Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid

Matthew J. Watt,1 Gregory R. Steinberg,1 G. J. F. Heigenhauser,2 Lawrence L. Spriet,1 and David J. Dyck1

Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1; and 2Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Submitted 15 January 2003; accepted in final form 25 April 2003

Watt, Matthew J., Gregory R. Steinberg, G. J. F. Heigenhauser, Lawrence L. Spriet, and David J. Dyck. Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid. Am J Physiol Endocrinol Metab 285: E412–E419, 2003.—Cyclopiazonic acid (CPA) is a sarcoplasmic reticulum Ca2+ ATPase inhibitor that increases intracellular calcium. The role of CPA in regulating the oxidation and esterification of palmitate, the hydrolysis of intramuscular lipids, and the activation of hormone-sensitive lipase (HSL) was examined in isolated rat soleus muscles at rest. CPA (40 μM) was added to the incubation medium to levels that resulted in subcontraction increases in muscle tension, and lipid metabolism was monitored using the previously described pulse-chase procedure. CPA did not alter the cellular energy state, as reflected by similar muscle contents of ATP, phosphocreatine, free AMP, and free ADP. CPA increased total palmitate uptake into soleus muscle (11%, P < 0.05) and was without effect on palmitate oxidation. This resulted in greater esterification of exogenous palmitate into the triacylglycerol (18%, P < 0.05) and phospholipid (89%, P < 0.05) pools. CPA decreased (P < 0.05) intramuscular lipid hydrolysis, and this occurred as a result of reduced HSL activity (20%, P < 0.05). Incubation of muscles with 3 mM caffeine, which is also known to increase Ca2+ without affecting the cellular energy state, reduced HSL activity (24%, P < 0.05). KN-93, a calcium/calmodulin-dependent kinase inhibitor (CaMKII), blocked the effects of CPA and caffeine, and HSL activity returned to pre-incubation values. The results of the present study demonstrate that CPA simultaneously decreases intramuscular triacylglycerol (IMTG) hydrolysis and promotes lipid storage in isolated, intact soleus muscle. The decreased IMTG hydrolysis is likely mediated by reduced HSL activity, possibly via the CaMKII pathway. These responses are not consistent with the increased hydrolysis and decreased esterification observed in contracting muscle when substrate availability and the hormonal milieu are tightly controlled. It is possible that more powerful signals or a higher [Ca2+] may override the lipid-storage effect of the CPA-mediated effects during muscular contractions.

FATTY ACIDS (FAs) derived from the plasma and intramuscular triacylglycerol (IMTG) constitute an important metabolic substrate for resting and contracting skeletal muscle (for review see Ref. 5). Evidence derived from resting isolated skeletal muscle preparations suggests that acute alterations in the extracellular hormonal milieu (insulin, epinephrine, leptin) (2, 22, 24) and the delivery of metabolic substrates (10) are important factors mediating the partitioning of FA between oxidative and nonoxidative (esterification) pathways. Compared with rest, ATP turnover in contracting skeletal muscle is markedly elevated, and is derived, in part, from increased oxidation of FA derived from extracellular locations and IMTG. Consequently, muscle contractions increase both exogenous FA and IMTG oxidation, while concomitantly enhancing FA esterification (9).

A key event of muscle contraction is the release of calcium (Ca2+) from the sarcoplasmic reticulum, and the resulting increase in cytosolic [Ca2+] has been suggested to potentiate a “feedforward” effect on fuel metabolism. Indeed, Ca2+ plays a key role in the regulation of carbohydrate metabolism in skeletal muscle by stimulating glucose transport (13, 28), activating the key regulatory enzymes glycogen phosphorylase (17) and pyruvate dehydrogenase (25) and thereby increasing its oxidation.

