Relationship between Lipolysis and Calcium in Epididymal Adipose Tissue of Obese-Hyperglycaemic Mice

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Summary. In the epididymal adipose tissue of lean (+/+) C57 BL/6J mice deprived of calcium by ethyleneglycol-bis-(β -aminoethylether) N,N'-tetraacetic acid (EGTA) treatment, corticotrophin-(1-24)-tetracosapeptide (ACTH)-stimulated lipolysis was abolished and peak levels of adenosine 3',5'-cyclic monophosphate (cyclic AMP¹) were reduced by 80%. Calcium omission was less effective on isoproterenol-stimulated lipolysis and was ineffective on theophylline- or dibutyl cyclic AMP-stimulated lipolysis. All EGTA effects were reversible. Lipolytic agents stimulated the net uptake of ⁴⁵Ca. This effect was inhibited by 2,4-dinitrophenol. The ⁴⁵Ca accumulated in response to lipolytic agents was largely La³⁺-nondisplaceable and was released with a half-life of 13 minutes under EGTA treatment. These results suggest that the mechanism promoting ⁴⁵Ca uptake in response to lipolytic agents was beyond cyclic AMP production in the adipose tissue of lean mice, but independent of free fatty acid release. In turn, intracellular as well as extracellular calcium facilitated maximal rates of lipolysis.

In the adipose tissue of obese-hyperglycaemic (ob/ob) mice, the response to the lipolytic agents of glycerol release and cyclic AMP levels was reduced by 65% and ⁴⁵Ca uptake was not stimulated. A 1–2 hour preincubation of adipose tissue increased markedly the effect upon lipolysis and ⁴⁵Ca uptake, but not that of peak cyclic AMP levels of isoproterenol. It is concluded that the adipose tissue of ob/ob mice displayed a reversible impairment of ⁴⁵Ca uptake in response to lipolytic agents.

Key words: Obese-hyperglycaemic mice, adipose tissue, lipolysis, calcium uptake, cyclic AMP, isoproterenol, ACTH-(1-24), theophylline.

Lipolytic hormones act upon adipose tissue via cyclic AMP and phosphorylation of hormone sensitive lipase [1]. ACTH at low concentration, however, can evoke a lipolytic response which is not accompanied by an increase in cyclic AMP levels [2, 3]. A group of antilipolytic agents, including α -adrenoceptor blocking agents, the tricyclic anti-depressant dibenzazepin, sulphonylurea drugs, the biguanide phenformin, local anaesthetics, and divalent cations interfering with calcium are able to inhibit hormone-stimulated lipolysis without decreasing cyclic AMP levels [4–6]. These observations support the suggestion that calcium, as well as cyclic AMP, is involved in the lipolytic process [7–11]. We have therefore studied the causal relationship between lipolysis and calcium movements in the adipose tissue of normal mice.

In the adipose tissue of ob/ob mice, previous data indicate in vitro defects in glycerol release [3] and adenylate cyclase activity [12, 13]. In the present work, we studied the effects of isoproterenol on calcium movements in the adipose tissue of ob/ob mice. A preliminary account of this work has appeared [14].

Materials and Methods

Male lean homozygous (+/+) and obese-hyperglycaemic (ob/ob) mice from our C57 BL/6J colony [4] were three to four months old at time of sacrifice and were fed ad libitum unless stated otherwise.

Theophylline, dibutyl cyclic AMP, isoproterenol, inulin, ATP and lyophilized firefly lantern extracts were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); bovine serum albu-

1 List of abbreviations: cyclic AMP: adenosine 3',5'-cyclic monophosphate; ACTH: corticotrophin-(1-24)-tetracosapeptide; 2,4-DNP: 2,4-dinitrophenol; HEPES: N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; EGTA: ethyleneglycol-bis-(β -aminoethylether)N,N'-tetraacetic acid; ob/ob: obese-hyperglycaemic

Table 1. Effects of isoproterenol, ACTH, theophylline and dibutyl cyclic AMP on glycerol release by adipose tissue of lean and obese-hyperglycaemic mice in the presence or in the absence of 2.5×10^{-3} mol/l calcium in the incubation medium

Additions	Concentration mol/l	Lean mice		Obese-hyperglycaemic mice	
		Ca +	Ca 0	Ca +	Ca 0
None		0.33±0.04	0.23±0.02 ^a	0.08±0.01	0.08±0.01
Isoproterenol	10 ⁻⁷	0.53±0.06	0.33±0.08 ^a	0.10±0.01	0.06±0.03
Isoproterenol	5 × 10 ⁻⁷	0.85±0.03	0.46±0.05 ^a	0.18±0.05	0.08±0.04
Isoproterenol	2.5 × 10 ⁻⁶	1.14±0.09	0.93±0.11 ^a	0.44±0.11	0.22±0.05 ^a
Isoproterenol	10 ⁻⁵	1.49±0.12	1.01±0.05 ^a	0.62±0.08	0.47±0.05 ^a
ACTH	10 ⁻⁵	1.53±0.13	0.28±0.07 ^a	0.67±0.05	0.10±0.03 ^a
Theophylline	5 × 10 ⁻³	1.49±0.14	1.29±0.09	0.63±0.06	0.51±0.04
Dibutyl cyclic AMP	5 × 10 ⁻³	1.31±0.10	1.27±0.15	0.72±0.04	0.71±0.05

