Control of endogenous triglyceride breakdown in the mouse diaphragm

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Abstract The control of endogenous triglyceride breakdown was studied in vitro, in the incubated intact mouse diaphragm. Isoproterenol (2 μg/ml) produced parallel increases in glycerol and free fatty acid release, and in tissue cyclic AMP levels, suggesting that cyclic AMP mediates the action of the catecholamine on triglyceride mobilization. In addition to cyclic AMP, calcium seems to be involved in the action of isoproterenol because preincubation of hemidiaphragms in the presence of the calcium ionophore A23187 decreased the lipolytic effect of the drug. Insulin (12.5 mU/ml) antagonized the action of isoproterenol on triglyceride breakdown (it decreased glycerol and free fatty acid release) without altering its stimulatory effect on cyclic AMP production, suggesting that the antilipolytic action of insulin is not mediated by a decrease in cyclic AMP levels. On the other hand, no detectable effect on lipolysis was observed with carbachol in control and denervated hemidiaphragms, although the latter possess acetylcholine receptors over the entire surface area of the muscle. It was concluded that catecholamines control triglyceride breakdown in muscle while the cholinergic system does not seem to be involved. Cyclic AMP, calcium, and insulin all affect lipolysis in muscle and the interrelationships remain to be elucidated—**Abumrad, N. A., H. M. Tepperman, and J. Tepperman.** Control of endogenous triglyceride breakdown in the mouse diaphragm. *J. Lipid Res.* 1980. **21:** 149–155.

Supplementary key words muscle lipolysis · isoproterenol · insulin · cyclic AMP · calcium · carbachol · glycerol · free fatty acids

Several studies have shown that skeletal muscle cells can store impressive amounts of lipids (1, 2). It has also been shown that muscle lipids can be an important energy reservoir, particularly in prolonged fasting (3, 4) and during muscular activity (5–7). Only a few studies have dealt with intramuscular lipid mobilization (8). It has been suggested that, in the heart (9), cyclic AMP (cAMP) is involved in catecholomine-stimulated lipolysis. No similar studies, to our knowledge, have been carried out in skeletal muscle. However, in an earlier report from this laboratory it was shown that isoproterenol can stimulate free fatty acid (FFA) release from the incubated rat diaphragm (3), and an increase in intracellular free fatty acid after addition of adrenaline was demonstrated in the same preparation by Garland and Randle (10). Therefore, it seemed of interest to investigate further the mechanism of skeletal muscle triglyceride (TG) hydrolysis, and to find out whether it responds to the controls that operate in adipose tissue. The growing interest in the metabolic role of intramuscular lipids (7, 11), and the numerous reports implicating muscle in blood lipid homeostasis (12–14), made such a study seem timely.

The following report, on the intact mouse diaphragm (which is a thinner preparation than the corresponding rat muscle) describes isoproterenol and insulin effects on glycerol and FFA release and on tissue cAMP levels. The effects of the calcium ionophore A23187 on lipolysis were also studied in an attempt to determine whether calcium was involved in the catecholamine effects.

MATERIALS AND METHODS

Diaphragm preparation

Male Swiss albino mice (Taconic Farms, Germantown, NY) were used at 6 weeks of age. They weighed between 20 and 30 g. They were killed by cervical dislocation and the intact diaphragm was excised rapidly according to the method of Kipnis and Cori (15). Hemidiaphragms were dissected by cutting along the midline tendinous insertions. From each mouse one hemidiaphragm was used as a control, the other as the experimental tissue. The incubations were carried out in Krebs Ringer bicarbonate buffer (1.28 mM Ca^{+2}) with 3% albumin (bovine albumin, Fraction V, FFA poor, 0.01% FA by weight) Pentex, Miles Lab, Kankakee, IL). The preparation was incubated in capped plastic vessels at 37°C and 110 oscillations per minute; the gas phase was 95% CO_2 and 5% O_2. All incubations lasted for 60 min unless stated otherwise.

Abbreviations: TG, triglyceride; FFA, free fatty acids; cAMP, cyclic adenosine monophosphate.

