 μl , or for MOME activity 7 μl , of muscle supernatant (protein concentration $\approx 3 \text{ mg} \cdot \text{ml}^{-1}$) or of adipose tissue infranatant (protein concentration $\approx 2.5 \text{ mg} \cdot \text{ml}^{-1}$) were incubated for 30 min at 37 °C with 100 μl of 5 mM (available acyl chains) TO (1.25×10^6 c.p.m.) or MOME (0.4×10^6 c.p.m.) and enzyme dilution buffer (20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithioerythritol and 0.2 $\text{mg} \cdot \text{ml}^{-1}$ BSA) in a total volume of 200 μl . Hydrolysis was stopped by addition of 3.25 ml of methanol/chloroform/heptane (10:9:7) followed, after vortexing for 10 s, by 1.1 ml of 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). The mixture was vortexed vigorously for 10 s and centrifuged at 1100 *g* for 20 min. Then, 1 ml of the upper phase containing the released fatty acids [19] was mixed with 10 ml of scintillation liquid. Radioactivity was determined in a Tri-Carb 2200 CA (Packard) scintillation counter. A unit of enzyme activity is equivalent to the amount needed for 1 μmol of fatty acids to be released per min at 37 °C. The intra-assay coefficient of variation calculated from eight determinations, each made in triplicate, on a crude muscle supernatant was 2.8% at 0.71 ± 0.008 m-unit $\cdot \text{mg}$ of protein $^{-1}$ for TO and 5.6% at 4.79 ± 0.095 m-unit $\cdot \text{mg}$ of protein $^{-1}$ for MOME activity.

Glycogen phosphorylase activity was measured in the direction of glycogen synthesis, being determined from the incorporation of [^{14}C]glucose 1-phosphate into glycogen [12]. Phosphorylase activities measured with and without AMP in the assay are referred to as total ($a+b$) and a activity, respectively. For estimation of triglyceride in muscle, extraction was performed with chloroform/methanol (2:1), hydrolysis with tetra-ethyl-ammonium hydroxide, and glycerol was measured by an enzymic fluorometric method. Recovery of added tripalmitate to muscle homogenate was $93 \pm 2\%$. Glycogen in muscle was determined by the hexokinase method after acid hydrolysis and expressed as

mmol of glucose·g of wet weight⁻¹. Muscle lactate was determined by an enzymic fluorometric method.

Treatment of muscle homogenate with phosphatase or anti-HSL antibody

In some experiments, immediately after soleus muscle homogenization and centrifugation, 182 units (10.7 μ l) of calf intestine alkaline phosphatase were added to 330 μ l of supernatant, and the mixture was divided into two portions. One portion was incubated at 37 °C, while the other was kept on ice. Aliquots were removed at various times and assayed for TO activity. In other experiments soleus muscle supernatant was incubated with an anti-HSL antiserum for 60 min at 37 °C before measurement of HSL activity was initiated by adding substrate.

The chicken anti-rat HSL antibodies used for the immunoinhibition assays have been shown not to neutralize the activity of lipoprotein lipase and monoglyceride lipase (results not shown). Anti-HSL antiserum or control serum (both 0.8 μ l) from a non-immunized hen, or 0.8 μ l of saline were added to 14 μ l (for later TO activity measurements) or 7 μ l (for MOME activity measurements) of soleus muscle supernatant diluted in enzyme dilution buffer to a final volume of 100 μ l.

Immunoprecipitation and Western-blot analysis of HSL

Aliquots of crude muscle supernatants, corresponding to 1.25 mg of total protein, were incubated with 20 μ g of affinity-purified rabbit anti-rat HSL for 2 h at 4 °C in a total volume of 272 μ l, followed by incubation with 30 μ l of Protein A–Sepharose slurry (Amersham Pharmacia Biotech) for 30 min at 4 °C. Immunoprecipitates were pelleted by centrifugation for 5 min at 3000 *g* at 4 °C, and the supernatants were used for determination of TO activity (17 μ l) and MOME activity (6 μ l), and for Western-blot analysis (13 μ l). Non-immune rabbit IgG (Sigma) was used for control ('sham') immunoprecipitations. For Western blotting, proteins were separated on 7.5% minigels (Bio-Rad) and electrophoretically transferred to PVDF membranes, incubated with affinity-purified rabbit anti-rat HSL followed by horseradish peroxidase-labelled secondary antibody, and visualized with an ECL[®] detection system (Amersham Pharmacia Biotech).

