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THE EFFECT OF ADRENALINE AND OF α- AND β-ADRENERGIC BLOCKING AGENTS ON ATP CONCENTRATION AND ON INCORPORATION OF $^{32}P_i$ INTO ATP IN RAT FAT CELLS

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Abstract—Isolated fat cells from rat epididymal adipose tissue were incubated in glucose-free medium. The effect of adrenaline and of adrenergic blocking agents on incorporation of $[^{32}P]P_i$ into fat cell ATP, and on the concentration of ATP was investigated. Fat cells incubated with adrenaline showed no change in specific radioactivity of ATP, but there was a fall in ATP concentration. Incubation with adrenaline together with the α-adrenergic blocking agent, phenoxybenzamine, resulted in a fall in the specific radioactivity of ATP, and also a fall in ATP concentration. Fat cells incubated with adrenaline together with the β-adrenergic blocking agent, propranolol, showed no change in ATP concentration but an increase in the specific radioactivity of ATP; this effect has been tentatively identified as an α-adrenergic effect.

Incubation of adrenaline and α- and β-adrenergic blocking agents with fat cells results in characteristic patterns of incorporation of $[^{32}P]P_i$ into phospholipids [1]. Little is known about pathways of phospholipid synthesis in adipose tissue, but it seems likely that they resemble those described in liver [2] and that the γ-phosphate of ATP is thus the phosphate donor. In order to interpret the effects of phospholipid synthesis that we have observed, it is necessary to have information about ATP concentration and incorporation of $[^{32}P]P_i$ into ATP under the conditions of our experiments. The effects of adrenaline on ATP concentrations in fat cells and on the incorporation $[^{32}P]P_i$ into ATP have been described [3], but there are no similar reports of the effects of adrenaline together with adrenergic blocking agents. The results of such studies are now presented.

MATERIALS AND METHODS

Materials. Adrenaline (Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K.) was made up in 0·1 M ascorbic acid (BDH Chemicals Ltd., Poole, Dorset, U.K.) Phenoxybenzamine hydrochloride [2-(*N*-benzyl-2-chloroethylamino)-1-phenoxy-propane hydrochloride; Dibenyline] was supplied by Smith, Kline & French Laboratories Ltd., Welwyn Garden City, Herts., U.K. Propranolol hydrochloride [1-isopropylamino-3-(1-naphthyloxy) propan-2-ol hydrochloride; Inderal] was from I.C.I. Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Purified yeast hexokinase (EC 2.7.1.1) was prepared by the method of Schulze [4]. ATP, ADP and AMP (sodium salts) were from Boehringer Corp. (London) Ltd. Firefly lantern extract and apyrase were from Sigma Chemical Co. Ltd., London, S.W.6., and bovine serum albumin (fraction V) from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Collagenase was from Worthington Biochemical Corp., Freehold, New Jersey, U.S.A., and Whatman DE 81 paper from W. & R. Balston Ltd., Kent, U.K. Radioisotopes were obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

Animals. Male Wistar rats (120–140 g) maintained on a stock laboratory diet were used, without previous starvation. They were killed by a blow on the head followed by decapitation.

Incubation medium. Krebs–Ringer bicarbonate buffer (1·3 mM Ca^{2+}), pH 7·4 [5], was used, containing bovine serum albumin which had previously dialysed overnight at 4° against Krebs–Ringer bicarbonate buffer. The final concn of albumin was 4% (w/v). The buffer was gassed with $O_2 + CO_2$ (95:5) and maintained at 37° before use. For incubations with ^{32}P, $KH_2\,^{32}PO_4$ was added to the incubation medium just before use, to give a final concn of 4 μCi/ml (109 μCi/mg of P). Glucose was not present in the incubation medium.

The hormonal sensitivity of each fat cell preparation was confirmed by measuring adrenaline-stimulated glycerol release in a portion of the cells, by the method of Garland and Randle [6]. The stimulation of glycerol release by adrenaline was increased in the presence of the α-adrenergic blocking agent phenoxybenzamine, and was abolished in the presence of the β-adrenergic blocking agent propranolol, as previously reported [1, 7].

Incorporation of $[^{32}P]P_i$ *into ATP.* Fat cells were incubated in medium containing $[^{32}P]P_i$ and the incubation stopped with trichloroacetic acid, final concn 5% (w/v). The extracts were centrifuged, and supernatants extracted three times with 3 vol of diethyl-ether. The samples were kept at 4° throughout this procedure, and stored overnight at −20°. ATP was separated from the extracts by the method of Morrison [8]. Fifty μl of each extract was applied to DEAE-cellulose paper (Whatman DE81) together with non-radioactive markers of ATP, ADP and

