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THE EFFECT OF ADRENALINE ON SYNTHESIS OF PHOSPHATIDYLCHOLINE IN FAT CELLS

JANET M. STEIN
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, England

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Abstract—(1) Isolated fat cells from rat epididymal fat pad when incubated *in vitro* in medium containing $^{32}P_i$, showed a time-dependent increase in specific radioactivity of microsomal phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol; incubation of fat cells in the presence of adrenaline resulted in an increased specific radioactivity only of phosphatidylcholine compared with phospholipids in incubated control cells. (2) Investigation of the incorporation of $^{32}P_i$ into the water-soluble precursors of phosphatidylcholine showed that the increase in radioactivity of phosphorylcholine was time-dependent, and was similar in both control and adrenaline-stimulated fat cells. Incorporation of $^{32}P_i$ into CDP-choline was less than into phosphorylcholine, and was markedly increased in fat cells incubated with adrenaline when compared with controls. (3) The concentration of phosphorylcholine in fat cells has been determined. (4) The fractional turnover rate of phosphatidylcholine has been calculated, and was found to be increased in adrenaline-stimulated cells when compared with incubated controls.

When isolated fat cells are incubated with adrenaline there is increased incorporation of $^{32}P_i$ into phospholipid when compared with incubated controls. This increase has been shown to be due to an increased specific radioactivity of phosphatidylcholine [1]. Subsequent work has been carried out with the aim of locating the point in the biosynthetic pathway of phosphatidylcholine at which stimulation caused by adrenaline occurs. Investigations of changes in ATP (the presumed phosphate donor in phospholipid synthesis) have shown that there is no increase in the specific radioactivity of ATP in fat cells incubated with adrenaline when compared with controls [2]; in addition, incubation with adrenaline results in a fall in fat cell ATP concentration [3, 4].

Clearly the stimulation of incorporation of $^{32}P_i$ into phosphatidylcholine caused by adrenaline is not related to any increase in ATP specific radioactivity or concentration. This suggests that the action of adrenaline on phosphatidylcholine labelling may be exerted at some point in the pathway of phospholipid synthesis distal to ATP, and the present study was undertaken to investigate this.

Preliminary studies were carried out in which plasma membranes were isolated from fat cells incubated with $^{32}P_i$. There was little incorporation of radioactivity into the plasma membrane phospholipids; studies with microsomal markers showed that this could be totally accounted for by contamination of the plasma membrane by the more highly labelled phospholipids of the microsomal fraction. Accordingly, microsomal preparations were used for this work.

MATERIALS AND METHODS

Adrenaline (Hopkin & Williams Ltd.) was made up in 0.1 M ascorbic acid. Collagenase was from Worthington Biochemical Corp., Freehold, New Jersey, bovine serum albumin (fraction V) from Armour Pharmaceutical Co., Eastbourne, Sussex. U.K.

Phosphorylcholine, phosphorylethanolamine, CDP-choline and CDP-ethanolamine were from Sigma London, Radiosotopes were from the Radiochemical Centre, Amersham, Bucks, U.K.

Animals. Male Wistar rats (120–140 g) were used). They were maintained on a stock laboratory diet.

Preparation of fat-cells. Fat cells were prepared from rat epididymal fat pad by the method of Rodbell [5]. Glucose was not present in the medium during the preparation of the cells.

Incubation medium. Incubations were carried out in Krebs–Ringer bicarbonate buffer (1.3 mM-Ca^{2+}), pH 7.4 [6] containing 4% w/v bovine serum albumin. $^{32}P_i$ was added to the medium just before the start of each incubation.

Microsomal preparations. Fat cell microsomal preparations were obtained by a method described by McKeel and Jarett [7]. The yield was 25–40% of the initial fat cell homogenate, and was estimated by assaying the recovery and specific activity of NADH-cytochrome *c* reductase [8], and the recovery of RNA [9, 10]. Assays of a plasma membrane marker enzyme, 5'-nucleotidase [11] showed that the microsomal fraction contained 15–20% of the total homogenate activity.