In contrast, there is little information regarding the effect of Ca2+ on the partitioning (oxidation and esterification) of FA, and specifically the role of Ca2+ influence on key regulatory enzymes of skeletal muscle FA metabolism. Recent findings in adipocytes suggest that Ca2+ promotes fat storage and inhibits oxidation secondary to reduced hormone-sensitive lipase (HSL) phosphorylation (32, 35). In contrast, FA utilization is augmented during skeletal muscle contractions without changes in the hormonal milieu or substrate availability, suggesting that Ca2+ may be important for stimulating IMTG hydrolysis and FA oxidation (9). Moreover, studies conducted in the rat soleus demonstrate the presence of HSL in skeletal muscle and transient activation of the enzyme by maximal tetanic contraction (19). Thus it would seem reasonable to hypothesize that Ca2+ would stimulate IMTG hydrolysis via increased HSL activ-
ity in skeletal muscle and, subsequently, FA oxidation.

One of the technical difficulties faced in ascribing direct effects of Ca^{2+} on metabolic responses in contracting skeletal muscle is the multitude of changes that accompany muscular contraction. Altered cytoplasmic energy charge ([ATP]−[ADP]/[Pi]), mitochondrial redox state, pH, and AMP-activated kinase (AMPK) activity have all been shown to independently alter fuel metabolism secondary to control through key regulatory enzymes.

In the present study, we have administered cyclopiazonic acid (CPA) to isolated skeletal muscle. CPA is a sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitor that increases intracellular calcium and at low concentrations (50 μM) does not cause contractions or alter the cellular energy charge. Our primary aim was to investigate the role of CPA in mediating changes in lipid metabolism in resting skeletal muscle, independent of changes in other stresses that compromise energy balance in skeletal muscle. Another aim was to assess the role of CPA on HSL activity. In the present study, we applied a pulse-chase technique to simultaneously assess FA hydrolysis from IMTG as well as esterification and oxidation of exogenous FAs in isolated soleus muscle. We hypothesized that CPA would 1) enhance IMTG hydrolysis via increased HSL activity, 2) increase both endogenous and exogenous FA oxidation, and 3) decrease FA esterification into endogenous pools.

METHODS

Animals and Muscle Preparation

Female Sprague-Dawley rats weighing 196 ± 3 g (mean ± SE) were used for all experiments. Animals were housed in a controlled environment on a 12:12-h reversed light-dark cycle so that they would be awake during the day when experiments were performed, and they were fed Purina rat chow ad libitum. All procedures were approved by the University of Guelph Animal Care Committee. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/kg). Animals were dissected into longitudinal strips from tendon to tendon by use of a 27-gauge needle. Two strips were utilized from each dissected muscle. The average strip mass was 33.5 ± 0.9 g.

strips were removed and placed in a 20-ml glass reaction vial containing 2 ml of warmed (30°C), pregassed (95% O_2-5% CO_2, pH 7.4), modified Krebs-Henseleit buffer containing 4% FA-free BSA (ICN Biomedicals), 6 mM glucose, and 0.5 mM palmitate. This was the base buffer used in all experiments. Temperature was maintained at 30°C, and the incubation medium was continuously gassed in all experiments, except for the experimental phase of the pulse-chase technique. Experiments were conducted in the following order: 1) dose-response experiments for increasing resting muscle tension, 2) determination of the cellular energy state, 3) pulse-chase studies, and 4) HSL activity (HSLα) determination.

Tension Development with CPA Experiments

Muscle strips were prepared as just described, and tendons were sutured with 5.0-gauge silk thread. The suture from the distal tendon was mounted to a fixed hook on a stimulating apparatus, and the proximal tendon was tied to a light stainless steel rod. The rod was connected to a Grass FT 03 force transducer (Grass Instruments, Quincy, MA). The muscle was mounted in a 10-ml incubation reservoir containing the base buffer and was equilibrated for 30 min. The resting tension was adjusted for optimal tetanic tension development under control conditions. In the present study, we used CPA (Calbiochem, La Jolla, CA) to increase cytosolic Ca^{2+}. CPA is a cell-permeable inhibitor of SR Ca^{2+}-ATPase activity and is commonly used to study calcium-signaling mechanisms (23). A 3 mM stock solution of CPA was prepared in dimethyl sulfoxide (DMSO). Tension development was monitored in muscles exposed to DMSO and various concentrations of CPA over 90 min. From these studies we determined that a CPA concentration of 40 μM was required for small increases in resting tension without causing a contraction or changing the cellular energy state (see RESULTS, Fig. 1), which is in the same concentration range determined in other studies to increase intracellular Ca^{2+} (8, 21, 27, 29). The small increase in resting tension is consistent with the premise that intracellular Ca^{2+} is also increased in this study in the rat epitrochlearis demonstrated increased Ca^{2+} release from the SR when muscle tension was increased minimally (34). Intracellular calcium was not directly measured in this study.