Epididymal adipose tissue fragments were preincubated for 30 min in Krebs-Ringer bicarbonate buffer with 2.5×10^{-3} mol/l CaCl_2 and 4 g/100 ml (w/v) albumin. They were then transferred into a medium of identical composition or to a medium without calcium but with 2×10^{-3} mol/l EGTA. Results are expressed as μmol glycerol/100 mg fresh tissue/90 min and are means of 5 experiments $\pm$ SEM. ^a $p < 0.05$ compared with Ca +

min (Fraction V, fatty acid poor) from Pentex (Miles Research Co., Kankakee, Ill., U.S.A.), and glycerol-3-phosphate dehydrogenase and glycerol kinase from Boehringer (Mannheim, FRG). ACTH (synthetic corticotrophin (1-24)-tetracosapeptide) was a gift from Ciba-Geigy Ltd. (Basel, Switzerland). Cyclic [8-³H] AMP (27 Ci/mmol), [³H]-inulin and ⁴⁵CaCl₂ were obtained from the Radiochemical Centre (Amersham, England). Lumasolve and Lumagel were from Lumac Systems A.G. (Basel, Switzerland).

In each experiment, the epididymal fat pads from six lean and two obese mice were cut in 20 mg fragments. Incubation techniques and the assays of glycerol in the medium and of cyclic AMP in the tissue were as previously described [3, 15, 16].

For ATP determination, the fragments were frozen at -70°C in 1 mol/l perchloric acid. After ultrasonication the homogenates were centrifuged and ATP was determined in the aqueous phase according to the luciferin-luciferase method of Bihler and Jeanrenaud [17]. ATP standards were prepared daily in 1 mol/l perchloric acid.

To allow the measurement of the net uptake of calcium, the tissue fragments were exposed at 37°C to a Krebs-Ringer bicarbonate medium (pH 7.4) containing ⁴⁵CaCl₂ (1 $\mu\text{Ci}/\text{ml}$) and [³H]-inulin (0.1 mg/ml; 1 $\mu\text{Ci}/\text{ml}$). At the end of the incubation, the fragments were blotted lightly and dissolved in 2 ml Lumasolve (4 hours at 50°C).

Radioactivity was assayed in adipose tissue by dual radioisotope counting in 12 ml of a scintillation medium (5.5 g Permablend III (Packard Instrument Co., Downers Grove, Ill., U.S.A.) in 1 l toluene) with a Packard 2450 Tri-Carb liquid spectrometer. Aliquots of the media were counted in 12 ml Lumagel. Quench corrections were applied by means of an external standard and the net uptake of calcium was corrected for ⁴⁵Ca present in the interstitial space available to [³H]-inulin:

$$\text{Net uptake of } ^{45}\text{Ca} = \frac{\left(\text{Ca}_T - H_T \times \frac{\text{Ca}_M}{H_M} \right) \times 100}{\text{s. a.} \times \text{fresh wt}} \quad \text{in nmol/100 mg}$$

where Ca_T = total dpm of ⁴⁵Ca; H_T = total dpm of [³H]; $\frac{\text{Ca}_M}{H_M}$ =

ratio of dpm of ⁴⁵Ca to dpm of ³H in the medium; s. a. = specific radioactivity of calcium in the medium; and fresh wt = tissue fresh weight in mg.

The lanthanum-wash technique was used to determine the proportion of total calcium accumulated which could be con-

sidered as "superficial" [18, 19]. After a 15 or 60 min preincubation, in the absence or presence of 10^{-5} mol/l isoproterenol, the tissue fragments were rinsed for 15 minutes at 37°C in a Hepes medium (pH 7.4) containing 2×10^{-3} mol/l LaCl_3 . The ⁴⁵Ca content of the fragments was determined as above.

Calcium efflux was measured on tissue fragments prelabelled for 60 min with ⁴⁵Ca (1 $\mu\text{Ci}/\text{ml}$) and [³H]-inulin (0.1 mg/ml; 1 $\mu\text{Ci}/\text{ml}$) in the presence or in the absence of 10^{-5} mol/l isoproterenol, rinsed for 1 min at 37°C with fresh medium, then transferred at 10 min intervals to a series of vials containing 2 ml of calcium-free Krebs-Ringer bicarbonate buffer (pH 7.4) with 4 g/100 ml albumin and 2×10^{-3} mol/l EGTA. The sum of radioactivity in each wash plus the radioactivity remaining in the fragments at the end of the efflux period was taken to represent the total initial radioactivity.

Results are given $\pm$ SEM and significance ($p < 0.05$) assessed with Student's test for grouped or ungrouped data.

Results

Lean Mice

Effects of Extracellular Calcium on in Vitro Lipolysis, Cyclic AMP and ATP. Constant rates of glycerol release from adipose tissue fragments were observed for at least 90 minutes in experiments conducted in the presence of 2.5×10^{-3} mol/l calcium under basal conditions as well as in the presence of 2.5×10^{-6} mol/l isoproterenol or with 10^{-3} mol/l theophylline (Fig. 1). The two lipolytic agents increased lipolysis 4–5 fold. Maximally effective concentrations [3] of isoproterenol, ACTH, and dibutyl cyclic AMP stimulated the release of glycerol 4 to 5-fold (Table 1).

In a calcium-free medium containing 2×10^{-3} mol/l EGTA the lipolytic response to 10^{-5} mol/l ACTH was abolished, and the response to 10^{-5} mol/l isoproterenol reduced by 40%, but the lipolytic response to 5×10^{-3} mol/l theophylline or 5×10^{-3} mol/l dibutyl cyclic AMP was not altered (Table 1).