Incorporation of $^{32}P_i$ into microsomal phospholipids. Samples of fat cells (4.5 ml), and approx. 200 mg dry wt cells/ml, were incubated at 37°, with shaking, in medium containing $^{32}P_i$, in polypropylene tubes. At the end of each incubation the fat cells were washed 4 times with 4 vol. 10 mM Tris pH 7.4 containing 0.25 M sucrose, and mM EDTA, the cells homogenised, centrifuged at 16,000 *g* and the resulting supernatant centrifuged at 160,000 *g* to give the microsomal fraction [7]. The microsomal pellet was dispersed in 0.5 ml NaCl (0.9%) and the lipids extracted overnight at 4° in 20 vol. chloroform–methanol (2:1 v/v) [12] 0.9% NaCl (0.2 vol.) was added to separate two

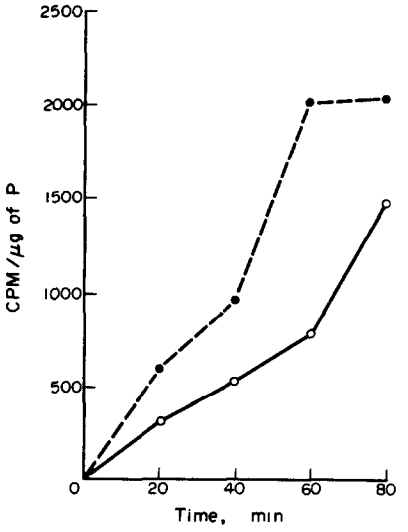

Fig. 1. Time course of incorporation of $^{32}P_i$ into fat cell microsomal phospholipid. Incubation medium (see Materials and Methods section) contained $^{32}P_i$ (10 μCi/ml). ——, control cells; -----, cells incubated with adrenaline (5.4×10^{-6} M). Each point represents microsomal phospholipid from 400 mg dry wt. of fat cells.

Table 1. Phospholipid composition of (a) fat cell microsomes, (b) liver microsomes, (c) whole fat cells

	(a)	(b)	(c)
Phosphatidylcholine + lysophosphatidylcholine + choline plasmalogen	62.8 ± 3.0	60.7	53.0
Phosphatidylethanolamine + ethanolamine plasmalogen	17.8 ± 0.9	25.5	17.4
Phosphatidylserine	6.6 ± 2.0	—	5.4
Phosphatidylinositol	7.4 ± 0.5	8.2	6.5
Sphingomyelin	4.0 ± 0.9	4.0	7.4
Phosphatidic acid	—*	1.6	2.0
Cardiolipin	—*	—	5.8
Phosphatidylglycerol	ND	ND	1.9
Glycerol ethers	ND	ND	2.8

(a) Present work. Figures given as phosphorus expressed as % total recovered phospholipid phosphorus, and are means ± S.E.M. of TLC analyses on three separate microsomal preparations.

(b) Analysed by TLC [20]. Value for phosphatidylethanolamine includes that for phosphatidylserine; value for phosphatidic acid includes value for cardiolipin.

(c) Analysed by alkaline hydrolysis method [1].

* Less than 1%.

N.D., not determined.

phases. The upper layer was discarded and the lower lipid-containing layer washed once with 0.5 vol. chloroform–methanol–10 mM KH_2PO_4 (3:48:47 by vol.), and three times with 0.5 vol. chloroform–methanol–0.9% NaCl (3:48:47 by vol. [13].

Total phospholipid phosphorus and radioactivity of the microsomal lipids was determined as previously described [1]. The specific radioactivities of individual microsomal phospholipids were determined after separating the phospholipids by two-dimensional thin layer chromatography [14]. The phospholipids were visualised with I_2 vapour, scraped into test-tubes, and digested at 180° for 30 min, with 0.2 ml conc. H_2SO_4–72% PCA (10:1 v/v) [15]. The samples were diluted with H_2O to total vol. 3 ml, radioactivity measured by Cerenčov counting, and phosphorus determined by the method of Bartlett [16].

Incorporation of $^{32}P_i$ into phosphoryl- and CDP-derivatives of choline and ethanolamine. Fat cells (1 ml), and approx 200 mg dry wt./ml, were incubated in polypropylene vials in medium containing $^{32}P_i$. TCA (final conc. 5% w/v) was added to terminate each incubation, the extracts kept at 4° for 30 min, and then centrifuged.

