Effects of adrenaline on triacylglycerol synthesis and turnover in ventricular myocytes from adult rats

Eleithyia M. S. SWANTON and E. David SAGGERSON[1]

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, U.K.

Ca^{2+}-tolerant myocytes were isolated with endogenous triacylglycerol (TAG) stores prelabelled with [^{3}H]palmitate and subsequently incubated for a 1 h chase period with [^{14}C]palmitate, 2 % albumin and 5 mM glucose. Measurements were then made of [^{14}C]palmitate conversion into TAG and phospholipids, of loss of [^{3}H]TAG, of glycerol release and of change in the total TAG content. Rates of *de novo* synthesis of TAG were calculated by a balance method. With 0.5 mM palmitate present, 5 μM adrenaline increased *de novo* synthesis of TAG by 81 % and incorporation of [^{14}C]palmitate into phospholipids by 59 %. Significant increases in these processes with adrenaline were also seen with 0.08, 0.14 and 0.26 mM palmitate. The β-agonist isoprenaline had little effect on *de novo* synthesis of TAG and had no effect on [^{14}C]palmitate conversion into phospholipids. The α_1-agonist phenylephrine mimicked adrenaline in increasing [^{14}C]palmitate conversion into phospholipids but had no effect on *de novo* synthesis of TAG. Adrenaline did not significantly alter the myocyte glycerol 3-phosphate content but caused a persistent 40 % increase in the activity of the form of glycerol-phosphate acyltransferase found predominantly in the sarcoplasmic reticulum. With 0.5 mM palmitate present, the value [^{14}C]TAG formed $-$decrease in [^{3}H]TAG consistently exceeded the enzymically measured change in cell TAG content. From this it was suggested that the specific radioactivity of [^{3}H]TAG pool(s) mobilized during the chase period was lower than that of the overall cell TAG. In the basal state, complete mobilization of TAG measured as glycerol release was low, but cycling of TAG to diacylglycerol or monoacylglycerol and back to TAG appeared to be high. With adrenaline present, glycerol release was increased 5–6-fold but recycling of lower acylglycerols to TAG was abolished. Glycerol release was inhibited by increasing extracellular palmitate from 0.08 to 0.5 mM. Adrenaline partially over-rode this effect.

INTRODUCTION

Cardiac myocytes contain an appreciable store of triacylglycerol (TAG) which is present as membrane-bound lipid particles [1], as free-floating cytoplasmic droplets and associated with lysosome-like structures [2]. Myocardial TAG is in a surprisingly dynamic state; for example, in the absence of exogenous fatty acid as much as 2 % of myocardial TAG can be turned over per minute [3] and the fatty acid derived therefrom can provide as much as 40 % of energy needs in a working heart experimentally perfused with glucose as sole exogenous substrate [4]. Even when exogenous fatty acid is present at high concentration, myocardial TAG turnover can still provide over 10 % of myocardial ATP production [4]. Despite this potential for turnover the myocardium normally does not drastically deplete or grossly over-expand this TAG reserve, suggesting that there must be very stringent control over the balance between *de novo* TAG synthesis and TAG lipolysis.

TAG lipolysis quantified as either glycerol release or disappearance of TAG has been extensively studied, particularly in the perfused heart, and to a lesser extent in isolated cardiac myocytes. The process is acutely increased by catecholamines [5–9], ischaemia or hypoxia [10,11], perhaps secondary to the local release of catecholamines [12–14], and by pressure development [15]. By contrast, exogenous long-chain fatty acids inhibit the mobilization of myocardial TAG reserves and/or also diminish its stimulation by catecholamines [6,16–18].

However, there is less information about conditions that may acutely alter the rate of *de novo* TAG synthesis (i.e. assembly of TAG starting from glycerol 3-phosphate) in heart cells or the mechanisms underlying such changes. Rather unexpectedly, changes in TAG synthesis that are documented are not opposite in direction to changes in lipolysis. Adrenaline has been reported to increase conversion of exogenous fatty acids into TAG and phospholipids by perfused hearts [5], and the β-adrenergic agonist isoprenaline to increase TAG synthesis *in vitro* [19] or to increase myocardial TAG accumulation *in vivo* [20,21]. Also there is enhanced incorporation of fatty acids into myocardial TAG during ischaemia and subsequent reperfusion [10,22].

The objective of this study was to investigate mechanisms regulating *de novo* synthesis of TAG and how this may be integrated with the regulation of lipolysis. We have used isolated Ca^{2+}-tolerant rat myocytes as the experimental system. Although these cells are quiescent, unlike those in the working heart, they permit investigations in which a number of parameters can be simultaneously varied and their use obviates possible problems of interpretation due to metabolic contributions from other cell types present in the intact heart.

MATERIALS AND METHODS

Chemicals

Routinely used chemicals were from British Drug Houses Ltd., Poole, Dorset, U.K. Phosphatidylcholine (from egg yolk), sodium palmitate, L-adrenaline bitartrate, L-isoprenaline hydrochloride, L-phenylephrine hydrochloride, oleoyl-CoA, *N*-ethylmaleimide, 1,2-*sn*-dioleoylglycerol and halothane (1-chloro-2-bromo-1,1,1-trifluoroethane) were from Sigma Chemical Co. Ltd, Poole, Dorset, U.K. Palmitoyl-CoA, various enzymes and

Abbreviations used: DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; PAP, phosphatidate phosphohydrolase; TAG, triacylglycerol.

[1] To whom correspondence should be addressed.

an enzymic test kit for determination of TAG were from Boehringer-Mannheim, Lewes, East Sussex, U.K. [U-^{14}C]Glycerol 3-phosphate and [1-^{14}C]palmitoyl-CoA were from Amersham International, Little Chalfont, Bucks, U.K. [U-^{14}C]Palmitic acid and [9,10-^{3}H]palmitic acid were from DuPont (U.K.) Ltd., Stevenage, Herts, U.K. Collagenase (Type 2 from *Clostridium histolyticum*) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. The Wako NEFA C test kit was from Alpha Laboratories, Eastleigh, Hants, U.K. Sodium phosphatidate was prepared by the action of phospholipase D on egg phosphatidylcholine followed by extraction and conversion of the product into the sodium salt. The phospholipase D was isolated from cabbage [23]. Sodium palmitate was bound to albumin using the method of Evans and Mueller [24], and the concentration of bound palmitate determined using the Wako NEFA C test kit.

Animals

These were male Sprague–Dawley rats (250–300 g). They were maintained at 20–22 °C on a 13 h light/11 h dark cycle with light from 06:00 to 19:00 h with constant access to drinking water and Rat and Mouse Breeding Diet (Special Diet Services, Witham, Essex, U.K.). The diet contained (w/w) 21 % protein, 39 % starches and sugars and 4 % fat.