Fig. 1. Effects of 2.5×10^{-6} mol/l isoproterenol ($\circ$) and 10^{-3} mol/l theophylline ($\blacktriangle$) on the time course of glycerol release (left panels) and net ^{45}Ca uptake in excess of $[^3\text{H}]$ -inulin space (right panels) in epididymal adipose tissue of 3–4 month old normal (upper panels) and obese-hyperglycaemic (lower panels) mice. Adipose tissue fragments were preincubated for 30 min with 2.5×10^{-3} mol/l calcium and incubated without ($\bullet$) or with isoproterenol ($\circ$) or theophylline ($\blacktriangle$). Values are means $\pm$ SEM of 7 experiments

Table 2. Reversibility of calcium deprivation on lipolysis, cyclic AMP levels, and ATP levels in the epididymal adipose tissue from normal mice. Epididymal adipose tissue fragments were preincubated for 30 min in calcium-free Krebs-Ringer bicarbonate buffer containing 2×10^{-3} mol/l EGTA. They were then incubated in the same medium ($0 \rightarrow 0$) or in a medium without EGTA but with 2.5×10^{-3} mol/l calcium ($0 \rightarrow +$). Controls were preincubated and incubated in the presence of 2.5 mol/l calcium throughout ($+ \rightarrow +$)

Conditions	Basal	Isoproterenol 10^{-5} mol/l	ACTH 10^{-5} mol/l
A/ Lipolysis			
$+ \rightarrow +$	0.24 ± 0.05	1.08 ± 0.09	1.22 ± 0.10
$0 \rightarrow 0$	0.10 ± 0.03^a	0.89 ± 0.07^a	0.08 ± 0.03^a
$0 \rightarrow +$	0.18 ± 0.03	1.08 ± 0.10	0.93 ± 0.10^a
B/ Cyclic AMP levels			
$+ \rightarrow +$	22 ± 4	301 ± 28	445 ± 57
$0 \rightarrow 0$	19 ± 4	253 ± 27	87 ± 13^a
$0 \rightarrow +$	25 ± 5	265 ± 30	283 ± 32^a
C/ ATP levels			
$+ \rightarrow +$	10.5 ± 1.2	6.3 ± 0.8	4.3 ± 0.6
$0 \rightarrow 0$	9.6 ± 1.1	9.2 ± 1.1	7.4 ± 0.9^a
$0 \rightarrow +$	10.2 ± 1.1	5.2 ± 0.7	3.6 ± 0.5

A/ Glycerol release was assayed after a 60 min incubation and expressed as $\mu\text{mol}/100\text{ mg}$ fresh tissue/60 min. B/ Cyclic AMP measurements were performed after a 7 min incubation and expressed as $\text{pmol}/100\text{ mg}$ fresh tissue. 10^{-3} mol/l theophylline was present during the preincubation and the incubation periods. In the absence of theophylline, basal cyclic AMP contents were 7 ± 2 $\text{pmol}/100\text{ mg}$. C/ ATP content in the tissue were assayed after a 60 min incubation and expressed as $\text{nmol}/100\text{ mg}$ fresh tissue. Results are means of 4 experiments $\pm$ SEM. ^a $p < 0.05$ compared with control ($+ \rightarrow +$) data

Cyclic AMP levels were determined after a 7 min exposure to ACTH or isoproterenol, i. e. at a time of maximal elevation of cyclic AMP in tissue fragments preincubated for 30 min with 10^{-3} mol/l theophylline [4]. In the presence of 2.5×10^{-3} mol/l calcium and with 10^{-3} mol/l theophylline alone, the peak concentration of cyclic AMP was increased 3-fold over the control value; this concentration increased 14- and 20-fold, respectively, when 10^{-5} mol/l isoproterenol or 10^{-5} mol/l ACTH were added in combination with 10^{-3} mol/l theophylline (Table 2).

After a 30 minute period of calcium deprivation in the presence of 10^{-3} mol/l theophylline, the basal levels of cyclic AMP were unaffected (Table 2). There was a dramatic decrease (-80%) in the peak elevation of cyclic AMP in response to 10^{-5} mol/l ACTH and a non-significant (-17%) reduction in the presence of 10^{-5} mol/l isoproterenol.

The tissue content of ATP was halved after a 60 minute exposure to 10^{-5} mol/l ACTH or to 10^{-5} mol/l isoproterenol in a normal medium (Table 2). In a medium in which calcium was omitted, the fall in ATP in response to the lipolytic hormones was lowered (-23% vs -59% with ACTH, and -4% vs -40% with isoproterenol: Table 2).

These effects of calcium depletion on lipolysis, and on the levels of cyclic AMP and ATP were reversible. After a 30 minute preincubation period in the presence of 2×10^{-3} mol/l EGTA, and relative to tissue fragments remaining in EGTA, the reintroduction of calcium increased the basal lipolytic rate such that over the next 60 minutes control values

Fig. 2. Dose-response curves of isoproterenol on glycerol release (left panels) and net ⁴⁵Ca uptake (right panels) in epididymal adipose tissue of 3–4 month old normal (upper panels) and obese-hyperglycaemic (lower panels) mice. Medium glycerol and ⁴⁵Ca uptake in excess of [³H]-inulin space were assayed after 90 min incubations. Values are means $\pm$ SEM of 6 experiments

were almost attained (Table 2). Under the same conditions, the ACTH-stimulated lipolysis was again almost fully restored upon reintroduction of calcium. With isoproterenol, the 18% elevation of lipolysis noted after calcium reintroduction corresponded to a normal rate of lipolysis (Table 2).