The intermediates of phospholipid synthesis were isolated by a modification of the method of Broad and Dawson [17]. The supernatants were extracted 3 times with 5 vol. of water-saturated ether, and stored

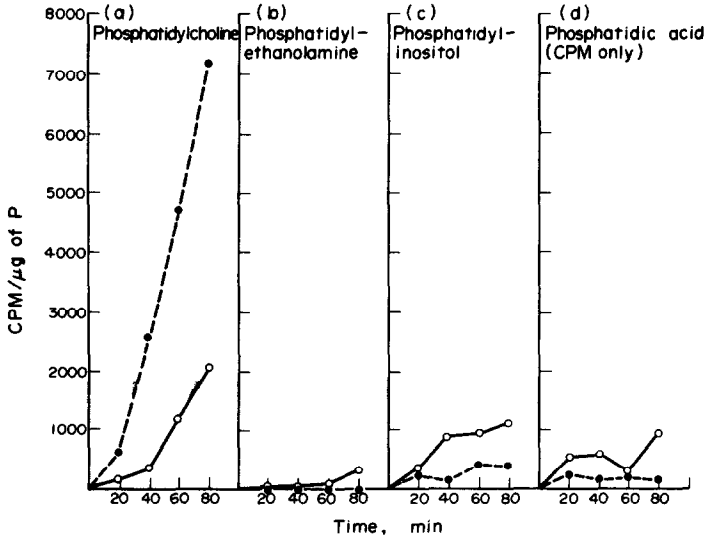

Fig. 2. Incorporation of $^{32}P_i$ into phospholipids of fat cell microsomes. ——, control cells; -----, cells incubated with adrenaline (5.4×10^{-6} M). Incubation medium (see Materials and Methods) contained $^{32}P_i$ 15 μCi/ml. Weight cells was 162 mg/ml.

at 4°, 50 μl of each extract was applied to Whatman No. 1 chromatography paper, and electrophoresis carried out for 3 hr at 55 V/cm at pH 3.5. Two spots corresponding to the phosphoryl- and CDP-derivatives of choline together with phosphoryl- and CDP-derivatives of ethanolamine, were located by the use of non-radioactive markers of these compounds. Each spot was cut out, attached to a fresh sheet of chromatography paper, and chromatographed in water-saturated phenol–acetic acid–ethanol (50:5:6 by vol.). This separated choline derivatives (R_f 0.95) from ethanolamine derivatives (R_f 0.49). The areas containing the spots were cut out, scintillation mixture added (PPO, 3.5 g; POPOP, 50 mg; in toluene (1 l.) with Triton X-100 (500 ml) containing 10% water), and radioactivity counted.

Measurement of pool size of phosphorylcholine. This was carried out by an isotope dilution method [18]. Phosphoryl[methyl-^{14}C]choline was added to a suspension of fat cells, which was deproteinised with TCA, final conc. 5%, and ether-extracted as in the previous section. The extract was lyophilised, dissolved in water and phosphorylcholine separated on Whatman No. 1 chromatography paper as described above. The phosphorylcholine spot was eluted with 0.04 M formic acid, evaporated, and redissolved in water. Samples were taken for estimation of phosphorus [16] and for radioactive counting, with an appropriate correction for quenching.

Protein was estimated in the initial fat cell sample by the method of Lowry *et al.* [19].

RESULTS

Fat cells were incubated *in vitro* in glucose-free medium containing $^{32}P_i$ in the presence or absence of adrenaline. Each fat cell sample was homogenised, the microsomal fraction prepared, and the specific radioactivity of microsomal total phospholipids determined. (Fig. 1).

There was a time-dependent increase in specific radioactivity of the microsomal phospholipids; the specific radioactivity of microsomal phospholipids from fat cells incubated with adrenaline was increased above that of microsomal phospholipids of corresponding controls at each time point. The magnitude of this increased specific radioactivity due to adrenaline was similar to that reported in phospholipids from whole fat cells incubated in presence and absence of adrenaline [1].

The composition of the phospholipids of the microsomal fraction from fat cells is given in Table 1, together with a TLC analysis of rat liver microsomal phospholipids [20] and an analysis of whole fat cell phospholipids [1]. The composition of fat cell microsomal phospholipids was similar to liver microsomal phospholipids. The proportion of phosphatidylcholine in fat cell microsomes was higher than that in whole fat cells, and the proportion of sphingomyelin was lower. Cardiolipin, normally found only in mitochondria, was absent from the microsomes.