Muscle Metabolite Experiments

Paired muscle strips were preincubated for 30 min in 2 ml of base buffer. One strip was removed and rapidly frozen (preincubation, or Pre-Inc). The second strip was incubated in the base buffer in the presence of 25 μl of DMSO (CON) or 40 μM CPA dissolved in 25 μl of DMSO (CPA) for 90 min. The concentration of DMSO did not exceed 1.25% of the total buffer volume. Muscle strips were rapidly frozen, freeze-dried, and powdered, and nonmuscle contaminants were removed. The freeze-dried muscle was extracted in 0.5 M HClO_4 (1 mM EDTA) and neutralized with 2.2 M KHCO_3. ATP, phosphocreatine (PCr), creatine, and lactate were subsequently determined by spectrophotometric assays (3, 13). An identical series of experiments (n = 8 for each condition) was performed to assess the effect of CPA on IMTG content. Briefly, muscle was freeze-dried and powdered, the IMTG was extracted, and the chloroform phase was evaporated. After reconstitution, phospholipids were removed upon the addition of silic acid. The IMTG was saponified, and the free glycerol was assayed fluorometrically (3).

Pulse-Chase Experiments

Preincubation and preexperimental labeling of the intramuscular lipid pools. Soleus (SOL) muscle strips were incubated in 2 ml of base buffer for 30 min to permit equilibration. The pulse-chase procedures used have been described previously (10). Briefly, muscle strips were removed from the preincubation buffer and transferred to a second vial containing a pulse buffer consisting of the base buffer plus 2 Ci of [9,10-3H]palmitate (Amersham Life Sciences, Oakville, ON, Canada). Muscles were pulsed with [9,10-3H]palmitate for 40 min to prelabel all endogenous lipid pools (triacylglycerol (TG); diacylglycerol (DG); phospholipid (PL)). The muscle strip was transferred to a third vial with incubation medium containing no radiolabeled palmitate to wash [9,10-3H]palmitate that did not enter the endogenous lipid pools (e.g., interstitium). At the end of the pulse and wash, one SOL strip from each pair was removed, blotted, weighed, and
extracted for endogenous lipids, as described below, to determine the incorporation of \([9,10-^3\text{H}]\text{palmitate}\).

**Experimental phase (chase).** The remaining muscles were incubated for an additional 90 min with 0.5 \(\mu\text{C} / \text{ml}\) of \([1-^{14}\text{C}]\text{palmitate}\) (Amersham Life Science). Strips were assigned to either CPA or CON as described. During the 90-min chase phase, exogenous palmitate oxidation and esterification were monitored by the production of \(^{14}\text{CO}_2\) and incorporation of \([1-^{14}\text{C}]\text{palmitate}\) into endogenous lipids. Intramuscular lipid hydrolysis was simultaneously monitored by measuring the decrease in lipid \([^{3}\text{H}]\text{palmitate}\) content. These experimental conditions are isolated and do not represent an intact physiological system.