Peak levels of cyclic AMP tested after a 7 minute exposure to lipolytic hormones in the presence of calcium and following a 30 minute calcium deprivation in the presence of 10^{-3} mol/l theophylline showed at least partial restoration (Table 2): with 10^{-5} mol/l ACTH the rise in cyclic AMP levels was 3.3-fold higher than in the continuous absence of calcium and corresponded to 64% of the levels observed in tissue fragments incubated in the uninterrupted presence of calcium; with 10^{-5} mol/l isoproterenol the elevation of cyclic AMP was normal.

The levels of ATP were again markedly affected by 10^{-5} mol/l ACTH or 10^{-5} mol/l isoproterenol treatment (–65% and –49%, respectively, Table 2).

Calcium Movements and Lipolysis. The basal net uptake of ⁴⁵Ca tested at a 2.5×10^{-3} mol/l extracel-

Table 3. Effects of isoproterenol on total ⁴⁵Ca uptake and on La³⁺-nondisplaceable ⁴⁵Ca after 15 or 60 minute incubation of adipose tissue from lean and obese-hyperglycaemic mice

Incubation time	Lean mice		Obese-hyperglycaemic mice	
	15 min	60 min	15 min	60 min
Without La ³⁺ wash				
Control	27 $\pm$ 4	39 $\pm$ 5	34 $\pm$ 5	43 $\pm$ 5
Isoproterenol 10^{-5} mol/l	29 $\pm$ 4	121 $\pm$ 14 ^a	36 $\pm$ 6	44 $\pm$ 5
With La ³⁺ wash				
Control	3 $\pm$ 1	15 $\pm$ 2	4 $\pm$ 2	15 $\pm$ 3
Isoproterenol 10^{-5} mol/l	12 $\pm$ 3 ^a	59 $\pm$ 7 ^a	4 $\pm$ 2	16 $\pm$ 3

Epididymal adipose tissue fragments were preincubated for 30 min and then incubated for 15 or 60 min in the presence of ⁴⁵Ca and [³H]-inulin as described under Materials and Methods. The La³⁺-washing was conducted for 15 min in a medium containing 10^{-2} mol/l HEPES, 140×10^{-3} mol/l NaCl, 0.5×10^{-3} mol/l MgCl₂, 6×10^{-3} mol/l KCl, 2×10^{-3} mol/l LaCl₃, and 4 g/100 ml albumin. Results are expressed as nmol ⁴⁵Ca/100 mg fresh tissue and are means of 4 experiments $\pm$ SEM. ^a $p < 0.05$ compared with control data

lular calcium concentration (Fig. 1) proceeded with an initial rapid phase of approximately 10 minutes duration (half-time 1–2 minutes) followed by a slower net uptake extending over 110 minutes. The first rapid phase of ⁴⁵Ca accumulation corresponded to about 70% of total ⁴⁵Ca accumulated. This phase was not significantly different in the presence of 10^{-5} mol/l isoproterenol or 5×10^{-3} mol/l theophylline, but a sustained accumulation of ⁴⁵Ca followed after 15 minutes so that the net uptake of ⁴⁵Ca was 3–5 times higher after 90 minutes than in the absence of lipolytic agents (Fig. 1). Half-maximal effects of isoproterenol on the net uptake of ⁴⁵Ca and glycerol release occurred at approximately 3×10^{-6} mol/l and 10^{-6} mol/l, respectively (Fig. 2).

Under basal conditions, the ⁴⁵Ca insensitive to La³⁺-washing represented 12% and 39% of the total ⁴⁵Ca accumulated after 15 minutes and 60 minutes, respectively. When 10^{-5} mol/l isoproterenol was present during the loading period, the proportion of ⁴⁵Ca accumulated that did not respond to the wash technique increased to 40% and 48%, respectively, after 15 and 60 minutes of incubation (Table 3). A 3.7-fold increase in the La³⁺ nondisplaceable ⁴⁵Ca could already be detected within 15 minutes of incubation with isoproterenol.

Maximally effective concentrations of the other lipolytic agents ACTH, theophylline and dibutyryl cyclic AMP induced a stimulation of net ⁴⁵Ca uptake comparable to that obtained with 10^{-5} mol/l isoproterenol (Table 4).

Table 4. Effects of isoproterenol, ACTH, theophylline and dibutyryl cyclic AMP on glycerol release and ^{45}Ca uptake by epididymal adipose tissue of lean and obese-hyperglycaemic mice

	Concentration mol/l	Lean mice		Obese-hyperglycaemic mice	
		Lipolysis	^{45}Ca uptake	Lipolysis	^{45}Ca uptake
Control		0.24±0.05	28±5	0.05±0.01	31±5
Isoproterenol	10 ⁻⁵	1.08±0.08 ^a	108±13 ^a	0.43±0.06 ^a	37±5
ACTH	10 ⁻⁵	1.22±0.09 ^a	123±15 ^a	0.47±0.06 ^a	33±5
Theophylline	5×10 ⁻³	1.12±0.08 ^a	126±15 ^a	0.41±0.06 ^a	41±6
Dibutyryl cyclic AMP	5×10 ⁻³	1.20±0.09 ^a	117±12 ^a	0.44±0.07 ^a	39±6

Epididymal adipose tissue fragments were preincubated for 30 min and incubated for 60 min in Krebs-Ringer bicarbonate buffer with 2.5×10^{-3} mol/l calcium and 4 g/100 ml albumin. Results are expressed as μmol glycerol/100 mg fresh tissue/60 min and as nmol ^{45}Ca /100 mg fresh tissue/60 min, and are means of 4 experiments $\pm$ SEM. ^a $p < 0.05$ compared with control data

The net uptake of ^{45}Ca was not saturable within a 10^{-4} to 2.5×10^{-3} mol/l concentration range of extracellular calcium, even when the highest concentration tested was offered in the presence of 10^{-5} mol/l isoproterenol or 5×10^{-3} mol/l theophylline (Fig. 3). On the other hand, a calcium concentration as low as 10^{-4} mol/l gave almost all the stimulated activities obtained normally with a 2.5×10^{-3} mol/l concentration.