Isolation of cardiac myocytes prelabelled with [^{3}H]palmitate

Cell isolation was essentially as described by Fuller et al. [25]. Rats were anaesthetized with halothane; the heart was then removed into ice-cold Krebs–Henseleit bicarbonate (KHB) medium (118.5 mM NaCl, 25 mM Na_2CO_3, 4.8 mM KCl, 1.2 mM Mg_2SO_4, 1.2 mM KH_2PO_4) containing 10 mM glucose and 2.5 mM $CaCl_2$. Hearts were trimmed to remove adhering fat and then cannulated for perfusion in a retrograde manner through the aorta. This was carried out for 4 min (37 °C; 80 cm hydrostatic pressure) until the heart was cleared of blood using Ca^{2+}-free KHB medium gassed with O_2/CO_2 (95 %/5 %). Collagenase (1 mg/ml), $CaCl_2$ (50 μM) and BSA (0.1 mg/ml) were then added to the perfusate, and recirculating perfusion continued for a further 10 min. The atria were then removed and the ventricular tissue cut into a star shape and placed in a 25 ml silicone-treated flask with 3.3 ml of KHB medium containing collagenase (2 mg/ml), $CaCl_2$ (50 μM), BSA (20 mg/ml) and sodium [^{3}H]palmitate (0.5 mM, 1 μCi/μmol). The flask was sealed and shaken (150 strokes/min) with gassing (95 % O_2/5 % CO_2) for 6 min at 37 °C. Myocytes liberated by the action of collagenase were then collected by filtration through a 250 μm mesh nylon gauze. The filtrate was made up to 10 ml with gassed KHB medium containing 50 μM $CaCl_2$, albumin (20 mg/ml)) and 0.5 mM sodium [^{3}H]palmitate and the cells allowed to sediment under gravity. The remaining tissue was re-incubated with collagenase and [^{3}H]palmitate as above a further three times until all the tissue was disaggregated. The four cell pellets were pooled and washed six times by resuspension in 10 ml of gassed KHB medium containing BSA (20 mg/ml) and 0.5 mM sodium palmitate. The Ca^{2+} concentration was increased stepwise with each washing. The first four washes also contained sodium [^{3}H]palmitate whereas the final two washes contained only unlabelled palmitate and served to remove any extracellular [^{3}H]palmitate. Finally the myocytes were suspended as a stock in KHB medium containing 1.3 mM $CaCl_2$, 5 mM glucose, BSA (20 mg/ml) and 0.5 mM sodium palmitate at a ratio of cells/medium of 1:20 (v/v). Viable cells exhibited a typical rod shape, had visible cross-striations and excluded Trypan Blue. Preparations with less than 70 % viable cells were discarded. The average viability of the preparations that were used was approx. 80 %.

Incubation of myocytes prelabelled with [^{3}H]palmitate

Portions (0.5 ml) of the myocyte stock (approx. 1.5 mg of cell dry weight) were added to silicone-treated glass flasks giving a final incubation volume of 2.5 ml consisting of KHB medium containing 1.3 mM $CaCl_2$ 5 mM glucose, albumin (20 mg/ml) and sodium [U-^{14}C]palmitate at various concentrations as indicated in Figures and Tables. Flasks were shaken at 50 strokes/min and 37 °C. Flasks were gassed for 5 min with O_2/CO_2 (95 %/5 %) before being sealed and left shaking for 1 h.

Measurement of ^{3}H and ^{14}C in myocyte lipids

At the end of 1 h of incubation flask contents were transferred to glass tubes and centrifuged for 20 s at 100 $\boldsymbol{g}_{av}$. The medium was removed for determination of non-esterified fatty acids (see below) and the cell pellets were extracted with 20 vol. (approx. 3 ml) of chloroform/methanol (2:1, v/v) [18]. The mixture was left for 1 h at room temperature with intermittent sonication to disperse any clumps of cellular material, this was followed by filtration through Whatman No. 1 filter paper. Phase separation was achieved by addition of 0.2 vol. of distilled water to the filtrate followed by centrifugation for 5 min at 2000 $\boldsymbol{g}_{av}$. The chloroform phase was removed and evaporated to dryness under a stream of O_2-free N_2. The residue of extracted lipids was then redissolved in a small known volume of chloroform and applied to a silica-gel (SIL G) TLC plate which was developed for 90 min in a solvent system of petroleum spirit (40–60 °C boiling point)/diethyl ether/acetic acid/methanol (90:20:3:2, by vol.). The plate was air-dried and lipid spots visualized by staining with I_2. The silica gel from each spot was scraped from the plate and added to Ecoscint A for liquid-scintillation counting using a dual-label programme to discriminate ^{3}H and ^{14}C. Individual lipid classes were identified by comparison with standards applied in parallel to the TLC plate. Using this method it was possible to separate tri-, di- and mono-acylglycerols, phospholipids and non-esterified fatty acids with a total recovery of radioactivity of more than 85 %. At the start of the incubation the contents of two flasks were also taken and used to determine the initial radioactivity in these lipid classes.

Measurement of the TAG content of myocytes

Lipids were extracted from cell pellets at the beginning and end of 1 h incubations as described above and the final chloroform phase was dried down under a stream of O_2-free N_2. The residue was resuspended in 1.2 ml of 74 mM Tris/citrate buffer, pH 8.2, containing 7.9 mM sodium cholate and 2 % (w/v) poly(ethylene glycol) followed by spectrophotometric assay of TAG using the kit supplied by Boehringer.

Measurement of non-esterified fatty acid in incubation media

Incubation media obtained at the beginning and end of 1 h incubations were assayed spectrophotometrically using the Wako NEFA C test kit. The assay was modified by reduction of the recommended assay volume from 3 ml to 210 μl so that assays could be performed in 96-well microtitre plates. Standards of oleic acid were run in parallel and the product of the assay was measured at 550 nm using a Titertek Multiscan MCC microplate photometer.

Measurement of glycerol and glycerol 3-phosphate

Incubations were terminated by addition of 0.2 ml of 60 % (v/v) $HClO_4$ followed by cooling in ice and neutralization with 200 μl of 1 M triethanolamine hydrochloride and 175 μl of

saturated K_2CO_3 solution. $KClO_4$ was removed by centrifugation and the supernatants stored at −70 °C before spectrophotometric assay for glycerol [26] and fluorimetric assay of glycerol 3-phosphate [27]. Duplicate flasks were treated in the same way at the start of incubations in order to determine the initial content of glycerol.

Measurement of the activities of enzymes of glycerolipid synthesis after incubation of myocytes

Myocytes were incubated for 1 h at 37 °C as above except that incubations were in a final volume of 5.5 ml of KHB medium containing 1.3 mM $CaCl_2$, 5 mM glucose, BSA (20 mg/ml) and 0.5 mM sodium palmitate. At the end of the incubation, the cells were collected by centrifugation at 100 g_{av} for 10 s in silicone-treated glass homogenizer tubes. The medium was rapidly removed by aspiration and the tubes were plunged into liquid N_2. Immediately before enzyme assay, the cell pellets were homogenized in 1 ml of ice-cold 10 mM Tris/HCl buffer, pH 7.4, containing 0.25 mM sucrose, 1 mM EDTA and 1 mM dithiothreitol using 2 × 15 s bursts of an UtraTurrax tissue disintegrator.

Glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) was assayed radiochemically at 30 °C as the incorporation of [^{14}C]glycerol phosphate into butanol-soluble products [28,29]. Assays were performed for 8 min in a final volume of 1.0 ml of 100 mM Tris/HCl buffer, pH 7.4, containing fatty acid-poor BSA (1.75 mg/ml), 0.7 mM dithiothreitol, 1 mM [U-^{14}C]glycerol phosphate (0.5 μCi/assay) and 100 μg of homogenate protein. Mitochondrial GPAT was discriminated by conducting the assay with 40 μM palmitoyl-CoA in the presence of 10 mM *N*-ethylmaleimide, and microsomal GPAT was discriminated by assay with 120 μM oleoyl-CoA in the absence of *N*-ethylmaleimide [29,30]. Parallel assays terminated at zero time were used as blanks. Diacylglycerol acyltransferase (DGAT: EC 2.3.1.20) was assayed radiochemically at 30 °C as the dioleoylglycerol-dependent incorporation of [1-^{14}C]palmitoyl-CoA into hexane-soluble product [31]. Assays were performed for 6 min in a final volume of 0.2 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 8 mM $MgCl_2$, fatty acid-poor BSA (0.1 mg/ml), 1.2 mM 1,2-*sn*-dioeoylglycerol, 30 μM [1-^{14}C]palmitoyl-CoA (0.25 μCi/assay) and 20 μg of homogenate protein. Dioleoylglycerol was dispersed in 0.5 % (w/v) sodium taurocholate at a concentration of 120 mM before use so that all DGAT assays in addition contained 0.005 % (w/v) sodium taurocholate. Dioleoylglycerol (but not taurocholate) was omitted from blanks. Phosphatidate phosphohydrolase (PAP; EC 3.1.3.4) was assayed at 37 °C as P_i release from an aqueous disperson of sodium phosphatidate [32]. All assays were performed for 20 min in a final volume of 100 mM Tris/maleate buffer, pH 6.8, containing 1 mM dithiothreitol, 1.4 mM sodium phosphatidate and 100 μg of homogenate protein. Assays of the Mg^{2+}-dependent (PAP1) activity also contained 3 mM $MgCl_2$ with or without 5 mM *N*-ethylmaleimide. The actual PAP1 activity was then calculated by difference as the *N*-ethylmaleimide-sensitive activity. Assays of PAP2 activity in addition contained 5 mM EDTA and 5 mM *N*-ethylmaleimide. Parallel assays terminated at zero time were used as blanks. Myocyte GPAT, DGAT and PAP activities were expressed relative to lactate dehydrogenase (EC 1.1.1.27) activity, which was assayed spectrophometrically at 25 °C [33].

Measurement of myocyte dry weight and protein content

Triplicate aliquots of myocyte stock suspensions and resuspension medium (minus cells) were placed in preweighed 10 ml beakers and heated at 70 °C until no further decrease in weight was observed. The dry weight of the myocytes was obtained by subtracting the dry weight of the resuspension medium. Protein contents were measured by the method of Bradford [34] with BSA as standard.

Statistical methods and expression of results

Values are presented as means ± S.E.M. Throughout, these are expressed as μmol of fatty acid equivalents/h per g dry weight, i.e. TAG and glycerol values have been multiplied by 3. *n* values indicate the numbers of separate preparations of myocytes. Statistical significance was calculated using Student's *t* test for paired samples.

RESULTS AND DISCUSSION

Characteristics of the myocyte experimental system

Saddik and Lopaschuk [18] have previously studied turnover and synthesis of TAG in isolated working rat hearts using a protocol in which hearts were initially 'pulsed' with [^{14}C]palmitate followed by a 'chase' perfusion with [^{3}H]palmitate. Our approach has been an adaptation of this; 0.5 mM palmitate (^{3}H-labelled if required) was present throughout cell isolation, and subsequent metabolic experiments were then performed in the presence of [^{14}C]palmitate. The TAG content of myocytes isolated in this way was 60 ± 6 μmol of fatty acid equivalents/g dry weight (n = 16), which is comparable with the values of 47 [35] or 50–60 [18] μmol of fatty acid equivalents/g dry weight found in freshly isolated rat hearts or the value of 48 μmol of fatty acid equivalents/g dry weight in hearts after a 1 h 'pulse' perfusion with 1.2 mM palmitate [18]. By contrast, rat myocytes that are isolated in the absence of fatty acid have severely depleted TAG contents of only 6–8 μmol of fatty acid equivalents/g dry weight [17,36]. Cells incubated during the 'chase' period with 0.5 mM palmitate not only maintained their TAG content but actually increased it (Table 1).

Preliminary experiments (n = 2) carried out under basal conditions with 0.5 mM palmitate present established that the following were linear with time for at least 1 h: conversion of

Table 1 Effects of adrenaline at 0.5 mM palmitate

Myocytes that had been prelabelled with [^{3}H]palmitate were incubated for 1 h in the presence of 5 mM glucose and 0.5 mM [^{14}C]palmitate. Values are expressed as μmol of fatty acid equivalents/h per g dry weight and are means ± S.E.M. (n = 16 except where indicated as n = 10). TAG contents at the start of the 'chase period' were 60 ± 6. ^{A} indicates $P < 0.0005$ for effects of adrenaline. ^{B,C} indicate $P < 0.025$, $P < 0.0005$ respectively compared with a value of 1.0. ^{D} indicates $P < 0.01$ for comparison of III versus I. ^{E,F} indicate $P < 0.025$, $P < 0.005$ respectively for comparison of IV versus II.

	Control	+ Adrenaline (5 μM)
Fate of exogenous [^{14}C]palmitate:		
TAG (I)	49 ± 3	58 ± 3^{A}
DAG	0.79 ± 0.05	1.43 ± 0.10^{A}
Phospholipid	1.88 ± 0.10	2.99 ± 0.13^{A}
Net utilization of fatty acid (n = 10)	126 ± 17	96 ± 19
Glycerol release	6.2 ± 1.6	36 ± 5^{A}
Increase in TAG content (II)	27 ± 4	26 ± 4
De novo TAG synthesis (III)	36 ± 4^{D}	65 ± 5^{A}
Loss of palmitate from [^{3}H]TAG	10.3 ± 1.8	16.6 ± 1.5^{A}
Glycerol release/loss of [^{3}H]TAG	0.65 ± 0.15^{B}	2.23 ± 0.27^{A,C}
[^{14}C]TAG − loss of [^{3}H]TAG (IV)	38 ± 3^{E}	42 ± 3^{F}
'Correction factor'	0.47	0.53