Statistics

The computer program SigmaStat for Windows version 1.0 was used for statistical analysis. Data are presented as means $\pm$ S.E.M. Three- and two-way analyses of variance were used to test differences in experiments comprising more than two conditions. The Student–Newman–Keuls test was used as a post-hoc test. Student's paired *t* test or the Wilcoxon signed rank test were used as applicable to test differences in experiments with only two conditions. A significance level of 0.05 in two-tailed testing was chosen *a priori*.

RESULTS

Stimulation of muscle lipase by contractions

During electrical stimulation, muscle tension was initially 39.6 $\pm$ 3.0 g (mean $\pm$ S.E.M., *n* = 9) and then showed an early rapid decrease (*P* < 0.05) to 30.9 $\pm$ 2.2 and 13.9 $\pm$ 0.8 g after 1 and 5 min, respectively, and subsequently a further slow decline (*P* < 0.05) to 11.6 $\pm$ 0.6 and 8.7 $\pm$ 1.2 g after 10 and 60 min, respectively. TO activity in crude muscle supernatant increased significantly within the first minute of contractions (Figure 1) and the increase of about 50% was maintained for 5 min. However, later during continued stimulation TO activity

Figure 1 Time course of effect of contractions on neutral lipase activity against TO and a diacylglycerol analogue (MOME) as well as on glycogen phosphorylase *a* activity in muscle

Incubated soleus muscles were stimulated electrically at resting length (one 200 ms train of 100 Hz per s) to perform repeated tetanic contractions for various periods of time. Measurements were made on crude supernatants (15800 *g* for 45 s). For lipase determinations three muscles were used per analysis, the two muscles from a given rat being either stimulated electrically or rested, for the same period of time. Values are means $\pm$ S.E.M. of 11–12 (lipase) and 5 (phosphorylase; expressed as the percentage of total activity) measurements during contractions (grey columns). Enzyme activities did not change during basal conditions (*P* > 0.05) and, accordingly, values were pooled (*n* = 45 for lipase and *n* = 5 for phosphorylase, white columns). However, because the TO/MOME activity ratio tended to increase with time in resting muscle, for this graph variable basal values are also shown for individual time points (with S.E.M. bars pointing downwards; *n* = 11–12). Asterisks denote value different from basal (*P* < 0.05).

Figure 2 Effect of okadaic acid on the response of neutral lipase (TO) activity to contractions in rat soleus muscle

Soleus muscles (from rats of 65–75 g) were incubated in the presence (OA) or absence of 150 nM okadaic acid for 35 min. They either rested (Basal) or were stimulated electrically to perform maximal tetanic contractions for the last 5 min (Contraction). Muscles were then frozen in liquid nitrogen and later homogenized and analysed for TO activity. Three muscles were used per analysis, the two muscles from a given rat being stimulated electrically or rested, and one incubated with and the other without okadaic acid. Columns represent means $\pm$ S.E.M. of three (basal) or four (contraction) analyses. *, Value different from corresponding value in rested muscle ($P < 0.05$). §, Value different from corresponding value obtained in muscle incubated without okadaic acid ($P < 0.05$).

Figure 3 Effect of phosphatase treatment on neutral lipase (TO) activity of rested and electrically stimulated soleus muscle

Incubated soleus muscles from 65–75 g rats were either rested (Basal) or were stimulated electrically to perform repeated maximal tetanic contractions for 5 min (Contraction). Muscles were then frozen in liquid nitrogen and later crude supernatants were prepared. The homogenization buffer contained 310 nM okadaic acid but, in contrast to standard procedures (see text), 20 mM Pipes (pH 7.0) instead of sodium pyrophosphate and glycerophosphate. Immediately after homogenization and centrifugation (15800 *g* for 45 s) 182 units (10.7 μ l) of calf intestine alkaline phosphatase were added to 330 μ l of supernatant, and the mixture was divided into two portions. One portion was incubated at 37 °C, while the other was kept on ice. Aliquots were removed at various times and assayed for TO activity. Values are means $\pm$ S.E.M. and $n = 3$ with three muscles in each sample. *, Value different from corresponding value in rested muscle ($P < 0.05$). §, Value in 37 °C experiment different from value at 0 min ($P < 0.05$).