2,4-Dinitrophenol (10^{-3} mol/l) did not alter net ^{45}Ca uptake and basal lipolysis (Table 5), but it inhibited by one third the response to 10^{-5} mol/l isoproterenol of net ^{45}Ca accumulation and glycerol release (Table 5).

The efflux of ^{45}Ca bound to cells or having entered intracellular spaces was recorded under EGTA treatment (2×10^{-3} mol/l in the absence of calcium), following a 60 minute preloading period in the presence of ^{45}Ca .

Semilogarithmic plots (Fig. 4) of values uncorrected for the amount of ^{45}Ca still present in the interstitial space after the 1 minute prewashing show that a rapid initial fall in tissue ^{45}Ca was followed by a steadily decreasing rate in ^{45}Ca efflux. Thus the process was multiphasic. When corrected for the ^3H -inulin space (Fig. 4) the data show that a large proportion of the rapidly exchangeable pool with half-time of 1–2 minutes observed during the net uptake experiments (Fig. 1) was not revealed by the efflux curve. This was due to the 1 minute prewashing in the efflux method, which prevented the detection of most of such a short-lived La^{3+} -displaceable interstitial pool. When added acutely, i.e. at the beginning of the efflux curve, isoproterenol had no apparent effect (data not shown), but when added together with ^{45}Ca during the 60 minute preloading period, the rate of efflux of ^{45}Ca that followed became apparently monoexponential (Fig. 4).

Fig. 3. Dose-response curves of calcium on glycerol release (left panels) and on the net uptake of ^{45}Ca (right panels) in epididymal adipose tissue of 3–4 month old normal (upper panels) and obese-hyperglycaemic (lower panels) mice. Medium glycerol and ^{45}Ca uptake in excess of ^3H -inulin space were determined after 90 min incubations in the absence (●) and in the presence of 10^{-5} mol/l isoproterenol (○) or 5×10^{-3} mol/l theophylline (▲). Values are means $\pm$ SEM of 6 experiments

Obese-Hyperglycaemic Mice

In obese mice, basal and stimulated lipolysis were also linear for at least 90 minutes, but all values were approximately 65% lower than in lean controls (Fig. 1). Maximally effective concentrations [4] of isoproterenol (10^{-5} mol/l), ACTH (10^{-5} mol/l), and dibutyryl cyclic AMP (5×10^{-3} mol/l) increased the release of glycerol 4 to 5 fold as in lean mice (Table 1). Also, with a medium free of calcium and enriched

Table 5. Effects of 2,4-dinitrophenol on basal- and isoproterenol-stimulated lipolysis and ^{45}Ca uptake in epididymal adipose tissue of lean and obese-hyperglycaemic mice

	Addition mol/l	Lean mice		Obese-hyperglycaemic mice	
		None	Isoproterenol 10^{-5} mol/l	None	Isoproterenol 10^{-5} mol/l
Lipolysis	—	0.13 ± 0.03	1.17 ± 0.09	0.07 ± 0.02	0.34 ± 0.05
	2,4-DNP 10^{-3}	0.11 ± 0.03	0.76 ± 0.07^a	0.08 ± 0.02	0.27 ± 0.05
^{45}Ca uptake	—	55 ± 9	114 ± 15	56 ± 8	57 ± 7
	2,4-DNP 10^{-3}	52 ± 7	82 ± 9^a	51 ± 8	55 ± 8

Epididymal adipose tissue fragments were preincubated for 30 min and incubated for 60 min in Krebs-Ringer bicarbonate buffer with ^{45}Ca and [^3H]-inulin, in the absence or in the presence of 10^{-3} mol/l 2,4-dinitrophenol. Results are expressed as μmol glycerol/100 mg fresh tissue/60 min and as nmol ^{45}Ca /100 mg fresh tissue/60 min and are means $\pm$ SEM of 9 experiments. ^a $p < 0.05$ compared with control data

Fig. 4. Effects of 10^{-5} mol/l isoproterenol on the time course of ^{45}Ca efflux from epididymal adipose tissue of 3–4 month old normal mice. Adipose tissue fragments were preincubated for 60 minutes in the presence of 2.5×10^{-3} mol/l ^{45}Ca and [^3H]-inulin, and in the absence (●) or presence (○) of 10^{-5} mol/l isoproterenol. They were then washed for 1 min and incubated without or with isoproterenol for 80 min. The amount of ^{45}Ca was expressed as percentage of initial ^{45}Ca (left panel) or initial calcium (right panel) remaining present in excess of [^3H]-inulin space. Two or three phases can be identified. Each point represents the means $\pm$ SEM of 3 experiments

with 2×10^{-3} mol/l EGTA, the lipolytic response to 10^{-5} mol/l ACTH was similarly abolished, and the response to isoproterenol was reduced by 40% whilst the lipolytic response to theophylline or dibutyryl cyclic AMP was unaffected (Table 1).

The net basal uptake of ^{45}Ca was identical to that observed in the adipose tissue of lean animals (Fig. 2). However, 2.5×10^{-6} mol/l isoproterenol and 5×10^{-3} mol/l theophylline were unable to increase this uptake (Fig. 2).