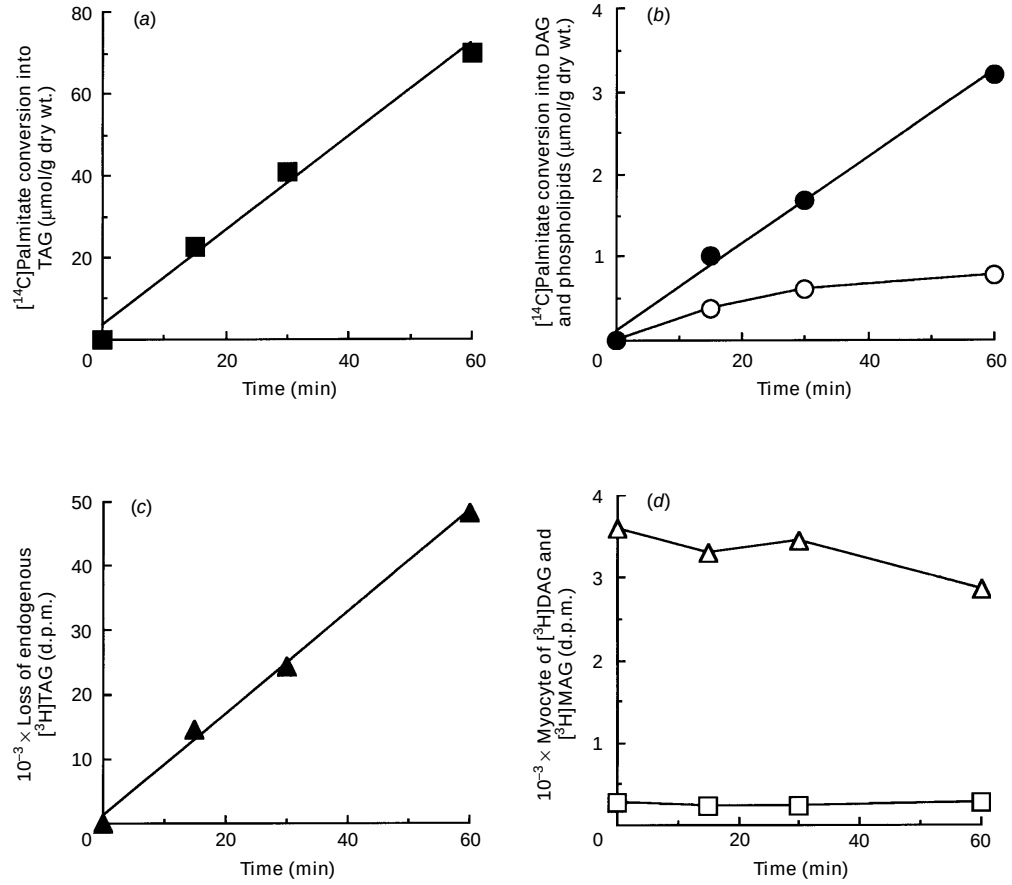


Figure 1 Time courses of glycerolipid synthesis and turnover under basal conditions

Myocytes that had been prelabelled with [^{3}H]palmitate were incubated for the indicated times with 5 mM glucose and 0.5 mM [^{14}C]palmitate. The values are means of two similar experiments. (**a**) and (**b**) ■, TAG; ●, phospholipids; ○, DAG. (**c**) and (**d**) ▲, TAG; △, DAG; □, MAG. The values are expressed as mean d.p.m. per myocyte incubation. The initial ^{3}H activity in TAG was 273000 d.p.m. per incubation.

[^{14}C]palmitate into TAG (Figure 1a), phospholipids (Figure 1b) and CO_2 (not shown); loss of ^{3}H from prelabelled TAG (Figure 1c) and conversion of prelabelled TAG into ^{3}H-labelled water-soluble products (not shown). By contrast, appearance of [^{14}C]palmitate in diacylglycerol (DAG) levelled off with time (Figure 1b). Although there was significant net utilization of fatty acids during the 'chase' phase (Table 1), this accounted for no more than 15% of the 0.5 mM palmitate that was present initially. In other preliminary experiments (not shown) it was established that conversation of [U-^{14}C]glucose into CO_2 and formation of lactate were linear with time. Although [^{3}H]DAG and [^{3}H]MAG could be detected in cells that had been prelabelled with [^{3}H]palmitate, the contents of these species were only 1% and 0.1% respectively of the initial content of [^{3}H]TAG and did not alter appreciably during the 'chase' period (Figure 1d).

Stimulation of glycerolipid synthesis by adrenaline

During a 'chase' period with 0.5 mM [^{14}C]palmitate (palmitate/albumin molar ratio = 1.65:1.0) a small (mean $+21\pm4$%, $n=16$) stimulation of conversion of exogenous palmitate into TAG was observed (Table 1). This slight effect was not always statistically significant (e.g. Table 4) and was not seen with lower concentrations of [^{14}C]palmitate (e.g. Table 2). For reasons discussed below this does not provide a true measure of the *de novo* synthesis of TAG. Compared with TAG, a much smaller flux of [^{14}C]palmitate into phospholipids was observed but this was enhanced to a greater extent (mean $=+64\pm9$%, $n=16$) by adrenaline and this effect was consistently observed (e.g. Tables 2, 3 and 4).

Paulson and Crass [35] have proposed the balance relationship:

Change in TAG content (ΔTAG) = TAG synthesis − lipolysis

i.e.

$$\textit{de novo}\ \text{TAG synthesis} = \Delta\text{TAG} + \text{lipolysis} \quad (1)$$

This is a very simple relationship which permits unambiguous quantification of *de novo* TAG synthesis. The value for *de novo* TAG synthesis encompasses synthesis from both endogenous (^{3}H) and exogenous (^{14}C) fatty acid and does not involve any errors from use of fatty acids in reacylation of DAG or monoacylgylcerol (MAG) products of partial lipolysis of TAG (see further discussion below). Using eqn. (1), we found that adrenaline significantly increased *de novo* synthesis of TAG by 79 ± 5% from 36 to 65 μmol of fatty acid equivalent/h per g dry weight (Table 1). Table 2 shows that a statistically significant stimulatory effect of adrenaline on *de novo* TAG synthesis was also seen over a 6-fold range of palmitate concentrations.

It was also noteworthy that variation of exogenous palmitate at lower concentrations (0.08–0.26 mM) did not greatly alter

Table 2 Effects of adrenaline and exogenous fatty acid concentration

Myocytes that had been isolated in the presence of 0.5 mM palmitate were incubated for 1 h in the presence of 5 mM glucose and the indicated concentration of [^{14}C]palmitate. Values are expressed as μmol of fatty acid equivalents/h per g dry weight and are means ± S.E.M. ($n = 5$ for 0.08, 0.14 and 0.5 mM palmitate, $n = 6$ for 0.26 mM palmitate). ^{A,B,C,D,E} indicate $P < 0.05$, < 0.025, < 0.01, < 0.005, < 0.0005 respectively for effects of adrenaline.