Figure 4 Effect of a hen anti-HSL antiserum on neutral lipase activity in incubated soleus muscle after 5 min of repeated tetanic contractions or rest

Crude supernatants were prepared from soleus muscle fibres, isolated by microdissection after freeze-drying, and treated with a hen anti-HSL antiserum, control serum or saline (Basal). Neutral lipase activity was measured against TO and a diacylglycerol analogue (MOME). Three muscles were used per analysis, the two muscles from a given rat being electrically stimulated or rested, but otherwise treated identically. Values are means $\pm$ S.E.M., $n = 6$. *, Value different from corresponding value in rested muscle ($P < 0.05$). §, Difference between supernatants incubated with and without anti-HSL respectively ($P < 0.05$).

decreased to basal levels (Figure 1). MOME activity in muscle did not change during contractions (Figure 1). Like TO activity, glycogen phosphorylase activity in muscle increased early during contractions and declined to basal levels during continued stimulation (Figure 1).

Effects of okadaic acid and alkaline phosphatase

The contraction-mediated increase in TO activity was increased by $\approx 110\%$ when muscle was stimulated in the presence of okadaic acid (Figure 2). Conversely, treatment of muscle homogenate with alkaline phosphatase at 37 °C rapidly and completely reversed the contraction-mediated increase in TO activity (Figure 3). At 0 °C the contraction-mediated increase in TO activity was

Table 1 Effect of anti-HSL immunoprecipitation (IP) on HSL protein and neutral lipase activity in crude supernatant from rested and electrically stimulated soleus muscle

Data represent means $\pm$ S.E.M. of four experiments. In each experiment one soleus muscle from each of six rats was stimulated electrically to perform repeated maximal tetanic contractions for 5 min while contralateral soleus muscles rested. Then pooled crude muscle supernatants were subjected to immunoprecipitation and to sham immunoprecipitation (see Figure 5). After pelleting, supernatants were used for Western-blot analysis (see Figure 5) and, in turn, densitometry, and for determination of neutral lipase activity (see Figure 1).

	HSL protein, densitometry (arbitrary units)		Lipase activity (m-units $\cdot$ mg of protein ⁻¹)			
	Basal	Contraction	TO		MOME	
			Basal	Contraction	Basal	Contraction
Sham IP	1938 $\pm$ 336	1778 $\pm$ 126	0.839 $\pm$ 0.041	1.019 $\pm$ 0.010*	1.734 $\pm$ 0.040	1.884 $\pm$ 0.050
α -HSL IP	280 $\pm$ 66†	452 $\pm$ 168†	0.165 $\pm$ 0.012†	0.230 $\pm$ 0.015*†	1.179 $\pm$ 0.031†	1.237 $\pm$ 0.074†

* Significantly different from basal value ($P < 0.05$).

† Significant difference compared with sham immunoprecipitation conditions ($P < 0.05$).

Figure 5 Effect of anti-HSL immunoprecipitation on HSL content of rested and electrically stimulated soleus muscle

Results of two experiments are shown. In each experiment one soleus muscle from each of six young rats was stimulated electrically to perform repeated maximal tetanic contractions for 5 min while contralateral soleus muscles rested. Then pooled crude muscle supernatants were subjected to immunoprecipitation with affinity-purified rabbit anti-rat HSL antibody (α -HSL IP) and to sham immunoprecipitation with non-immune rabbit IgG (sham IP). After pelleting, 13 μ l of supernatant was used for Western-blot analysis with affinity-purified rabbit anti-rat HSL antibody. The four lanes to the left represent one and the four lanes to the right the other experiment. Molecular-mass markers were run in an adjacent lane (results not shown).

maintained throughout incubation of homogenate with alkaline phosphatase (Figure 3). TO activity also remained constant if homogenate was incubated at 37 °C without addition of alkaline phosphatase (results not shown).