**Extraction of muscle lipids.** Muscles were placed in 13-ml plastic centrifuge tubes containing 5.0 ml of ice-cold 1:1 chloroform-methanol (vol/vol) and were homogenized using a polytron (Brinkman Instruments, Mississauga, ON, Canada). After homogenization, samples were centrifuged at 2,000 \(g\) (4°C) for 10 min. The supernatant was removed with a glass Pasteur pipette and transferred to a clean centrifuge tube. Distilled water (2.0 ml) was added, and samples were shaken for 10 min and centrifuged as before to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of \(^{14}\text{C}\)-labeled oxidative intermediates resulting from isotopic fixation. This represented a twofold correction for exogenous \([^{14}\text{C}]\text{palmitate}\) oxidation, as previously described (10). The chloroform phase, which contains the total lipids extracted from muscle, was gently evaporated under a stream of \(N_2\) and redissolved in 100 \(\mu\text{l}\) of 2:1 chloroform-methanol. Small amounts of phosphatidylcholine, dipalmitin, and tripalmitin (Sigma Chemical, St. Louis, MO) were added to the 2:1 chloroform-methanol to facilitate the identification of lipid bands on the silica gel plates. Fifty microliters of each sample were spotted on an oven-dried silica gel plate (Fisher Scientific Canada, Mississauga, ON). Silica gel plates were placed in a sealed tank containing solvent (60:40:3, heptane-isopropyl ether-acetic acid) for 40 min. Plates were then dried, sprayed with dichlorofluorescein dye (0.02% wt/vol in ethanol), and visualized under long-wave ultraviolet light. The individual lipid bands were marked on the plate with a scalpel and scraped into vials for liquid scintillation counting.

**Measurement of exogenous oxidation.** Gaseous \(^{14}\text{CO}_2\) produced from the exogenous oxidation of \([1-^{14}\text{C}]\text{palmitate}\) during the incubation was measured by transferring 1.0 ml of the chase incubation medium to a 20-ml glass scintillation vial containing 1.0 ml of 1 M \(\text{H}_2\text{SO}_4\) and to a 0.5-ml Fisher microcentrifuge tube containing 1 M benzethonium hydroxide. Liberated \(^{14}\text{CO}_2\) was trapped in the benzethonium hydroxide over 60 min, the microcentrifuge tube containing trapped \(^{14}\text{CO}_2\) was placed in a scintillation vial, and radioactivity was counted.

**HSL Experiments**

SOL muscle strips were prepared as previously described and incubated in 2 ml of base buffer for 30 min to permit equilibration. Some muscles were removed at 30 min and rapidly frozen (Pre-Inc). The remaining muscles were then subjected to either 25 \(\mu\text{l}\) of DMSO (CON) or 40 \(\mu\text{M}\) CPA dissolved in 25 \(\mu\text{l}\) of DMSO (CPA). In a second series of experiments, we evaluated the role of increased \(\text{Ca}^{2+}\) on HSLa by adding caffeine (CAF) to a final concentration of 3 mM. Caffeine is structurally unrelated to CPA and, at the concentrations used, has previously been shown to increase \([\text{Ca}^{2+}]\) without causing contraction or lowering inorganic phosphate (P) (34).

In separate experiments, we examined the possible involvement of \(\text{Ca}^{2+}\)/calmodulin-dependent kinase II (CaMKII) in the regulation of HSLa. KN-93 (Calbiochem), an inhibitor of CaMKII, was added to a final concentration of 50 \(\mu\text{M}\) during CON, CPA, and CAF. In all experiments, muscle strips were incubated for 90 min, temperature was maintained at 30°C, and the incubation medium was continuously gassed. Muscles were removed and rapidly frozen in liquid nitrogen at the conclusion of each experiment.