Figure 2 depicts a time course experiment in which fat cells were incubated for varying times in the presence and absence of adrenaline. At each time point, a portion of the fat cells was homogenised, the microsomal pellet prepared, and the specific radioactivities of the phospholipids determined. The specific radioactivities of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in microsomes from control cells all increased with time. In microsomes from fat cells incubated with adrenaline there was a marked increase in the specific radioactivity of phosphatidylcholine (Fig. 2a), whereas the specific radioactivities of phosphatidylethanolamine and phosphatidylinositol were reduced (Fig. 2b, c). Incorporation of $^{32}P_i$ into phosphatidic acid is presented as total counts incorporated, since the amount of phosphorus present was too small to be measured accurately; incorporation of $^{32}P_i$ into phosphatidic acid in cells incubated with adrenaline was lower than in incubated controls. There was negligible incorporation of $^{32}P_i$ into sphingomyelin and phosphatidylserine. Phosphatidylcholine was thus the only phospholipid to show an increased specific radioactivity in the fat cells incubated with adrenaline, and is moreover the major component of the fat cell microsomal phospholipids (Table 1).

The influence of adrenaline on incorporation of $^{32}P_i$ into the water-soluble precursors of phosphatidylcholine was next examined; this was compared with incorporation of $^{32}P_i$ into the precursors of phosphatidylethanolamine, a phospholipid which shows a decrease in specific radioactivity in adrenaline-stimulated cells. Figure 3 shows a time course experiment in which incorporation of $^{32}P_i$ into phosphorylcholine, phosphorylethanolamine, CDP-choline, CDP-ethanolamine and total lipid was measured in fat cells incubated in the presence and absence of adrenaline. $^{32}P_i$ was incorporated into phosphorylcholine and phosphorylethanolamine in a time-dependent fashion, and there was no difference between cells incubated with or without adrenaline. $^{32}P_i$ incorporated into CDP-choline and CDP-ethanolamine was much lower than in their corresponding phosphoryl-derivatives. Incorporation of $^{32}P_i$ into CDP-choline was enhanced in fat cells incubated with adrenaline compared with incubated controls (Fig. 3b). There was no significant difference between the radioactivity of CDP-ethanolamine in cells incubated with adrenaline or in control cells. Incorporation of $^{32}P_i$ into total phospholipid was increased in the adrenaline-stimulated fat cells (Fig. 3e) and confirmed the hormonal sensitivity of the preparation.

The pool size of phosphorylcholine was determined in a fat cell preparation, containing 6.7 g dry wt cells and 77 mg total protein, to which was added 1 μCi (19.2 nmol) of phosphoryl[methyl-^{14}C]choline, sp. act. 1.14×10^8 dis/min per μmol. After isolation from the fat cell extract, the final specific activity was 1.7×10^6 dis/min per μmol. The phosphorylcholine content of the extract was calculated to be 16.3 μmol/g fat cell protein.

DISCUSSION

There is little information about pathways of phospholipid synthesis in adipose tissue. Experiments carried out in this laboratory to study incorporation of $^{32}P_i$ into fat cell phospholipids [1, 21] suggest that the pathways of phospholipid synthesis may resemble those described in liver [22], and that synthesis of phosphatidylcholine is therefore via phosphorylcholine and CDP-choline.