Immuno-inhibition

In the presence of antibody against HSL, TO and MOME activities in resting muscle were reduced by 58 and 36 %, respectively (Figure 4). Furthermore, anti-HSL completely prevented detection of the contraction-induced increase in TO activity (Figure 4). Control serum did not influence neutral lipase activities (Figure 4).

Immunoprecipitation

Anti-HSL immunoprecipitation reduced the HSL protein concentration by 81 $\pm$ 4 % ($n = 8$) identically in rested and electrically stimulated muscle ($P > 0.05$; Table 1, Figure 5). Correspondingly, TO activity was reduced by 81 $\pm$ 1 % in rested muscle

($n = 4$) and by 79 $\pm$ 1 % when calculated for both rested and stimulated muscle ($n = 8$; Table 1). Accordingly, the contraction-induced increase in TO activity was also significantly reduced by immunoprecipitation (55 $\pm$ 16 %, $n = 4$, $P < 0.05$). Furthermore, the fractional reduction in TO response to contractions did not differ significantly from the fractional reduction in HSL protein concentration ($P > 0.05$). These findings indicate that the contraction-induced increase in neutral lipase activity reflects stimulation of HSL. In line with this view, before and after immunoprecipitation the contraction-induced increase in TO activity was significantly correlated with HSL protein concentrations ($r = 0.82$, $P < 0.01$). Immunoprecipitation also reduced MOME activity (33 $\pm$ 2 %, $n = 8$) but less than HSL protein concentration ($P < 0.05$; Table 1).

Adrenergic blockade and destruction

After 5 min of stimulation both TO and MOME activity in muscle were identical ($P > 0.05$), whether contractions were performed in the absence or presence of 10⁻⁷ M propranolol (TO, 0.57 $\pm$ 0.09 versus 0.56 $\pm$ 0.09 m-unit $\cdot$ mg of protein⁻¹; MOME, 5.77 $\pm$ 0.62 versus 5.22 $\pm$ 0.36 m-unit $\cdot$ mg of protein⁻¹, $n = 6$). Also sympathectomy did not influence the contraction-induced increase in muscle TO activity (Figure 6). Direct electrical stimulation had no effect ($P > 0.05$) on TO [9.81 $\pm$ 0.36 (stimulated) versus 10.31 $\pm$ 1.0 (unstimulated) m-unit $\cdot$ mg of protein⁻¹, $n = 5$] and MOME [53.84 $\pm$ 2.57 (stimulated) versus 57.20 $\pm$ 0.97 (unstimulated) m-unit $\cdot$ mg of protein⁻¹, $n = 5$] activities in epididymal fat tissue.

Energy stores in muscle

During incubation, glycogen concentration was constant ($P > 0.05$) in resting muscle (21.0 $\pm$ 3.9 mmol of glycosyl units $\cdot$ kg of wet weight⁻¹, $n = 12$) but decreased ($P < 0.05$) to 15.9 $\pm$ 2.2 and 11.7 $\pm$ 2.0 mmol $\cdot$ kg⁻¹ ($n = 6$) after 5 and 60 min of contractions respectively. Muscle lactate concentration increased ($P < 0.05$) from 2.9 $\pm$ 0.5 mmol $\cdot$ kg⁻¹ ($n = 9$) at rest to 14.8 $\pm$ 0.9 ($n = 5$) and 6.1 $\pm$ 1.2 ($n = 6$) mmol $\cdot$ kg⁻¹ after 5 and 60 min of contractions respectively. No significant change in muscle triglyceride content was found whether measurements were done on undissected [5.1 $\pm$ 0.5 (rest), 4.2 $\pm$ 0.5 (5 min of contractions) and 4.3 $\pm$ 0.7 (60 min) mmol $\cdot$ kg⁻¹, $n = 6$, $P > 0.05$] or dissected [17.9 $\pm$ 2.1

Figure 6 Influence of sympathectomy on the effect of 5 min of repeated tetanic contractions on neutral lipase activity in incubated soleus muscle

Prior to incubation (7 days) 1.2 cm of the left sympathetic chain was removed at the kidney level. Three muscles were used per analysis, the two muscles from a given rat being either stimulated electrically or rested. Values are means $\pm$ S.E.M., $n = 6$. *, Value different from resting value ($P < 0.05$). Values from sympathectomized and control muscles did not differ ($P > 0.05$).