Skeletal muscle was freeze-dried, dissected free of connective tissue, blood, and fat under magnification, and powdered. Powdered muscle was used for the determination of HSLa as described by Langfort et al. (20) with minor modifications (31). The powdered muscle was homogenized on ice by using a rotating Teflon pestle on glass in 20 volumes of homogenizing buffer consisting of 0.25 M sucrose, 1 mM dithioerythritol, 40 mM \(\beta\)-glycerophosphate, 10 mM sodium pyrophosphate, 31 mM okadaic acid, 20 \(\mu\text{g/ml}\) leupeptin, 10 \(\mu\text{g/ml}\) antipain, and 1 \(\mu\text{g/ml}\) pepstatin, pH 7.0. After centrifugation, the supernatant was removed and stored on ice for immediate analysis of HSL activity. A substrate consisting of 5 mM triolein, 14 \(\times\) 10\(^6\) dpm \([9,10-^{3}\text{H}]\text{triolein}\), 0.6 mg phospholipid (phosphatidylethanolamine-phosphatidylinositol, 3:1, wt/wt), 0.1 M potassium phosphate, and 20% BSA was emulsified by sonication. The muscle homogenate supernatant (14 \(\mu\text{l}\)) was incubated at 37°C with enzyme dilution buffer (86 °C) for 10 min. The supernatant was removed with a glass Pasteur pipette and transferred to a clean centrifuge tube. The mixture was vortexed and centrifuged at 1,100 \(g\) for 20 min, and 1 ml of the upper phase containing the released FAs was removed for determination of radioactivity on a beta spectrometer (Beckman LS 5000TA). All values are normalized to total protein (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). It is important to note that the in vitro HSL assay represents the optimal catalytic rate of HSL at the time of snap-freezing and does not permit the determination of phosphorylation state because of the existence of numerous phosphorylation sites that are stimulatory or inhibitory in nature. Thus this assay measures HSL activity and does not detect potential allosteric effects of calcium or other metabolites.

**Calculations and Statistics**

The quantity of palmitate esterified and oxidized was calculated from the specific activity of labeled palmitate in the incubation medium (i.e., radiolabeled palmitate in dpm/total palmitate in nmol). Hydrolysis of intramuscular lipids at rest was calculated from the loss of preloaded \([^{3}\text{H}]\text{palmitate}\) (in nmol/g wet wt) from each pool. Free ADP and AMP concentrations were calculated with the assumption of equilibrium of the adenylate kinase and creatine kinase reactions (7). Free ADP was calculated using the measured ATP, creatine, and PCR values, an estimated \(\text{H}^+\) concentration (28), and the creatine kinase equilibrium constant of 1.66 \(\times\) 10\(^6\). Free AMP concentration was calculated from the estimated free ADP and measured ATP with the adenylate kinase constant of 1.05. Results were analyzed using a one-way ANOVA, and specific differences were located using a Student-Newman-Keuls post hoc test. Statistical significance was set at \(P \leq 0.05\).
RESULTS

Tension Development during Incubation with CPA

CPA induced a dose-dependant increase in muscle tension in the rat soleus (Fig. 1). DMSO had only minor effects on muscle tension, and no significant increase occurred over 90 min. At high CPA concentrations (1 mM), muscle tension developed rapidly and was increased to ~8 g by 3 min. This is consistent with rapid entry and action of CPA on reducing SR Ca^{2+}-ATPase activity and accumulation of cytosolic Ca^{2+}. Moreover, this is comparable to the tension developed by the addition of 20 mM CAF to the incubation medium (Fig. 1), which is frequently used to induce muscle contractions.

Muscle Metabolite Responses to CPA

Although ATP and PCr declined modestly during surgery, muscle contents of ATP and PCr were not different from Pre-Inc at the conclusion of 90 min of incubation in CON and CPA (Table 1). Calculated free AMP and free ADP contents were not changed from Pre-Inc by either treatment (Table 1). These data suggest that our preparation was stable during the experiment.

IMTG

IMTG content averaged 30.3 ± 5.0 mmol/kg dm after 30 min of Pre-Inc. IMTG content was not different from Pre-Inc after 90-min incubation in either trial and averaged 30.2 ± 3.2 and 35.5 ± 6.8 mmol/kg for CON and CPA, respectively.

Exogenous Palmitate Uptake, Esterification, and Oxidation

CPA increased (P < 0.05) total exogenous palmitate uptake (palmitate oxidation + total esterification; Fig. 2). Esterification into the TG and PL pools was greater (P < 0.05) in CPA compared with CON; however, there were no differences between treatments for DG esterification (Table 2). Total palmitate esterification into endogenous pools was greater (P < 0.05) in CPA (Fig. 2). CPA had no effect on exogenous palmitate oxidation (CON: 77.1 ± 6.2, CPA: 76.4 ± 6.8 nmol/g wm, Fig. 2).