The concentration of isoproterenol giving an half-maximal effect on glycerol release was approximately the same as in lean mice (Fig. 2). On the other hand,

this agent was unable to stimulate the uptake of ^{45}Ca significantly (Fig. 2).

In the adipose tissue of obese mice, the lanthanum nondisplaceable ^{45}Ca was unaffected by the presence of 10^{-5} mol/l isoproterenol and represented 12–13% and 35–36% of the total ^{45}Ca accumulated after 15 and 60 minutes, respectively (Table 3). A reduction in medium calcium concentration did not affect the rate of lipolysis (Fig. 3). The basal net uptake of ^{45}Ca increased in a dose-dependent manner (as in normal mice), but was not stimulated by 10^{-5} mol/l isoproterenol or by 5×10^{-3} mol/l theophylline at any of the medium calcium concentrations tested (Fig. 3).

In the adipose tissue of obese mice, 10^{-3} mol/l 2,4-dinitrophenol (Table 5) did not affect either glycerol release or net ^{45}Ca uptake.

Effect of Preincubating Fat Pads and Fasting Lean and Obese Mice

In adipose tissue fragments of normal mice, a 2 hour preincubation significantly increased basal lipolysis and net ^{45}Ca uptake (Table 6), but the effects of 10^{-5} mol/l isoproterenol on both parameters were similar in absolute value to those observed in tissue without preincubation. The significant increase of basal- and isoproterenol-stimulated levels of cyclic AMP after a 1–2 hour preincubation probably reflected the time required for more effective cyclic AMP phosphodiesterase inhibition.

In adipose tissue fragments of obese mice, a one-hour preincubation increased the basal- and isoproterenol-stimulated production of glycerol. This effect was confirmed after two hours of preincubation and in addition there was now a significant response of ^{45}Ca uptake to isoproterenol (Table 6). By contrast, preincubation did not increase basal- and isoproterenol-stimulated levels of cyclic AMP.

In the adipose tissue of lean mice submitted to a

Table 6. Effects of preincubation on lipolysis, ^{45}Ca uptake, and cyclic AMP levels in epididymal adipose tissue from lean and obese-hyperglycaemic mice

Prewashing period hours	Isoproterenol 10^{-5} mol/l	Lean mice			Obese-hyperglycaemic mice		
		Lipolysis	^{45}Ca uptake	Cyclic AMP	Lipolysis	^{45}Ca uptake	Cyclic AMP
0	0	0.14 ± 0.04	20 ± 7	17 ± 4	0.07 ± 0.04	8 ± 3	10 ± 2
0	+	1.38 ± 0.10^a	139 ± 33^a	127 ± 11^a	0.60 ± 0.15^a	13 ± 4	35 ± 9^a
1	0	0.15 ± 0.08	29 ± 4	28 ± 5	0.19 ± 0.07	20 ± 3^b	16 ± 2
1	+	1.46 ± 0.12^a	142 ± 17^a	381 ± 39^a	$0.97 \pm 0.10^{a,b}$	33 ± 4^b	27 ± 3^a
2	0	0.53 ± 0.16^b	48 ± 10^b	28 ± 7	0.40 ± 0.10^b	32 ± 8^b	15 ± 1
2	+	1.21 ± 0.16^a	129 ± 8^a	313 ± 41^a	$1.07 \pm 0.28^{a,b}$	$70 \pm 16^{a,b}$	26 ± 4^a

Epididymal adipose tissue fragments were either incubated directly or preincubated for one or two periods of 60 min in Krebs-Ringer bicarbonate buffer before incubation. Glycerol release was assayed after a 60 min incubation and expressed as $\mu\text{mol}/100$ mg fresh tissue. ^{45}Ca uptake was measured after a 60 min incubation period in the presence of ^{45}Ca and $[^3\text{H}]$ -inulin and expressed as $\text{nmol}/100$ mg fresh tissue. Cyclic AMP levels in the tissue were assayed after a 7 min incubation and expressed as $\text{pmol}/100$ mg fresh tissue. 10^{-3} mol/l theophylline was present during the preincubation and the incubation period. Results are means of 8 experiments $\pm$ SEM. ^a $p < 0.05$ compared with isoproterenol free medium. ^b $p < 0.05$ compared with no prewashing period

Table 7. Effects of fasting on lipolysis, ^{45}Ca uptake, and cyclic AMP levels in the epididymal adipose tissue from lean and obese-hyperglycaemic mice

	Lean mice		Obese-hyperglycaemic mice	
	Control	Isoproterenol 10^{-5} mol/l	Control	Isoproterenol 10^{-5} mol/l
A/ Glycerol release				
Fed	0.25 ± 0.03	1.39 ± 0.02	0.10 ± 0.01	0.44 ± 0.06
Fasted	0.47 ± 0.06^a	1.35 ± 0.07	0.08 ± 0.01	0.36 ± 0.03
B/ Calcium uptake				
Fed	13 ± 2	81 ± 20	21 ± 1	30 ± 5
Fasted	54 ± 6^a	150 ± 65^a	23 ± 5	32 ± 5
C/ Cyclic AMP				
Fed	20 ± 1	152 ± 17	20 ± 5	37 ± 4
Fasted	28 ± 5	141 ± 22	12 ± 4	29 ± 5

Lean and obese-hyperglycaemic mice were either fasted for 24 h or fed ad libitum. Epididymal adipose tissue was preincubated for 30 min in a Krebs-Ringer bicarbonate buffer. Glycerol release, ^{45}Ca uptake and cyclic AMP levels were assayed and expressed as detailed in legend of Table 6. Results are means of 4 experiments $\pm$ SEM. ^a $p < 0.05$ compared with fed ad libitum data

24 hour fasting period (Table 7), in vitro basal lipolysis was increased by 86%, basal cyclic AMP levels were unaltered and the basal uptake of ^{45}Ca increased by 232%. The effects of 10^{-5} mol/l isoproterenol on glycerol release and cyclic AMP levels were not affected, whereas that on net ^{45}Ca uptake was greater by 88% than in mice fed ad libitum. In obese animals a similar fasting period did not modify basal lipolysis, cyclic AMP levels or basal net ^{45}Ca uptake, either in the absence or the presence of 10^{-5} mol/l isoproterenol.