	Without adrenaline				With adrenaline (5 μM)			
Palmitate concn. (mM) ...	0.08	0.14	0.26	0.50	0.08	0.14	0.26	0.50
Fate of exogenous [^{14}C]palmitate:								
TAG	15 ± 3	21 ± 3	37 ± 5	44 ± 4	13 ± 2	23 ± 4	38 ± 5	55 ± 3^{D}
Phospholipid	1.1 ± 0.3	1.4 ± 0.4	1.9 ± 0.3	1.9 ± 0.2	1.4 ± 0.3^{D}	2.0 ± 0.5^{D}	2.9 ± 0.7^{A}	3.0 ± 0.2^{E}
Glycerol release	19 ± 3	17 ± 3	9.8 ± 2.3	6.8 ± 3.7	55 ± 5^{D}	42 ± 6^{C}	35 ± 6^{C}	32 ± 6^{D}
Change in total TAG content	−3 ± 8	+8 ± 6	+7 ± 8	+35 ± 6	−10 ± 9	+3 ± 9	+7 ± 5	+36 ± 4
De novo TAG synthesis	16 ± 6	24 ± 6	19 ± 7	42 ± 5	41 ± 10^{A}	43 ± 8^{A}	42 ± 5^{B}	68 ± 4^{B}

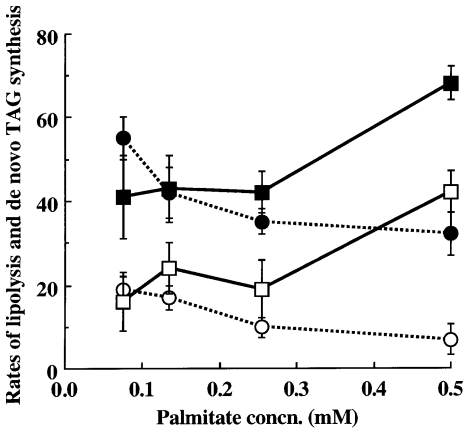


Figure 2 Effects of adrenaline and palmitate concentration on rates of glycerol release and *de novo* TAG synthesis

The values are from Table 2. .... Glycerol release [○, basal; ●, with adrenaline (5 μM)]; ——, TAG synthesis [□, basal; ■, with adrenaline (5 μM)].

rates of *de novo* synthesis of TAG in the basal or adrenaline-stimulated states and that these reasonably matched rates of TAG mobilization as measured by glycerol release (Figure 2). Clearly, in the presence of adrenaline, calculated rates of *de novo* synthesis of TAG at 0.08 or 0.14 mM palmitate greatly exceeded rates of incorporation of [^{14}C]palmitate, leading to the conclusion that there was considerable capability to recycle previously mobilized ^{3}H-labelled fatty acid back to TAG under these conditions.

Significant stimulation of conversion of [^{14}C]palmitate into phospholipids by adrenaline was seen over a 6-fold range of palmitate concentrations (Table 2) although the magnitude of this effect was greater at the higher fatty acid concentrations (+63 ± 8% at 0.5 mM compared with +37 ± 6% at 0.08 mM). We do not know to what extent the incorporation of [^{14}C]palmitate into phospholipids represents *de novo* synthesis compared with contribution to a cycle of partial deacylation and reacylation. However, we suggest that the latter may be small under our conditions because in cells 'pulsed' with [^{3}H]palmitate there was no significant subsequent loss of ^{3}H from phospholipids over a 1 h 'chase' with 0.08, 0.14 or 0.26 mM [^{14}C]palmitate with or without adrenaline (5 μM) present ($n = 5$, results not shown).

The effects of isoprenaline (β-adrenergic agonist) were tested (Tables 3 and 4). Although all tested concentrations of isoprenaline substantially increased glycerol release, there was no effect of this agonist on *de novo* TAG synthesis except at 10 μM. Isoprenaline had no effect whatever on conversion of [^{14}C]palmitate into phospholipids. Phenylephrine had no effect on *de novo* TAG synthesis but generally caused significant stimulation of conversion of [^{14}C]palmitate into phospholipids. Overall we conclude that the effect of adrenaline to stimulate *de novo* TAG synthesis cannot be satisfactorily mimicked by either of these

Table 3 Effects of isoprenaline

Myocytes that had been isolated in the presence of 0.5 mM palmitate were incubated for 1 h in the presence of 5 mM glucose and 0.5 mM [^{14}C]palmitate. Values are expressed as μmol of fatty acid equivalents/h per g dry weight and are means ± S.E.M. ($n = 4$ throughout). ^{A,B,C,D} indicate $P < 0.05$, < 0.025, < 0.01, < 0.005 respectively for effects of adrenaline or isoprenaline.

		Adrenaline	Isoprenaline			
	No agonist	5 μM	0.3 μM	1 μM	3 μM	10 μM
Fate of exogenous [^{14}C]palmitate						
TAG	58 ± 6	74 ± 2^{B}	50 ± 3	53 ± 3	51 ± 3	55 ± 4
Phospholipid	2.0 ± 0.3	3.6 ± 0.2^{C}	1.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.4	2.2 ± 0.2
Glycerol release	9 ± 3	52 ± 13^{C}	35 ± 6^{D}	39 ± 9^{C}	46 ± 9^{D}	47 ± 10^{C}
Increase in TAG content	27 ± 6	31 ± 11	9 ± 9	14 ± 5	5 ± 6	20 ± 5
De novo TAG synthesis	36 ± 7	83 ± 10^{B}	44 ± 14	53 ± 9	52 ± 12	67 ± 13^{A}

Table 4 Effects of phenylephrine

Myocytes that had been isolated in the presence of 0.5 mM palmitate were incubated for 1 h in the presence of 5 mM glucose and 0.5 mM [^{14}C]palmitate. Values are expressed as μmol of fatty acid equivalents/h per g dry weight and are means ± S.E.M. ($n = 5$ throughout). ^{A,B,C,D} indicate $P < 0.05$, < 0.025, < 0.01, < 0.005 respectively for effects of adrenaline or phenylephrine.