Intramuscular Lipid Hydrolysis

Intramuscular lipid hydrolysis was decreased (P < 0.05) from 9.3 ± 2.5 nmol/g wm in CON to barely detectable rates in CPA (Fig. 3).

Table 1. Muscle metabolite contents in soleus strip muscle preparation immediately after excision and following 30-min of Pre-Inc and 90 min of incubation with or without CPA

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP</th>
<th>PCr</th>
<th>Free AMP</th>
<th>Free ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid freeze</td>
<td>17.0 ± 2.0*</td>
<td>39.7 ± 5.2*</td>
<td>0.92 ± 0.28</td>
<td>127 ± 35</td>
</tr>
<tr>
<td>Pre-Inc</td>
<td>12.8 ± 0.8</td>
<td>27.8 ± 2.1</td>
<td>1.04 ± 0.31</td>
<td>107 ± 19</td>
</tr>
<tr>
<td>CON</td>
<td>12.2 ± 1.0</td>
<td>23.7 ± 3.0</td>
<td>1.26 ± 0.34</td>
<td>115 ± 15</td>
</tr>
<tr>
<td>CPA</td>
<td>11.5 ± 1.0</td>
<td>25.6 ± 3.4</td>
<td>1.04 ± 0.43</td>
<td>103 ± 17</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 for ATP and phosphocreatine (PCr); n = 6 for free AMP and free ADP. ATP and PCr are expressed as mmol/kg dry mass. Free AMP and ADP are expressed as μmol/kg dry mass. Pre-Inc, preincubation in modified Krebs-Henseleit buffer. Incubation was without (CON) or with cyclopiazonic acid (CPA). Rapid freeze values obtained from Ref. 9. *Significantly different (P < 0.05) from other time points.
Effects of Increased CPA and CAF on HSLa

HSLa averaged $1.35 \pm 0.04 \text{ nmol/min}^{-1} \cdot \text{mg protein}^{-1}$ at the conclusion of Pre-Inc and was not different after 90 min in CON (Fig. 4). In contrast, HSL activity was decreased ($P < 0.05$) by 22% in CPA and to a similar magnitude in CAF. To explore the mechanism whereby Ca$^{2+}$ inhibits HSL, we added 50 μM KN-93, a CaMKII inhibitor, to the incubation medium. KN-93 had no effect on HSLa during CON or when added alone (1.34 ± 0.05 nmol/min$^{-1} \cdot$mg protein$^{-1}$), but it eliminated the inhibitory effects of CPA and CAF such that HSLa was not different from Pre-Inc (Fig. 4).

To confirm that HSL was activated during contraction, we stimulated muscle strips ($n = 4$) with 150-ms trains comprised of 0.1-ms impulses (20–40 V, 60 Hz) for 2 min at 20 tetani/min. HSLa was increased from 1.32 ± 0.06 at rest to $1.84 \pm 0.16 \text{ nmol/min}^{-1} \cdot \text{mg protein}^{-1}$ during contraction.

DISCUSSION

The release of Ca$^{2+}$ during contraction has been proposed to be an important early signal in promoting

Table 2. Palmitate esterification into various lipid pools during 90-min incubation with or without CPA

<table>
<thead>
<tr>
<th>Lipid Pool</th>
<th>CON</th>
<th>CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>2.8 ± 0.4</td>
<td>5.3 ± 1.2*</td>
</tr>
<tr>
<td>DG</td>
<td>21.2 ± 4.0</td>
<td>26.1 ± 3.5</td>
</tr>
<tr>
<td>TG</td>
<td>65.7 ± 5.8</td>
<td>77.1 ± 8.4*</td>
</tr>
<tr>
<td>Total</td>
<td>89.1 ± 8.4</td>
<td>108.6 ± 9.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 10$. PL, phospholipid; DG, diacylglycerol; TG, triacylglycerol. All units are expressed as nmol/g wet mass. *Significant difference from CON ($P < 0.05$).
control necessary to study metabolic/ enzymatic regulation, it is recognized that the results obtained may not necessarily be indicative of the metabolism/ regulation in human skeletal muscle.