(rest) and 17.0 ± 2.2 (5 min) $\text{mmol} \cdot \text{kg}$ of dry weight⁻¹, $n = 9$, $P > 0.05$ muscle.

DISCUSSION

The major new finding of the present study is that contractions stimulate the activity of HSL in skeletal muscle. The contraction-mediated activation of HSL probably involves phosphorylation of the enzyme. Furthermore, during contractions the time course of the increase in HSL activity is similar to that of the increase in glycogen phosphorylase activity. In crude muscle supernatant the neutral lipase activity against triglyceride substrate (TO) was increased after just 1 min of contractions, whereas the activity towards diglyceride substrate (MOME) did not change in response to contractions (Figure 1). A similar pattern is seen in adipose tissue in response to adrenaline and is thought to reflect activation of the neutral lipase by

phosphorylation while the total enzyme concentration is not influenced [5,19].

The fact that detection of the contraction-induced increase in TO activity could be prevented if analysis was carried out in the presence of antibody specifically inhibiting HSL shows that HSL is the neutral lipase activated by contractions (Figure 4). This conclusion is supported by the finding that an 80% reduction in muscle-HSL protein concentration by specific immunoprecipitation was accompanied by similar reductions in TO activity and in the contraction-induced increase in this activity (Table 1, Figure 5). However, HSL may not be the only neutral lipase in muscle. Thus, in agreement with previous findings [6,20], immuno-inhibition of MOME activity was not complete (Figure 4), and immunoprecipitation reduced MOME activity significantly less than muscle-HSL protein concentration (Table 1). In addition to neutral lipase skeletal muscle also contains an acid lysosomal lipase as well as the alkaline lipoprotein lipase, but these enzymes cannot be involved in physiological regulation of the intramuscular triglyceride stores [3]. This fact, the finding that adrenaline stimulates muscle HSL [6], and the present findings strongly indicate that HSL is the enzyme responsible for hydrolysis of triglyceride in skeletal muscle. In line with this view HSL content correlates directly with triglyceride concentrations in the various muscle-fibre types, it being highest in slow-twitch oxidative and lowest in fast-twitch glycolytic muscle [2,4,6,9,10].

Apparently, as is the case for glycogen phosphorylase [12,13], muscle HSL is also under dual control by adrenaline [6] and contraction. The increase in TO activity elicited by the applied intense exercise protocol was of the same magnitude as the increase seen previously in homogenate of muscle [6] and adipose tissue [15] in response to a maximally effective concentration of adrenaline [6]. The same was true for the increase in glycogen phosphorylase *a* activity (Figure 1) [6], as also reported previously [12]. The effect of contractions on both enzymes was transient (Figure 1). This has been shown previously to apply to glycogen phosphorylase [12,13]. Furthermore, it has been shown that, in the presence of adrenaline, glycogen phosphorylase *a* activity may be increased throughout contractions [12]. In accordance with these findings we have shown recently that in adrenalectomized patients muscle glycogen phosphorylase *a* activity is increased at the end of prolonged exercise only if adrenaline is infused to mimic physiological conditions. Interestingly, responses of TO activity in muscle paralleled those of glycogen phosphorylase (J. Langfort, J. Ihlemann, M. Kjær and H. Galbo, unpublished work).