Discussion

The use of intact tissue pieces instead of isolated fat cells avoids the problem of differential lysis of large fragile adipocytes in obese mice. This more "physiological" preparation involves the usual questions concerning slow diffusion in the interstitial space, the

contribution of structures and cells other than fat cells, and the mode of expression of results collected from lean and obese animals [3, 12]. Even if the present data could be expressed in terms of cellularity and/or subcellular water spaces rather than tissue weight, the major differences in the response to lipolytic agents in the adipose tissue of obese and non-obese mice would still be apparent.

1. The Role of Calcium in Lipolysis in Lean Mice

The lipolytic response to ACTH was highly dependent on the presence of extracellular calcium, whereas that induced by isoproterenol was less dependent, and that due to theophylline and dibutyryl cyclic AMP was unaffected by the absence of extracellular calcium (Tables 1 and 2). This variable sensitivity can be partially explained by the requirement for extracellular calcium of ACTH binding [20], whereas isoproterenol binding does not involve calcium.

The modest effect of calcium deprivation (Fig. 3 and Table 1) on isoproterenol-induced lipolysis occurred in spite of there being a normal elevation of cyclic AMP levels. These results are mostly in accord with those of others [6, 7, 8, 11, 21, 22]. They suggest that exogenous calcium acted as an additional messenger for isoproterenol at a site distal to the production of cyclic AMP. If this were true, then the normal lipolytic response obtained with theophylline or dibutyryl cyclic AMP in the presence of extracellular calcium might reflect the capacity of these agents to redistribute intracellular stores of calcium and compensate for the lack of exogenous calcium as previously suggested [11].

The reduction of ATP levels in response to isoproterenol and ACTH (Table 2) could be the consequence of uncoupling of oxidative phosphorylation by intracellular free fatty acids [17, 23]. After a period of calcium deprivation, ATP levels were not altered any more in response to lipolytic agents. However, glycerol release, cyclic AMP levels and ATP levels were again affected by readdition of calcium to the incubation medium (Table 2), consistent with data of Efendic et al. [11] on human adipose tissue, but not with those of Siddle and Hales [24] on isolated rat fat cells.

The kinetics of ^{45}Ca uptake suggested the presence of at least three types of calcium pool: 1/ the interstitial space which equilibrated with $[^3\text{H}]\text{-inulin}$ with a half-time of 1–2 minutes only. At equilibrium this space was equivalent to about 17 $\mu\text{l}/100\text{ mg}$ wet weight (data not shown); 2/ a La^{3+} -displaceable pool allowing rapid accumulation, independent of the preceding one [19], and presumably corresponding to the large binding capacity of negative charges located at the outer surface of plasma membranes (inter alia phospholipids and glycosaminoglycans [25]). Its extent was about 30 nanomoles/100 mg wet weight; 3/ presumably intracellular pool(s) with slower filling. These pools were not La^{3+} -displaceable (Table 3) and were related to energy production (Table 5). Their size was expanded 4-fold under isoproterenol and theophylline (Table 3 and Fig. 1).

From a methodological point of view, the precise estimate of ^{45}Ca uptake into the intracellular space depended on the accurate determination of the extracellular space. If the diffusion of $[^3\text{H}]\text{-inulin}$ in the latter space was slower and/or less complete than that of ^{45}Ca , an overestimation of the amount of ^{45}Ca gaining access to the intracellular compartments would be unavoidable. In the present experiments, $[^3\text{H}]\text{-inulin}$ diffused in adipose tissue fragments of both normal and ob/ob mice with a half-time of 1 min. An apparent steady state was reached after 15 min only (data not shown), suggesting increasing difficulty for this marker (with a molecular weight of around 5000) to gain access to some extracellular

compartment(s) and/or a progressive oedema of the extracellular space [26]. Hence, the "superficial" calcium determined by the La^{3+} -wash technique (Table 3) might correspond in part to an extracellular space not readily available to $[^3\text{H}]\text{-inulin}$.

If all the ^{45}Ca taken up by the slowly equilibrated pool(s) was present in an intracellular water space occupying 5% of adipose tissue fragments [26–28], the ^{45}Ca concentration would amount to 3 mmol/l and 12 mmol/l after 60 min without and with isoproterenol, respectively. When compared to the concentration in the incubation medium (2.5 mmol/l) and the estimated $0.1\text{--}1.0 \times 10^{-6}\text{ mol/l}$ cytosol free ionized calcium concentration in most cells, this illustrates the capacity of adipose cell structures for concentrating calcium. Among potential locations are the extensive endoplasmic reticulum of fat cells [29, 30] and mitochondria [25, 31], both of which store calcium in an ATP-dependent manner. The finding that 10^{-3} mol/l 2,4-dinitrophenol impaired the isoproterenol-stimulated accumulation of ^{45}Ca as well as the lipolytic response (Table 5) is consistent with this mechanism and confirms previous data [25, 32].