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TABLE 1. Isoproterenol effect (2 μg/ml) on lipolysis and cAMP in the mouse diaphragm

	Glycerol	FFA	cAMP
	μmol/g/hr[a]	*μmol/g/hr*[a]	*pm/mg/hr*[b]
Basal	5.1 ± 0.4[d]	3.2 ± 0.1	2.3 ± 0.1
+2 μg/ml Isoproterenol	9.8 ± 0.8	5.5 ± 0.4	4.5 ± 0.2
% Change[c]	+95 ± 12	+72 ± 4	+89 ± 9
P[e]	<0.005	<0.005	<0.005

[a] Glycerol and FFA (μmol/g wet weight/hr) were measured in the medium. The glycerol release after 1 min was found to be less than 3% of the basal 60 value. The FFA release after 1 min was below the sensitivity of the FFA method used. They were both considered negligible.
[b] cAMP was measured in combined tissue and medium.
[c] % Change refers to the average change above basal.
[d] Values are means of six observations and are represented ± their standard errors.
[e] Comparisons of basal and isoproterenol treated preparations were made by the paired t test. ($P < 0.05$ is considered statistically significant).

Lipolysis studies

The glycerol and FFA released into the medium were measured in aliquots taken at the end of each incubation and kept frozen at −20°C until analyzed. Glycerol was estimated according to Korn (16). All glycerol values were subsequently corrected for the contribution of the albumin, since Joseph DeGeorge,[2] working in our laboratory, found that albumin substantially added to the glycerol measure. FFA were determined by the colorimetric procedure of Falholt, Lund, and Falholt (17). For determination of tissue triglycerides, hemidiaphragms were extracted by chloroform–methanol 2:1 (18). Triglycerides were measured in the extract after transesterification with sodium ethoxide according to the procedure of Soloni (19). Dibutyryl cAMP was obtained from Calbiochem (La Jolla, CA) and theophylline (aminophylline) from Sigma (St. Louis, MO).

cAMP measurements

cAMP was measured in perchloric acid extracts of the combined tissue and medium at the end of the incubations. Isolation of cAMP (20) was carried out prior to the assay by column chromatography on Dowex 50Wx8, 100–200 mesh (Bio Rad Lab., Richmond, CA). Cyclic AMP was measured by means of the Gilman assay (21). Tritium-labeled cAMP was purchased from New England Nuclear (Boston, MA) and the protein kinase binding protein was a gift from Dr. Robert Richman (Pediatrics Dept., State University of New York, Upstate Medical Center). Unlabeled cAMP was obtained from Calbiochem (La Jolla, CA).

[2] DeGeorge, J. Unpublished observations.

A23187 effect on lipolysis

Hemidiaphragm pairs were incubated at 37°C for 2 hr in Krebs Ringer bicarbonate buffer without albumin. To one hemidiaphragm, ionophore A23187 (a generous gift from Dr. Robert Hamill, Eli Lilly Co., Indianapolis, IN) was added at a final concentration of 1.9 μm. At the end of this first incubation hemidiaphragms were transferred to fresh buffer containing 3% albumin and reincubated for 1 hr in the presence of isoproterenol (2 μg/ml).

Carbachol effect on lipolysis

Carbachol (Sigma, St. Louis, MO) was added to the incubation medium at the two concentrations 1×10^{-5} and 1×10^{-3} M. In one experiment carbachol was added to chronically denervated hemidiaphragms (20 days past denervation). Left hemidiaphragms were denervated by cutting the left phrenic curve at the base of the neck under ether anesthesia. The mice recovered quickly. The effectiveness of phrenic nerve section could be assessed easily by observing the respiratory excursion on the operated and unoperated sides of the intact mouse.

RESULTS

Isoproterenol effect of lipolysis and cAMP

Table 1 shows that isoproterenol (2 μg/ml) increased glycerol and FFA release as well as cAMP production by the incubated mouse diaphragm. Basal glycerol release expressed as micromoles per gram tissue per hour was higher than the basal free fatty acid release. However, the stimulatory effect of isoproterenol was significant on both parameters. To find out whether the glycerol release into the medium is a true measure of endogenous triglyceride breakdown in the incubated diaphragm muscle, the following experiment was done. Five hemidiaphragms were extracted right after surgery for TG determination, and their matched

TABLE 2. Glycerol release as a measure of endogenous TG breakdown

Preincubation TG[a]	Postincubation TG[b]	TG Hydrolyzed[c]	Glycerol Released[d]
μmol/g		*μmol/g/hr*	
15.5 ± 1.7[e]	6.5 ± 0.7	8.9 ± 1.8	9.3 ± 0.7

[a] Tissue TG determined in hemidiaphragms right after surgery.
[b] TG levels in hemidiaphragms following their incubation for 60 min in the presence of 2 μg/ml isoproterenol.
[c] Difference in TG levels between hemidiaphragms in [a] and their matched halves in [b].
[d] Total glycerol release during the 60 min incubation.
[e] Values are means of five observations ± their standard errors.