	No agonist	Adrenaline	Phenylephrine			
		5 μM	0.3 μM	1 μM	3 μM	10 μM
Fate of exogenous [^{14}C]palmitate						
TAG	44 ± 4	47 ± 1	44 ± 34	47 ± 4	49 ± 4^{D}	51 ± 4^{D}
Phospholipid	1.8 ± 0.2	2.5 ± 0.1^{D}	2.5 ± 0.4^{A}	2.2 ± 0.3	2.2 ± 0.2^{A}	2.6 ± 0.2^{C}
Glycerol release	2.0 ± 0.9	26 ± 5^{D}	3.0 ± 1.6	7.3 ± 2.4^{A}	12 ± 3^{B}	12 ± 3^{C}
Increase in TAG content	24 ± 8	17 ± 8	15 ± 12	20 ± 11	22 ± 9	26 ± 9
De novo TAG synthesis	26 ± 8	45 ± 8^{A}	21 ± 12	27 ± 12	33 ± 10	38 ± 8

Table 5 Effect of adrenaline on activities of enzymes of TAG synthesis and contents of glycerol 3-phosphate

Myocytes were incubated for 1 h in the presence of 5 mM glucose and 0.5 mM palmitate followed by measurements of glycerol 3-phosphate in incubations or freeze-stopping of cells, homogenization and enzyme assays as described in the Materials and methods section. Enzyme activities are expressed as (nmol/min per unit of lactate dehydrogenase activity) × 100. Glycerol 3-phosphate contents are shown as μmol/g dry weight of cells. ^{A} indicates $P < 0.025$ for the effect of adrenaline.

	n	Control	Adrenaline (5 μM)
Glycerol 3-phosphate content	8	1.3 ± 0.1	1.6 ± 0.2
Enzyme activity			
Microsomal GPAT	4	12.9 ± 1.9	18.1 ± 3.3^{A}
Mitochondrial GPAT	4	6.9 ± 0.7	7.4 ± 0.6
PAP1	5	27.8 ± 4.9	30.1 ± 4.0
PAP2	5	24.1 ± 1.5	28.2 ± 5.9
DGAT	5	3.2 ± 0.4	2.7 ± 0.5

agonists, suggesting that α- and β-mediated pathways together are necessary to achieve the complete effect. By contrast, the effect of adrenaline to stimulate phospholipid synthesis appears to be predominantly mediated through an α-adrenergic pathway. In this respect it is noteworthy that phenylephrine is a stimulator of hypertrophy in cardiac myocytes [37], a condition in which increased *de novo* synthesis of phospholipids would be expected. Phenylephrine also had a small but significant effect on glycerol release (Table 4). We do not know whether this is due to some activity of the agonist at β-receptors or whether it is possible in myocytes to activate this process through an α-adrenergic pathway. We are unaware of any previous studies of effects of phenylephrine on cardiac lipolysis.

Since adrenaline stimulated the pathway of *de novo* TAG synthesis in the presence of a wide range of fatty acid concentrations, it seemed likely that this must be due either to an increase in glycerol 3-phosphate content or to activation of one or more of the enzymes of the pathway. In various experiments ($n = 4$, results not shown) adrenaline caused the following changes in glycerol 3-phosphate content: with 0.08 mM palmitate, 0 % change; with 0.14 mM palmitate, −6 %; with 0.26 mM palmitate, −6 %; variation in palmitate concentration had no effect on glycerol 3-phosphate content. Furthermore Table 5 shows that adrenaline had no significant effect on glycerol 3-phosphate content at 0.5 mM palmitate. For various reasons GPAT, PAP and DGAT activities have been implicated in acute regulation of TAG synthesis in adipose tissue or liver. Adipocyte GPAT is inactivated through an adrenergic mechanism [29,38], possibly involving an unidentified cAMP-independent protein kinase [39], and is activated by insulin [40,41]. Two forms of PAP are now recognized [42]. PAP1 (Mg^{2+}-activated, *N*-ethylmaleimide-inhibited PAP) is thought to be involved in TAG synthesis [43], whereas PAP2 is thought to be primarily involved in signal transduction. PAP1 is translocated between the cytosol and the endoplasmic reticulum in liver and adipocytes [32,43,44], and it is argued that translocation to the endoplasmic reticulum will enhance TAG synthesis [43]. Also, PAP1 in adipocytes is inactivated through an adrenergic mechanism [38]. DGAT activity in adipocytes [38] and hepatocytes [45] is inactivated by catecholamines and glucagon respectively, and there is evidence for regulation of DGAT in these tissues by a cAMP-independent protein phosphorylation mechanism [46,47]. By contrast, in fibroblasts where TAG synthesis is increased by dibutyryl cAMP, DGAT activity is also increased [48]. Table 5 shows that adrenaline had no acute effects on PAP1, PAP2 or DGAT that could be detected after freeze-stopping of myocytes and rapid assay. Nor could any evidence be obtained for translocation of PAP1 to membrane fractions in response to adrenaline; in fact, adrenaline significantly ($P < 0.05$) decreased the ratio of membrane-bound/soluble PAP activity from 0.51 to 0.38 (results not shown). We have shown [49] that, like liver and adipose tissue, cardiac myocytes contain two forms of GPAT. There is a small proportion that is mitochondrial and the majority, which is enriched in the sarcoplasmic reticulum fraction from heart extracts, has properties similar to that found in microsomal fractions from other tissues. When GPAT activities were assayed using 1 mM glycerol 3-phosphate as substrate (this is very close to the measured content in the cells), this microsomal/sarcoplasmic reticulum GPAT activity was found to be significantly increased by 40 % with adrenaline whereas mitochondrial GPAT activity was unchanged (Table 5). This finding is at variance with Heathers et al. [50], who observed a decrease in microsomal GPAT after perfusion of hearts with adrenaline. However, the experimental conditions of Heathers et al. [50] differed from ours in significant respects in that there was no fatty acid present in their perfusate (net catabolism of TAG would therefore be expected), hearts were not freeze-stopped and GPAT assays were performed with a much higher than physiological concentration of glycerol 3-phosphate.

TAG turnover

A number of previous studies have measured either glycerol release or loss of prelabelled TAG as an index of myocardial

lipolysis. However, Table 1 shows that these measurements did not correlate with each other in that the ratio (glycerol release/loss of [^{3}H]TAG) was significantly less than 1.0 under basal conditions and significantly greater than 1.0 in the presence of adrenaline. This raised the possibility that glycerol release and loss of ^{3}H-labelled fatty acid from the TAG pool are measurements of distinct events and also raised the possibility that one or other of these measurements is in error. In order to calculate molar rates of loss of [^{3}H]TAG shown in Table 1, we assumed that the specific radioactive of TAG was d.p.m. in TAG/TAG content at the start of the 'chase' period. As discussed below, this assumption is open to question. Glycerol is not reutilized to any significant extent by the myocardium. This is because of the absence [51] or very low activity [52] of glycerokinase and is supported by studies demonstrating minimal incorporation of [^{14}C]glycerol into glycerolipids in perfused heart [53] or cardiac myocytes [54]. Although de Groot et al. [55] have criticized the validity of glycerol release as a measure of cardiac lipolysis on the grounds that sonicated heart homogenates can catalyse the dephosphorylation of glycerol 3-phosphate, the experimental conditions used by de Groot et al. [55] would have disrupted lysosomes and are not relevant to whole-cell conditions. In any case, other studies with perfused hearts [56] or isolated myocytes [36] have clearly shown that [^{14}C]glucose is not converted into free glycerol to any extent in the short term. In principle, breakdown of phospholipids could also contribute to glycerol production. This has been demonstrated to be the case for myocytes deprived of both oxygen and endogenous substrates [57]. However, under less extreme, normoxic, conditions rates of phospholipid turnover are very small compared with those of TAG lipolysis in perfused hearts [16,56,58,59] or isolated myocytes [60] and, as discussed above, we also observed minimal loss of [^{3}H]palmitate from myocyte phospholipids in the presence or absence of adrenaline. Considering all of these factors we have confidence in glycerol production as a measure of the complete mobilization of TAG (as opposed to partial degradation to DAG or MAG).