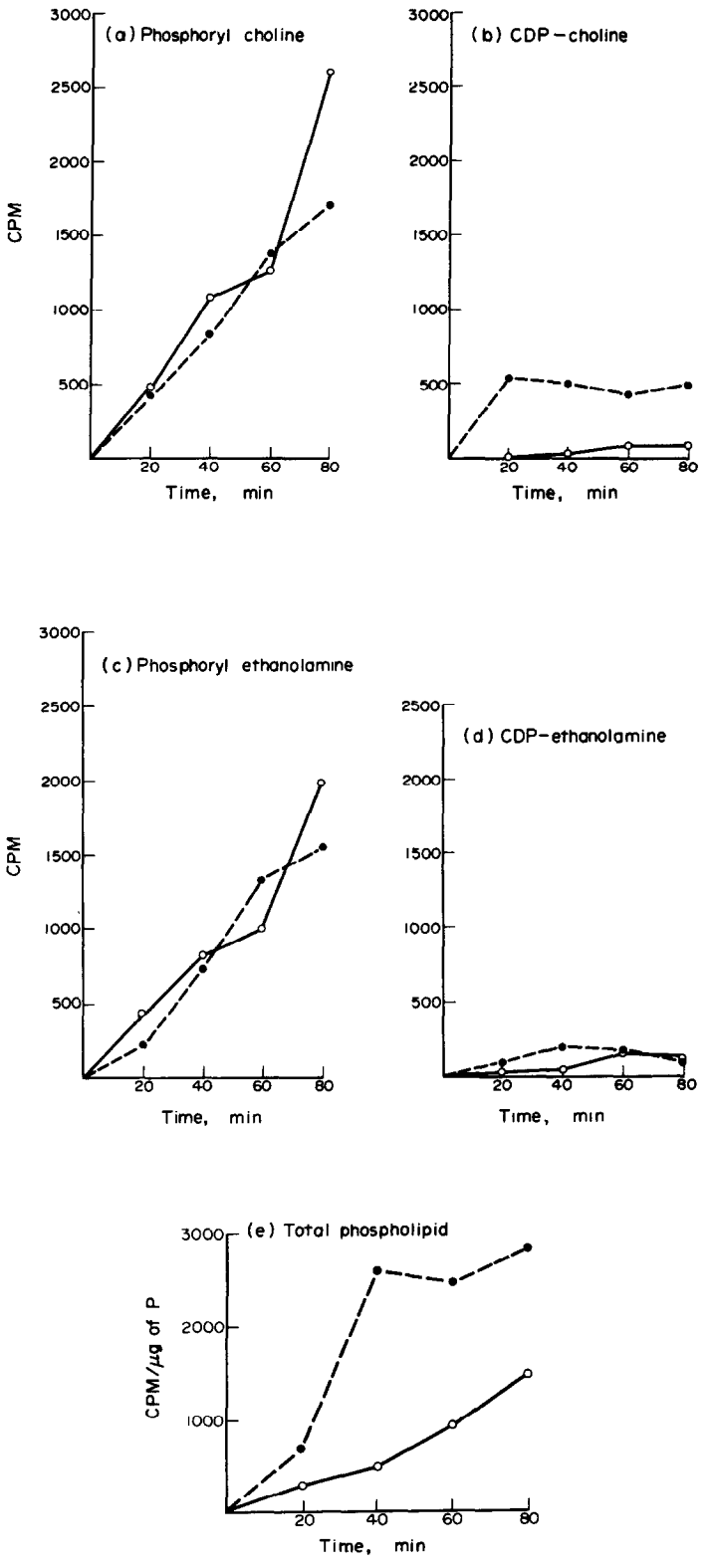

Fig. 3. Incorporation of $^{32}P_i$ into (a) fat cell phosphorylcholine; (b) CDP-choline; (c) phosphorylethanolamine; (d) CDP-ethanolamine; (e) Total phospholipid. Data of a, b, c and d represents CPM in 50 μl deproteinised extract. Specific radioactivity of total phospholipid (e) was determined as in Materials and Methods section. Specific radioactivity of medium was 27.7×10^6 counts/min per μatom P_i. ——, control cells; -----, cells incubated with adrenaline (5.4×10^{-5} M). Weight cells was 230 mg/ml.

Table 2. Fractional turnover rate of phosphatidylcholine calculated from data of Fig. 3

	Phosphorylcholine relative specific radioactivity	Phosphatidylcholine relative specific radioactivity	Fractional turnover rate of phosphatidylcholine (fraction of pool replaced/hr).
Control			
20 min	1.03×10^{-2}	0.46×10^{-3}	
60 min	2.69×10^{-2}	1.72×10^{-3}	0.19
Adrenaline			
20 min	0.88×10^{-2}	1.18×10^{-3}	
60 min	2.94×10^{-2}	4.32×10^{-3}	0.62

Values for relative specific radioactivities are expressed as counts/min per μatom P in compound ÷ counts/min per μatom P in incubation medium.

A study of the pattern of incorporation of $^{32}P_i$ into the water-soluble precursors of phosphatidylcholine should give information about the point(s) in the pathway which may be affected in fat cells stimulated with adrenaline. Ideally, such a study would include measurements of the specific radioactivities of all precursors of phosphatidylcholine both from fat cells incubated with adrenaline and from control cells so that turnover times could be calculated for each. In practice, not all of the intermediates could be measured accurately. Previous work has shown that the specific radioactivity of ATP in fat cells does not change in the presence of adrenaline [2]; the similarity of the time courses of incorporation of radioactivity into phosphorylcholine in fat cells incubated with and without adrenaline (Fig. 3a) is some confirmation of this, and also provides evidence that the activity of choline kinase (EC2.7.1.32) is not changed under these conditions.