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TABLE 3. Glycerol release from hemidiaphragms in response to dibutyryl cAMP, cAMP, theophylline, and theophylline plus isoproterenol

Drug Concentration	Control	Experimental	% Change	P^b
		μmol/g/hr		
dbcAMP 0.5 mM	4.8 ± 0.4	10.8 ± 0.3	+123 ± 21	<0.005
dbcAMP 1 mM	5.3 ± 0.9	19.3 ± 1.0	+318 ± 75	<0.001
dbcAMP 5 mM	5.3 ± 0.4	16.5 ± 1.4	+213 ± 10	<0.001
dbcAMP 10 mM	6.6 ± 1.2	21.2 ± 1.5	+260 ± 75	<0.001
cAMP 0.5 mM	5.5 ± 0.2	5.5 ± 0.5	+8 ± 7	>0.05
cAMP 1 mM	6.6 ± 0.4	8 ± 0.6	+20 ± 9	>0.05
cAMP 5 mM	5.7 ± 0.4	6.9 ± 1.0	+11 ± 8	>0.05
cAMP 10 mM	5.5 ± 0.9	15 ± 1.8	+188 ± 33	<0.005
cAMP 15 mM	6.1 ± 0.8	18 ± 2.0	+210 ± 9	<0.005
Theophylline 1 mM	5.5 ± 0.7	6.2 ± 0.4	+6 ± 4	>0.05
(1 mM) Theophylline + Isoproterenol (2 μg/ml)[a]	8.4 ± 0.5	13.3 ± 0.2	+60 ± 11	<0.005

[a] The controls in this experiment had 2 μg/ml isoproterenol.
[b] All values are means of six observations ± standard errors. Comparisons between control and experimental were made by the paired t test ($P < 0.05$ is statistically significant).

controls were incubated in the presence of 2 μg/ml isoproterenol for 60 min. The total glycerol released during this period was measured. The incubated hemidiaphragms were then extracted and post-incubation TG levels were determined. It is seen in **Table 2** that diaphragm TG levels drop following the incubation and that the drop can be accurately assessed by measuring glycerol release into the medium.

Effect of dibutyryl cAMP, cAMP and theophylline on lipolysis

It can be seen in **Table 3** that stimulation of lipolysis occurs with dibutyryl cAMP with a maximum effect obtained at 1 mM. On the other hand, no effect could be obtained with cAMP at concentrations below 10 mM and no further stimulation was observed with 15 mM. Table 3 also shows that theophylline at the concentration used (1 mM) did not affect basal glycerol release. However it potentiated the action of isoproterenol inasmuch as a higher stimulation of lipolysis was obtained with theophylline (1 mM) and isoproterenol (2 μg/ml) than with isoproterenol alone.

Effect of the ionophore A23187 on lipolysis

The calcium ionophore A23187 (**Table 4**) at a final concentration of 1.9 μM, affected glycerol and FFA release only under specific experimental conditions. The hemidiaphragms had to be preincubated with the ionophore in buffer free of albumin. No effect was observed when no preincubation was carried out even at a higher ionophore concentration (6 μM), or when albumin was included in the preincubated medium, whether the extracellular calcium concentration was 1.28 mM or 2.56 mM. As seen in Table 4 the ionophore increased basal FFA release. On the other hand, the lipolytic activity of isoproterenol (2 μg/ml) was dimin-

TABLE 4. Ionophore A23187 (1.9 μM) effect on basal and isoproterenol-stimulated lipolysis

Assay Conditions	Glycerol Release		FFA Release	
	μmol/g/hr		*μmol/g/hr*	
2nd Incubation[a]	−Isop.	+Isop.	−Isop.	+Isop.
Preincubation − Ionophore	5.6 ± 0.2	11.8 ± 0.9	1.4 ± 0.1	5 ± 0.2
Preincubation + Ionophore	6.8 ± 0.5	9 ± 0.8	2 ± 0.2	3.9 ± 0.2
% Change	+20 ± 6	−21 ± 3	+41 ± 3	−23 ± 3
P^b	$P > 0.05$	$P < 0.005$	$P < 0.001$	$P < 0.001$

[a] Hemidiaphragms were preincubated for 2 hr, with or without the ionophore, in Krebs Ringer bicarbonate without albumin added. Following preincubation the muscles were transferred to fresh medium with or without isoproterenol (2 μg/ml) with 3% albumin.
[b] All values are means of six observations ± SEM. Comparisons were made using the paired t test ($P < 0.05$ is statistically significant).