In this study, CPA had no effect on absolute exogenous palmitate oxidation but altered the relative partitioning of lipids within the cell. CPA increased total exogenous palmitate uptake and promoted esterification of exogenous palmitate into endogenous lipid pools. Endogenous lipid hydrolysis was also blunted with CPA, and this was likely to be the result of attenuated HSL activity. In addition, we demonstrated that the CPA- and CAF-induced inhibition of HSL activity may be mediated via the CaMKII pathway. Taken together, these data suggest that CPA (presumably Ca$^{2+}$ at subcontraction levels) promotes lipid storage in resting skeletal muscle by simultaneously increasing FA uptake and esterification and decreasing endogenous lipid breakdown.

**Evidence of Increased Intracellular [Ca$^{2+}$]**

Low concentrations of CPA (40 $\mu$M) induced very small increases in muscle tension that progressively increased over 90 min (Fig. 1). This pattern of tension development is indirect evidence of increased cytosolic [Ca$^{2+}$] due to reduced uptake by the SR. Previous work conducted in skinned mammalian fibers demonstrated increased isometric tension development in myofibrils at CPA concentrations $\geq$10 $\mu$M (18). These small increases in muscle tension are also consistent with those observed in rat epitrochlearis exposed to small concentrations of W-7, a compound that induces Ca$^{2+}$ release from the SR (34). Thus, as in other studies (34), we did not directly measure intracellular calcium. However, our findings of a dose-response increase in muscle tension development, as well as evidence from previous studies that have demonstrated increased muscle Ca$^{2+}$ with CPA (8, 18, 21, 26, 27, 29), strongly argue that CPA increased Ca$^{2+}$ in this study.

**CPA Decreased IMTG Hydrolysis and HSL Activity**

A major finding of the present study was the reduced IMTG hydrolysis with CPA. The decreased IMTG hydrolysis in skeletal muscle is entirely consistent with studies in human adipocytes that demonstrated reduced lipidolysis when Ca$^{2+}$ was increased by agouti protein, an obesity gene product (33), or the addition of KCl to the incubating medium (32). There is also considerable indirect evidence to support a stimulatory role of Ca$^{2+}$ on skeletal muscle HSL activity. HSL activity was increased during maximal tetanic contractions in rat skeletal muscle, although it decreased to resting levels after 10 min (19). In human skeletal muscle, HSL activity was increased at the onset of exercise (1 min), suggesting that factors related to the contractile process (e.g., Ca$^{2+}$) may be involved with the rapid activation of HSL (31). Furthermore, there is evidence to suggest that protein kinase C, a Ca$^{2+}$ target, is involved in the contraction-mediated increase of HSL activity (6).

In contrast to our original hypothesis, but consistent with the measured reduction in IMTG hydrolysis (Fig. 3), HSL activity was decreased in resting skeletal muscle with CPA. To further explore the putative mechanism(s) mediating the decreased HSL activity, we inhibited the activity of CaMKII, which is a target for Ca$^{2+}$ and has been shown to bind and phosphorylate the “inactive” Ser$^{565}$ site and prevent further phosphorylation and activation of HSL in adipose tissue (11). When the antagonist KN-93 blocked the action of CaMKII, we observed reversal of the inhibitory CPA-induced effect and the return of HSL activity to baseline values (Fig. 2). Moreover, this effect was demonstrated by using two independent methods (CPA and CAF) that are known to increase [Ca$^{2+}$]. Although the use of a protein kinase inhibitor cannot exclude the involvement of other protein kinases in the control of a cellular process, these are the first data to indicate an inhibitory role for CaMKII on HSL activity in resting skeletal muscle. However, the current conclusions must be tempered by the possibility that differences in strength and frequency of the Ca$^{2+}$ signal during contraction (Ca$^{2+}$ spike) compared with the present study (slow accumulation) may alter the behavior of CaMKII.