In washout experiments, the tissue fragments could be almost totally depleted of their preloaded ^{45}Ca with EGTA (Fig. 4). The multiphasic ^{45}Ca efflux was a consequence of interstitial space, and of the retention of ^{45}Ca in intracellular exchangeable pools of increasing affinity. The one minute washing period preceding the recording of ^{45}Ca efflux sufficed to deplete the interstitial space of 33% of the $[^3\text{H}]\text{-inulin}$ contained in this space at the beginning of the washout experiment (data not shown). The monoexponential efflux seen after 60 minutes of isoproterenol stimulation of ^{45}Ca uptake was a result of ^{45}Ca being then retained in a core of the tissue from which efflux was relatively slow.

We confirm previous data showing the stimulatory effects of lipolytic hormones on ^{45}Ca influx in tissue pieces [9, 10] and adipocytes [6, 33], but could not confirm the stimulation of ^{45}Ca efflux observed at late stages of washout curves [33]. In our experiments the apparent opposite effect, i. e. a decreased release of ^{45}Ca from fat tissue, is in doubt since the hormone effect on ^{45}Ca efflux was tested, before the equilibration with the isotope was complete (after 60 minutes: Figs. 1 and 4). At any rate, the net increase in the amount of ^{45}Ca accumulated after exposure to isoproterenol certainly reflected an increased rate of entry and not simply an increased exchange of calcium across plasma membranes. Any over-estimation of basal ^{45}Ca uptake tended to under-estimate the stimulatory effects of lipolytic agents on this parameter.

To define the intricate relationship between lipolysis and ^{45}Ca movements in the adipose tissue of lean mice, primary and secondary effects must be

carefully delineated since cyclic AMP and calcium are interrelated intracellular messengers in a variety of cells [34]. First, it appears that the precipitation of calcium soaps of free fatty acids was not directly responsible for the calcium content of tissue. At low calcium concentration in the medium, lipolysis could indeed be stimulated maximally, although the net uptake of ^{45}Ca by the tissue was low (Fig. 3); furthermore, the isoproterenol concentration provoking half-maximal stimulation of lipolysis was three times lower than that required for half-maximal stimulation of ^{45}Ca uptake (Fig. 2).

On the other hand, the effects of theophylline and dibutyryl cyclic AMP (Table 1) suggest that intermediary events taking place before the release of free fatty acids, but beyond adenylate cyclase activation, induced a marked accumulation of cytosolic calcium.

In fact, calcium movements in both directions appeared capable of regulating the lipolytic process: a/ The availability of exogenous calcium was required for the specific action of ACTH (Tables 1 and 2, and ref. [20], for an optimal response to isoproterenol (Tables 1 and 2, and refs. [6, 8, 11, 21, 22]), and probably for lipolytic steps beyond cyclic AMP accumulation (Table 2 and ref. [6]); b/ The mobilisation of intracellular calcium [35] might also contribute to an optimal lipolytic response to isoproterenol (Tables 1 and 2), and cover calcium requirements in the presence of theophylline or dibutyryl cyclic AMP (Table 1) as previously shown [8, 10].

II. Lipolysis-Calcium Relationship in the Adipose Tissue of Obese-Hyperglycaemic Mice

The poor lipolytic response of the adipose tissue of obese mice has been related to a defect in adenylate cyclase activity [3, 12]. The present data imply that an alteration in calcium movements might also contribute to reduce a futile cycle of lipolysis and re-esterification in this tissue. At equilibrium ^{45}Ca was distributed in the extracellular space (9 $\mu\text{l}/100\text{ mg}$ wet weight: data not shown) and a mostly La^{3+} -displaceable space of normal size (Fig. 1). However, unlike in normal mice, the entry of ^{45}Ca was not stimulated by isoproterenol and by dibutyryl cyclic AMP (Figs. 1–3; Table 3). In addition the stimulation of ^{45}Ca uptake, in response to an increase in calcium concentration from 0.1 to 2.5 mmol/l, was lower than in lean mice (Fig. 3).

These observations stress again that there was no direct relationship between lipolysis and the net uptake of ^{45}Ca , i. e. the latter does not result directly from the presence and concentration of free fatty acids. More importantly they allow an extension to adipose tissue of the hypothesis [36] of a defect in calcium movements in the liver of ob/ob mice. These

authors observed that hepatic glycogen breakdown was poorly stimulated by vasopressin. Since vasopressin is thought to act on glycogenolysis by modifying the movements of calcium in the cell [36], alterations in hepatic calcium handling might contribute to the syndrome. The observation [37] of a defective $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity in plasma membranes of liver and kidney suggests that more than one alteration in the control of ionic distribution are present in obese mice.

Attempts to correct the present alterations were only partly successful. The lower in vitro sensitivity of lipolysis to isoproterenol was not increased by a one day fast, but was fully restored by a 1–2 hour preincubation in a glucose-free buffer, as previously shown [38]. Furthermore, the net calcium uptake became sensitive and the cyclic AMP elevation remained refractory to isoproterenol after preincubation (Table 7). This is in line with previous data showing that a preincubation period also increased the response to insulin of both catecholamine-stimulated lipolysis [38] and glucose uptake [39]. One possible explanation of these results would be that the removal of an inhibitor present in excess in the adipose tissue of obese mice and acting beyond adenylate cyclase contributed to this recovery. Insulin saturating adipose tissue, because of the high circulating insulin level, might not represent this labile unidentified inhibitor, considering the reduced insulin-binding capacity of the tissue [40] and the failure of an anti-insulin serum to correct in vitro the depressed response of lipolysis to epinephrine or theophylline [41].

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