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TABLE 5. Insulin effect (12.5 mU/ml) on lipolysis and cAMP production in the presence of isoproterenol (2 μg/ml)

Additions	Glycerol	FFA	cAMP
	μmol/g/hr	*μmol/g/hr*	*pm/mg/hr*[a]
Isoproterenol	8.7 ± 0.9	5.2 ± 0.3	4.8 ± 0.2
Isoproterenol Insulin	6.4 ± 0.6	3.4 ± 0.1	4.6 ± 0.3
% Change	−28 ± 3.0	−33 ± 2.0	+8 ± 7.5
P[b]	<0.005	<0.005	>0.05

[a] cAMP was measured in combined tissue and medium (glycerol and FFA in the medium only).
[b] Values are means of six observations ± SEM. Comparisons were made using the paired t test ($P < 0.05$ is statistically significant).

ished in hemidiaphragms preincubated with the ionophore compared to those preincubated without the drug.

Lack of carbachol effect on lipolysis

The carbachol effect was studied in control and denervated hemidiaphragms in an attempt to find out whether the cholinergic system has any influence on TG breakdown in muscle. Carbachol (10^{-5} and 10^{-3} M) did not produce a detectable change in the glycerol release. A negative response was also observed in chronically denervated hemidiaphragms. However, a small increase in basal glycerol release was noted with denervation (5.2 ± 0.4 in right innervated versus 7.2 ± 0.6 in left denervated hemidiaphragms, $P < 0.05$).

Insulin effect on lipolysis

Table 5 shows that while insulin antagonized the isoproterenol effect on lipolysis as seen by the lowering of glycerol and FFA release, it did not affect the elevated cAMP levels which were similar to those in controls incubated in the presence of isoproterenol alone. The concentration of insulin used was 12.5 mUnits/ml (1 mU/ml was found ineffective and 6 mU/ml did not produce a consistent effect).

DISCUSSION

Mechanism of action of isoproterenol

The fact that basal glycerol release in μmol/g per hr exceeded basal FFA release may be due to the combined effect of FFA reesterification and oxidation. On the other hand, most if not all of the glycerol produced is released because glycerokinase activity of skeletal muscle is low (22).

cAMP involvement. The increase in levels of cAMP (approximately twofold) after isoproterenol, correlates well with the elevated lipolysis. Others have shown previously that isoproterenol can elicit a rapid increase in cAMP in cardiac muscle (23). An increased adenylate cyclase activity of skeletal muscle could be measured within 5 min after exposure to the hormone (24). In a detailed study on the time course of cAMP response to adrenaline in rat intact diaphragms, Eden et al. (25) described an early cAMP peak followed by a fall and leveling off of the nucleotide to values which were 2 to 5 times the control values at 60 min after exposure to the catecholamine. Our cAMP data agree well with the plateau values of Eden et al. (25). The fact that cAMP production and lipolysis are both increased 60 min after the start of the incubation is consistent with the possibility that cyclic AMP mediates the lipolytic action of isoproterenol. The results with dibutyryl cAMP, cAMP, and theophylline are also consistent with this hypothesis. In effect, the dibutyryl derivative of cAMP increased glycerol release up to a maximum obtained with a concentration of 1 mM. This is similar to the optimal concentration Crass (26) found effective in mobilizing TG in the rat heart. Cyclic AMP also had a lipolytic action but only at 10 times the concentration required for the dibutyryl derivative. These differences in the relative potency of the two drugs could be attributed to the fact that dibutyryl cAMP is thought to penetrate biological membranes more readily than the parent compound (27). In addition it is thought to be less sensitive to the degradative action of the enzyme phosphodiesterase (28) which it might also inhibit (29). Theophylline, a potent inhibitor of this enzyme, potentiated the lipolytic action of isoproterenol, providing further evidence for the involvement of cAMP in the action of the catecholamine. Theophylline has also been shown to potentiate the effect of isoproterenol on cAMP production in frog skeletal muscle (30). We confirmed these findings in the mouse diaphragm.[3]

Calcium involvement. In our preparation, preincubation of hemidiaphragms with the calcium ionophore resulted in a stimulatory effect on basal FFA release, while reducing the subsequent lipolytic response to isoproterenol. Interpretation of these results depends on whether the ionophore is thought to produce a net influx into (31) or efflux from cells (32). Recently it has been suggested (32, 33) that the ionophore released calcium from intracellular stores by acting on internal membranes and that this release of calcium activates cellular processes. The increase in basal FFA release in hemidiaphragms preincubated with the ionophore could represent an activation by calcium of the lipolytic process. In this context it has been proposed recently that a mechanism of action of

[3] Abumrad, N. A. Unpublished observations.