In fairness, it should be noted that small decreases in ATP and PCr (~24 and 30%, respectively) occurred between the procurement of the SOL strips and the experimental (chase) phase. However, phosphagen concentrations were completely stable during the chase phase when all metabolic measurements were determined. Also, any significant impairment in the energy charge would have resulted in the activation of AMPK, which causes FA to be partitioned toward oxidation and away from storage in skeletal muscle (23). Given our findings of increased esterification and unaltered oxidation, it is very unlikely that there was any physiologically significant change in energy charge. Finally, our findings in skeletal muscle differ with those in human adipocytes that did not find an effect of CaMK inhibitors on KCl-inhibited lipolysis (32). Instead, increased Ca$^{2+}$ in adipose tissue exerted an anti-lipolytic effect by increasing phosphodiesterase activity, resulting in decreased cAMP and HSL phos-
phorylation (32). Such a mechanism in skeletal muscle cannot be discounted, and further studies to investigate the regulation of HSL activity are clearly warranted.

**CPA Promoted Lipid Storage in Skeletal Muscle**

Given the postulated feedforward regulation of fuel metabolism in skeletal muscle, we hypothesized that CPA (and presumably Ca^{2+}) would repartition FA toward oxidation and away from esterification. Instead, we observed increased total palmitate uptake and enhanced esterification of exogenous palmitate into the endogenous TG pool. There are no data to our knowledge pertaining to the effect of Ca^{2+} on palmitate uptake and lipid esterification in skeletal muscle. The finding of increased total palmitate uptake in the present study was not entirely unexpected, because fatty acid translocase and FA uptake are increased in electrically stimulated skeletal muscle, which suggests a possible role for Ca^{2+} (4). Palmitate esterification increases proportionally with oxidation in contracting rat skeletal muscle; however, these data are confounded by the altered intracellular milieu induced by contraction (9). The increased esterification agrees with data in adipose tissue suggesting that Ca^{2+} promotes TG storage by simultaneously reducing lipolysis and stimulating lipogenesis (35).

The mechanism mediating the increased lipid storage in skeletal muscle is unclear. Glycerol-3-phosphate acyltransferase is a logical target, because it catalyzes the initial and committed step in lipid biosynthesis (5). The increased lipid storage. The physiological significance of Ca^{2+}-induced FA esterification is not readily apparent but may occur secondary to increased FA transport. Given that the increase in FA uptake (Fig. 2) and the energy demand of the cell are low, the logical destination of excess lipid is storage. Alternatively, it is possible that the partitioning of lipids toward esterification with CPA at rest is a mechanism similar to the regulation of lipid storage in adipose tissue, and that, during muscular contractions, more powerful mediators controlling endogenous hydrolysis and esterification override the effects of Ca^{2+}. Consistent with this notion, endogenous hydrolysis is elevated markedly, and esterification is decreased during tetanic contractions (increased Ca^{2+}) compared with rest (9).

**Conclusion**

The results of the present study show that CPA administration to subcontraction concentrations simultaneously promotes lipid storage and decreases IMTG hydrolysis in isolated, intact soleus muscle. The decreased IMTG hydrolysis is likely to be mediated by reduced HSL activity, which is attenuated via the CaMKII pathway. These responses are not consistent with the increased hydrolysis and decreased esterification observed in contracting muscle when substrate availability and the hormonal milieu are tightly controlled. Accordingly, more powerful signals or a higher [Ca^{2+}] must override the lipid-storage effect of CPA during muscular contractions.

We are grateful to Stacey Reading for technical assistance, Dr. Coral Murrant for technical advice and use of the force transducer, and Dr. Jake Barclay for invaluable discussions.

**DISCLOSURES**

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada (D. J. Dyck and L. L. Spriet) and the Canadian Institute of Health Research (G. J. F. Heigenhauser). G. R. Steinberg was supported by an NSERC postgraduate scholarship.

**REFERENCES**