The phosphorylcholine concentration of fat cells under control conditions (16.3 μmol/g protein) is similar to that of rat liver, when published values are recalculated on a basis of liver protein (14.5 μmol/g protein [23], 20.6 μmol/g protein, [24]. Attempts to measure the CDP-choline content of fat cells by an isotope dilution method were unsuccessful in this laboratory. However, several lines of indirect evidence suggest that, as in liver, the CDP-choline content of fat cells incubated under control conditions is about 3–5% that of phosphorylcholine, i.e. approx. 0.5–0.8 μmol CDP-choline/g fat cell protein. Fat cells incubated in [^{14}C]choline under control conditions for 1 hr showed a distribution of radioactivity of about 1:24 between CDP-choline and phosphorylcholine (Stein, unpublished observations) and a similar ratio is obtained in control fat cells incubated in $^{32}P_i$ (Fig. 3a, b). An estimate of the CDP-choline content of fat cells may also be made from the data of Fig. 3, if it is assumed that the specific radioactivity of CDP-choline at each time point is equal to that of the precursor, phosphorylcholine. A calculation carried out on this basis for control fat cells gives an average figure of 0.5 μmol CDP-choline/g fat cell protein, and agrees with the figure of 5 μmol/100 g liver, i.e. approx. 0.5 μmol/g protein determined by Wilgram *et al.* [25]. In contrast, a similar estimate of the CDP-choline content of adrenaline-stimulated fat cells gives a value that is increased above the control value and may indicate that the pool size of CDP-choline is increased as a result of adrenaline stimulation.

The fractional turnover rate of phosphatidylcholine in adrenaline-stimulated and control fat cells has been calculated, from the data of the experiment depicted in Fig. 3, using a procedure described by Pumphrey [26] and based on Rescigno and Segre [27]. The following assumptions have been made: (1) the system is at steady-state; (2) the precursor for the purpose of calculation is phosphorylcholine (since there was no direct measurement of the pool size of CDP-choline); (3) the pool size of phosphorylcholine in adrenaline-stimulated fat cells and control cells is the same, and relative specific radioactivities calculated on this basis; (4) the phosphatidylcholine content is calculated as 63% of the total phospholipid content (Table 1) and from this a figure for the relative specific radioactivity of phosphatidylcholine is derived.

From the calculated value of the fractional turnover rate of phosphatidylcholine in control fat cells, shown in Table 2, it follows that in adrenaline-stimulated fat cells the fractional turnover rate of phosphatidylcholine is increased 3-fold over that of control cells. The fat cells used in these experiments contain approximately 90 μmol phosphatidylcholine/g protein and synthesis of phosphatidylcholine may therefore be calculated to be approximately 17 μmol/g protein per hr in control fat cells and 55 μmol/g per hr in adrenaline-stimulated fat cells.

Some suggestions may be made about the mechanism of the increased synthesis of phosphatidylcholine in adrenaline-stimulated fat cells. The results show that there is increased incorporation of $^{32}P_i$ into CDP-choline in fat cells stimulated with adrenaline; this can be due to increased activity either of CTP-choline phosphate cytidyltransferase (EC2.7.7.15) or of choline phosphotransferase (EC2.7.8.2). In rat liver, activation of CTP-choline phosphate cytidyltransferase by lysolecithin [28] and of choline phosphotransferase by a fall in ATP concentration [29] have been shown. In fat cells during adrenaline-stimulated lipolysis there is a fall in ATP concentration which together with an increased production of diglycerides may favour activation of choline phosphotransferase.

What is the physiological significance of the increased turnover of phosphatidylcholine in rat cells incubated with adrenaline? In many tissues, adrenaline stimulation when mediated by the α-receptors causes increased turnover of phosphatidylinositol [30] and this effect also occurs in fat cells incubated with adrenaline in presence of a β-adrenergic blocking agent [1].

It is suggested that the increased turnover of phosphatidylcholine in fat cells may be related to their remarkable ability to respond to adrenaline by hydrolysis of triglyceride, and to increase export of fatty acids (20–40 times) across the plasma membrane in presence of adrenaline. Phosphatidylcholine, a major component of both microsomal and of plasma membranes may be involved in facilitating this transport.

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