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adrenaline on adipose tissue lipolysis, in addition to that involving cAMP and independent of it, involves a substrate activation of endogenous triglycerides (possibly by calcium) making them more susceptible to the action of lipases (34). The decreased response to isoproterenol from hemidiaphragms preincubated with the ionophore is in agreement with observations made with hepatocytes by Chen, Babcock, and Lardy (32), where preincubation with A23187 reduced the calcium efflux as well as the glycogenolysis produced by norepinephrine. If the ionophore in our preparation produced a release and consequently some depletion of intracellularly stored calcium that may be involved in the action of isoproterenol, this might result in a diminished lipolytic response to the catecholamine. Complex interrelationships between cAMP and calcium have been described in cardiac and skeletal muscle and they were related to glycogen metabolism (35), establishing a connection between membrane effects modulating intracellular calcium concentration for contraction and relaxation and muscle metabolism. In this context a possible involvement of calcium in triglyceride breakdown in muscle could represent an alternate way in which mechanical and metabolic events in skeletal muscle might be related.

Cholinergic influence on muscle lipolysis

The results obtained with carbachol suggest that the cholinergic system is not involved in TG mobilization. The experiments done with denervated hemidiaphragms suggest that the lack of effect was not due to the absence of acetycholine receptors in the incubated tissue, since denervated hemidiaphragms are known to develop cholinergic receptors over the entire surface area of the muscle within a few days (36). The lack of effect of carbachol on glycerol release is compatible with the report that carbachol does not affect cAMP levels in rat cardiac tissue (23). The small increase in basal glycerol release we observed in left denervated hemidiaphragms (20 days post denervation) could be a result of the fluctuating adenylate cyclase activity after denervation (37). More studies are necessary to find out whether the catecholamine sensitive lipolysis in muscle is modulated by some trophic nerve influence.

Insulin effect on lipolysis and cAMP

Ruderman, Houghton, and Hems (38) showed that insulin causes a depression of glycerol output by the perfused rat hindquarter. Reimer et al. (39), using the same preparation, described a lowering by insulin of FFA but not of glycerol release which they explained as a result of increased FFA reesterification. In our experiment, insulin (Table 3) antagonized isoproterenol action on both FFA and glycerol release, which makes it unlikely that the insulin effect could be explained exclusively by a stimulation of FFA reesterification. The insulin concentration we found effective is unphysiologically high (12.5 mU/ml). However, high insulin concentrations are often used in order to obtain the metabolic effects of insulin in muscle incubations (40) or perfusions (38), and may be related to the high tissue proteolytic activity. The fact that increased cAMP levels were observed while lipolysis was diminished suggests that insulin's antilipolytic action is not mediated by changes in cAMP. Our results are similar to those of Craig, Rall, and Larner (40) who found an effect of insulin on glycogen metabolism in circumstances when insulin had no demonstrable effect on cAMP concentration in rat diaphragms. It is possible that insulin in our experiment could have affected the cAMP production during the fast peak described by Eden et al. (25). However this is unlikely since the effect of insulin on cAMP in adipose tissue seems small and delayed between 40 to 60 min after exposure to the hormone (41, 42). It remains controversial whether decreased cAMP mediates the antilipolytic effect of insulin in adipose tissue. Kissebah et al. (41) described a temporal dissociation between insulin's action on lipolysis and cAMP concentration. However Burns, Langley, and Robison (42) reported simultaneous decreases in lipolysis and cAMP in rat fat tissue 60 min after exposure to isoproterenol and insulin. It is also interesting that calcium (41) and, more recently, cyclic IMP (43) have been suggested as mediators of insulin action.

The interrelationships among cAMP, calcium and insulin, their roles in lipolysis of muscle triglycerides, and in coordinating mechanical and metabolic events in muscle certainly merit further study.

The authors wish to thank Dr. Robert Richman for generously supplying the cAMP binding protein, and Dr. Naji Abumrad for his help with the phrenic nerve sections. This work was supported by Grant No. AM-05410 from the National Institute of Arthritis, Metabolism and Digestive Disease, and No. 5 S07RR0402, Biomedical Research Support Grant from U.S. Public Health Service.

Manuscript received 6 November 1978 and in revised form 1 August 1979; accepted 21 August 1979.

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