In theory, the present finding of an increase in HSL activity upon electrical stimulation of muscle might not be due to a direct effect of contractions upon HSL within muscle cells. Adipocytes may be interlaced between muscle fibres [2], and stimulation of HSL in adipocytes might occur secondarily to muscle contraction. However, the studied soleus muscles from young rats contained very few adipocytes [6] and, in addition, meticulous muscle fibre isolation was carried out before analysis. Furthermore, contracting muscle does not release any substance known to stimulate adipocytes. Another possibility is that the electrical stimulation elicited activity in intramuscular sympathetic nerve fibres which, in turn, increased the HSL activity in adipocytes or muscle cells. However, this was not the case, because the increase in HSL activity upon electrical stimulation was not impaired by a β -adrenergic blocking agent in a dose known to abolish the adrenaline-induced increase in HSL activity in muscle [6]. Furthermore, the response to electrical stimulation was intact in muscle that had been sympathectomized prior to study (Figure 6). Finally, the curious possibility that electrical stimulation directly activated adipocytes within the stimulated muscle was

excluded by the finding that electrical stimulation did not influence HSL activity in isolated epididymal fat tissue.

In agreement with the supposition that the mechanism involves phosphorylation, the contraction-induced increase in neutral lipase activity was markedly enhanced when muscle was stimulated in the presence of the protein phosphatase inhibitor okadaic acid (Figure 2). Furthermore, the contraction-mediated lipase activation rapidly vanished when homogenate of electrically stimulated muscle was treated with alkaline phosphatase (Figure 3). Bearing in mind the similarity of HSL and glycogen phosphorylase responses in muscle, it is tempting to speculate that the mechanism for contraction-induced HSL activation involves calcium/calmodulin-dependent protein kinase. However, this kinase has been shown to phosphorylate adipocyte HSL *in vitro* at a site that should inhibit rather than enhance HSL activity [19]. Still, distinct isoforms of adipocyte HSL exist, which may be regulated differently [21,22]. In line with the possibility of a specific muscle-HSL isoform, HSL mRNA is slightly larger in muscle than in adipose tissue (3.5 versus 3.3 kb) [23]. Of note is also the recent finding of novel phosphorylation sites in adipocyte HSL that are phosphorylated in response to protein kinase A and which necessitate a new view on molecular HSL regulation [24]. It follows that, even for adipocyte HSL, novel phosphorylation sites regulated by Ca^{2+} may be discovered.

In agreement with previous studies of incubated or perfused muscle stimulated electrically at fixed rates, muscle tension showed an early rapid decrease followed by a further slow decline [16,25]. As expected, the rate of muscle glycogen breakdown and muscle lactate concentrations varied directly with force production. However, no significant decrease in muscle triglyceride concentration was found, indicating that the fractional breakdown was low relative to the variation in prevailing concentrations between rats and to the sensitivity of the triglyceride assay. Tracer methods might have revealed triglyceride breakdown even though this was not detectable by chemical methods [4]. Furthermore, in previous studies of muscle contracting *in vitro* or *in vivo*, a decrease in triglyceride concentration has been a much more frequent finding in type-2a than in other muscle-fibre types [2,9,10]. So, the fact that young soleus muscles contain type-1 in addition to type-2a fibres may have complicated detection of triglyceride breakdown in the present study [26]. Lack of detectable intramuscular triglyceride depletion, despite ongoing lipolysis, would also be envisaged if released non-esterified fatty acids were subjected to recycling. This might well have been the case when HSL was activated early during exercise, because a high rate of glycogen breakdown is accompanied by accumulation of glycerophosphate and probably non-esterified fatty acid acyl-CoA [27,28]. Esterification of non-esterified fatty acid in muscle contracting *in vitro* has in fact been demonstrated, even with less intense stimulation than used in the present study [29]. Alternatively, accumulation of metabolites may also have impaired lipolysis by causing allosteric feedback inhibition of activated HSL [19,30]. Finally, it cannot be excluded that activation of HSL had no functional consequence during the applied conditions *in vitro*, if these interfered with proper regulation of other components involved in regulation of lipolysis. While HSL controls the rate-limiting step in triglyceride hydrolysis, proteins coating the lipid droplets (e.g. perilipin in adipocytes and adipose-differentiation-related protein in muscle) may impose an adjustable barrier to HSL action *in vivo* [19,31]. However, the molecular mechanisms that govern the catabolism of stored triglyceride are far from fully clarified [31].

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