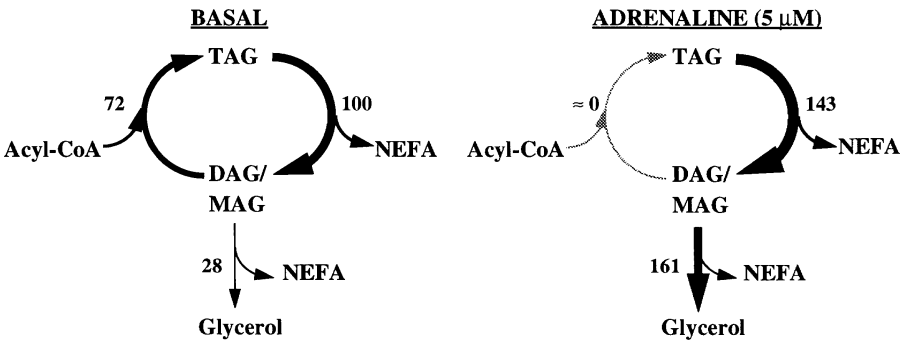
In principle the following relationship should hold:

$$\Delta\text{TAG} = [^{14}\text{C}]\text{TAG formed} - \text{loss of } [^3\text{H}]\text{TAG} \quad (2)$$

provided that partial acylglycerols (DAG or MAG) do not accumulate. As shown in Figure 1d there was no accumulation of these species during the 'chase' period under basal conditions nor did adrenaline cause any accumulation of the same (not shown). The validity of eqn. (2) would not be affected by partial lipolysis of TAG to DAG (or MAG) followed by reconversion back to TAG. For example, if ^{3}H-labelled fatty acid was lost by partial lipolysis and then replaced by ^{14}C-labelled fatty acid these events would cancel each other out in eqn. (2). Likewise replacement of ^{3}H-labelled fatty acid by ^{3}H-labelled fatty acid during partial lipolysis/resynthesis would not show up in eqn. (2). As shown in Table 1 the values of ΔTAG obtained by direct measurement were significantly less than those calculated by eqn. (2) (27 ± 4 compared with 38 ± 3 in the basal state and 26 ± 4 compared with 42 ± 3 with adrenaline). Since ΔTAG and [^{14}C]TAG are straightforward measurements, we conclude that the measurement of loss of [^{3}H]TAG is in error (see above). The measured and calculated values for ΔTAG were brought into agreement by application of 'correction factors' to the assumed specific radioactivity of [^{3}H]TAG. In the basal case this needed to be multiplied by 0.47 and by 0.53 with adrenaline. The implication is that lipolysis during the 'chase' period preferentially hydrolyses 'older' TAG rather than TAG that was newly formed and labelled during the 'pulse' period. Previous studies have suggested that there is metabolic heterogeneity of TAG in the myocardium [35,58], and Stein and Stein [60] pulse-labelled perfused hearts with [^{3}H]oleate and showed changes in the subcellular localization of [^{3}H]TAG with time. Also Saddik and Lopaschuk [18] noted that, when perfused hearts were 'pulsed' with 1.2 mM [^{14}C]palmitate and then 'chased' with 0.4 mM palmitate, a greater proportion of unlabelled rather than labelled fatty acids was lost from TAG during the 'chase' (although this was not the case if fatty acids were omitted from the 'chase' [18]).

In the basal state, even without application of the specific radioactivity 'correction factor', it is clear from the ratio glycerol release/loss of [^{3}H]TAG that there must be some loss of [^{3}H]palmitate that is not associated with complete degradation of [^{3}H]TAG to glycerol. With application of the 'correction factor', loss of [^{3}H]TAG becomes 21.9 μmol of fatty acid equivalents/h per g dry weight (10.3/0.47) meaning that $21.9 - 6.2 = 15.7$ μmol of fatty acid equivalents/h per g dry weight must be recycled back to TAG when DAG or MAG products of partial lipolysis (which do not themselves accumulate) are re-esterified. The extent to which this partial lipolysis/resynthesis is simply a TAG to DAG to TAG cycle or involves conversion of TAG to MAG followed by resynthesis of TAG is uncertain. The former involves only DGAT for the resynthesis arm and this activity is clearly measurable in cardiac myocytes (see [49] and Table 5). The latter also involves MGAT. Tamboli et al. [54] have shown that the 1-hexadecyl glycerol ether analogue of MAG is not esterified by myocytes or perfused heart, a finding that might suggest the absence of MGAT activity. However, although good substrates for the intestinal form of MGAT [61], 1- or 2-alkylglycerols are very poor substrates for the form of MGAT that is expressed in liver from suckling rats [61–63] and which is very specific for 2-acylglycerol substrates [61]. Soluble fraction from whole heart contains a small amount of a CoA-independent MGAT activity [64], and whole homogenate of heart from 11–14 day-old rats has a small amount of MGAT activity assayed with a 2-acylglycerol substrate [62]. Whether MGAT is expressed in cardiac myocytes to a small extent and whether it is of the intestinal or hepatic type remains to be established. Therefore, at present, recycling of MAG back to TAG as well as of DAG to TAG has to be considered as a possibility. Scheme 1 shows that 72% of DAG/MAG generated from TAG is recycled back to TAG in the basal state. We also noted that conversion of [^{14}C]palmitate into TAG in the basal state (49 ± 3 μmol of fatty acid equivalents/h per g dry weight) was significantly greater, than the value for *de novo* synthesis of TAG (36 ± 4 μmol of fatty acid equivalents/h per g dry weight). This implies that approx. 13 μmol of fatty acid equivalents/h per g dry weight must be being used in the reacylation of partial acylglycerols and provides further support for the operation of a deacylation/reacylation cycle.

With application of the specific radioactivity 'correction factor,' loss of [^{3}H]TAG when adrenaline was present becomes 31.3 μmol of fatty acid equivalents/h per g dry weight (16.6/0.53), which is quite comparable with the value of 36 μmol of fatty acid equivalents/h per g dry weight for glycerol release (the ratio glycerol release/loss of [^{3}H]TAG now becomes close to 1.0). Therefore, with adrenaline present, TAG lipolysis to DAG/MAG is accelerated by approx. 40% and essentially all DAG/MAG is degraded rather than being recycled back to TAG (Scheme 1). The high value of the ratio glycerol release/loss of [^{3}H]TAG with adrenaline might at first sight be interpreted in terms of extensive recycling of mobilized ^{3}H-labelled fatty acid back to TAG. However, this cannot be so with 0.5 mM [^{14}C]palmitate present (although it is quite feasible with lower concentrations of [^{14}C]fatty acid) for two reasons. First, with adrenaline present, *de novo* synthesis of TAG (65 ± 5 μmol of fatty acid equivalents/h per g dry weight) was only marginally and non-



Scheme 1 Effect of adrenaline on dynamics of TAG turnover

A value of 100 is taken as the basal rate of loss of [^{3}H]TAG (after application of the appropriate 'correction factor') and all other values are expressed relative to this. NEFA, non-esterified fatty acids.

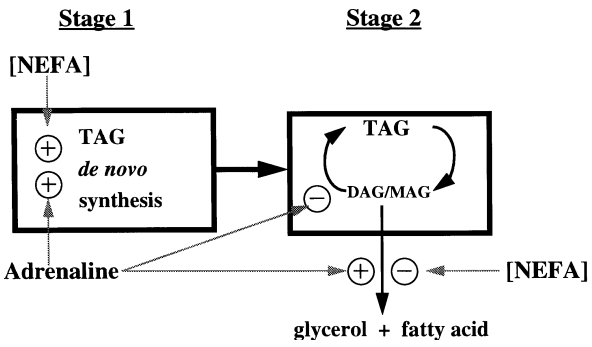
significantly greater than the conversion of [^{14}C]palmitate into TAG (58 ± 3 μmol of fatty acid equivalents/h per dry weight). Second, in this experiment cells were incubated with 1250 nmol of [^{14}C]palmitate. With approx. 1.5 mg dry weight of cells present the maximum contribution of mobilized [^{3}H]palmitate to the fatty acid pool could only have been 40–50 nmol (even after 'correction' of specific radioactivity).

As shown in Table 2, provision of exogenous palmitate caused a concentration-dependent inhibition of glycerol release. This was seen to a lesser extent when adrenaline was present (raising palmitate from 0.08 to 0.5 mM decreased glycerol release by 64 and 42 % in the absence and presence of adrenaline respectively). Broadly similar effects were reported by Larsen and Severson [17] using TAG-loaded myocytes incubated with oleate and isoprenaline. This would seem to be a fundamentally important mechanism which enables the myocyte to adjust its mobilization of endogenous fatty acids very sensitively in accord with exogenous availability. The same phenomenon is not seen with adipoyctes, which will continue lipolysis at a steady rate until exogenous fatty acids begin to reach a 3:1 molar ratio with incubation medium albumin (E. D. Saggerson, unpublished work). It has been proposed that there is direct feedback inhibition of myocyte lipase(s) by non-esterified fatty acid [2,65]. However, a free concentration of 100 μM oleate was required to cause 50 % inhibition of lipase activity in 100000 ***g*** supernatant from rat heart whereas 25 μM non-esterified oleate had minimal effect [66]. By contrast, it can be calculated that we observed extensive inhibition of myocyte lipolysis at concentrations of non-esterified palmitate that never exceeded 5 μM. Furthermore there is unlikely to be appreciable unbound non-esterified fatty acid in rat heart, since cytosolic fatty acid-binding protein content is reported to be 49 nmol/g wet weight [67] and fatty acid content is 15–45 nmol/g wet weight depending on the type of extracellular substrate supplied [3,11,68]. We therefore dispute previous interpretations of this phenomenon and suggest that inhibition of cardiac lipas(s) might involve indirect effects. For example, the AMP-activated protein kinase is expressed to a high level in heart [69]. This enzyme can deactivate hormone-sensitive lipase [70] and is itself activated by a separate protein kinase (AMP-activated protein kinase kinase), which itself can be activated by submicromolar concentrations of fatty acyl-CoA [71,72]. Furthermore, conversion of non-esterified fatty acid in to fatty acyl-CoA also involves the generation of AMP from ATP. We therefore speculate on a possible involvement of the AMP-activated protein kinase in this phenomenon, which merits further investigation.

General discussion

Previous studies have shown that a cycle of TAG synthesis/lipolysis occurs in the myocardium [65,73]. This cycling is increased in the initial phase of ischaemia [10,11] or when hearts are perfused with elevated concentrations of lactate [3,74]. Increased TAG synthesis in these cases would be expected because glycerol 3-phosphate content will be raised. Here we find that adrenaline simultaneously enhances both sides of this cycle in a manner that involves no change in glycerol 3-phosphate content (Table 5) and which is essentially independent of fatty acid concentration (Table 2). This suggested that the intrinsic activity of one or more enzymes of the pathway of *de novo* TAG synthesis must be increased, e.g. GPAT (Table 5). Other previous studies have suggested that fatty acids entering the myocardium are preferentially converted into TAG and are then mobilized by intracellular lipolysis before oxidation [75,76]. If so, increased *de novo* synthesis of TAG is a likely prelude to the increased oxidation of fatty acid that occurs on adrenergic stimulation. We now suggest (Scheme 2) that this initial *de novo* synthesis of TAG ('stage') and its subsequent turnover ('stage 2') need to be spatially separated within the myocyte and that there must be two pools of DGAT activity. For *de novo* synthesis of TAG to occur when adrenaline is present (or absent), DGAT at 'stage 1' must be active. By contrast, Scheme 1 indicates that the DGAT pool involved in recycling of DAG back to TAG ('stage 2') must be inactivated when adrenaline is present. If this is the case, the pool of DGAT used at 'stage 1' must actually be activated by adrenaline because total cell DGAT activity is not significantly changed by adrenaline (Table 5). Arguments about the specific radioactivity of [^{3}H]TAG (see above) would also favour the notion that there is some degree of separation between newly synthesized TAG and the turnover pool of TAG.

We suggest that the quite extensive cycling between TAG and DAG/MAG with no net release of fatty acid represents an 'anticipatory poise' which could facilitate a rapid turn-on of net mobilization of TAG on demand, e.g. on adrenergic stimulation. How many lipases participate in these events is unclear. Myocardial cells contain TAG lipase(s) with acid pH optima [77,78] and at least two with neutral optima which appear to be lipoprotein lipase [79] and hormone-sensitive lipase [80–85].



Scheme 2 Depiction of two hypothetical stages of TAG metabolism in cardiac myocytes

It is suggested that stage 1 constitutes the *de novo* pathway of TAG synthesis. This is increased with increasing concentration of fatty acid (NEFA) and by adrenaline. State 2 involves the turnover of TAG which may have a lower specific radioactivity than the TAG synthesized immediately before stage 1 during a 'pulse' incubation. Adrenaline inhibits DAG/MAG to TAG recycling at stage 2 and stimulates net mobilization of TAG to glycerol + fatty acid. Increasing concentrations of NEFA inhibit net mobilization.

Conversion of TAG into DAG/MAG was only increased by adrenaline by 43% (Scheme 1), suggesting that most of this conversion could be performed by a constitutive TAG lipase. By contrast conversion of DAG/MAG into glycerol was increased nearly 6-fold by adrenaline (Scheme 1). We suggest that hormone-sensitive lipase, which shows a 10-fold higher rate of lipolysis of DAG than of TAG [86], could perform most of the DAG lipolysis together with a small proportion of the TAG into DAG conversion. Further studies are needed to substantiate these suggestions.

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