AMP. The chromatograms were developed with 0·6 M ammonium formate pH 3·1, and nucleotides located under ultraviolet light. R_f values were:- ATP 0·12; ADP 0·36; AMP 0·60. The R_f of inorganic phosphate was 0·57, so that the radioactivity of the incubation medium phosphate was completely separated from ATP. The area containing ATP was cut into strips (10 × 40 mm), scintillation mixture added (PPO, 3·5 g; POPOP, 50 mg; in toluene (1 l.) with Triton X-100 (500 ml), containing 10% water. The ATP in the spot was characterised further, by the following methods:

(1) *Treatment with hexokinase.* Fat cells were incubated for 1 hr in medium containing [^{32}P]P_i. Deproteinised fat cell extracts (200 μl) containing 5–10 nmoles AT^{32}P were incubated for 15 min at 37° with yeast hexokinase (2 units), glucose (1 mM), $MgSO_4$ (1 mM), ATP (1 mM) and triethanolamine–HCl buffer, pH 7·4 (5 mM) in a final vol of 0·4 ml. Control extracts were incubated similarly, but without added glucose. The extracts were deproteinised, and chromatographed on DEAE–cellulose paper. Incubation with hexokinase and glucose caused a loss of 82·5 ± 0·9% of radioactivity from the ATP spot (16 estimations).

(2) *Treatment with luciferase.* Luciferase specifically degrades ATP to AMP and pyrophosphate [9]. Fat cell extracts containing AT^{32}P were digested with firefly lantern extract (2 mg/ml) contained in the standard buffer for the luciferase assay. Simultaneously a fat cell extract of equivalent cell concentration was incubated with luciferase. Production of luminescence by this latter sample fell to zero after 4 hr. The luciferase-treated extracts and incubated controls were then deproteinised, and the extracts chromatographed on DEAE–cellulose paper. Incubation with luciferase removed 98·1 ± 0·4% of radioactivity from the mononucleotide triphosphate spot (six estimations).

In each experiment the recovery of ATP was determined by the addition of [U-^{14}C]adenosine-5′-triphosphate to samples of fat cells, and isolation by the paper chromatographic procedure. Recoveries averaged 87 per cent and radioactivity in each experiment was corrected for this.

Fat cell ATP concentration. Fat cell extracts were deproteinised and ether-extracted as in the previous section, but without isotope in the medium. ATP in the extracts was measured by the luciferase method [10, 11] and is described in Stein and Hales [12].

Determination of the specific radioactivity of ATP. In each experiment a series of fat cells were incubated in medium containing [^{32}P]P_i and a parallel series incubated simultaneously under identical conditions but without isotope. The measurements of radioactivity of ATP and of ATP concn were combined to calculate the specific radioactivity of ATP.

Measurements of oxygen uptake by fat cells. Rates of oxygen consumption were measured polarographically with an oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Fat cells (3 ml) were suspended in the standard Krebs–Ringer bicarbonate buffer containing 4% bovine serum albumin at 37°. Since ascorbic acid is itself a reducing agent, the adrenaline was dissolved in approx 10^{-2} N HCl, just before the start of the experiment.

RESULTS

When fat cells were incubated with [^{32}P]P_i and the β-adrenergic blocking agent propranolol [3·4 × 10^{-5} M] together with adrenaline [5·4 × 10^{-6} M], there was a significant increase in the specific radioactivity of ATP compared with incubated controls, but no change in ATP concentration. (Table 1, Expts. 1–3). Incubation of fat cells with the α-adrenergic block-

Table 1. Effect of adrenaline and of α- and β-adrenergic blocking agents on fat cell ATP specific radioactivity and on ATP concentration

Expt.	Concn of fat cells (mg/ml)		Control	Adrenaline	Adrenaline + propranolol	Propranolol	Adrenaline + phenoxybenzamine	Phenoxybenzamine
1	73	Specific radioactivity	615·6 ± 20·3 (5)	612·7 ± 49·9 (5)	947·9 ± 50·3 (5)*			
		ATP concn	153 ± 6 (4)	108 ± 8 (3)†	150 ± 15 (4)			
2	49	Specific radioactivity	796·4 ± 26·8 (5)		1391·4 ± 35·3 (5)*	940·7 ± 24·9 (5)†		
		ATP concn	182 ± 5 (4)		156 ± 5 (4)‡	165 ± 6 (4)		
3	89	Specific radioactivity	336·4 ± 20·7 (4)	311·8 ± 14·0 (4)	593·6 ± 45·2†	389·3 ± 30·3 (4)		
		ATP concn	279 ± 18 (4)	173 ± 11 (4)†	260 ± 16 (4)	264 ± 23 (4)		
4	113	Specific radioactivity	574·3 ± 11·3 (5)	552·0 ± 24·8 (5)		595·4 ± 6·1 (5)		
		ATP concn	191 ± 4 (4)	161 ± 3 (4)†		191 ± 3		
5	94	Specific radioactivity	736·6 ± 49·6 (5)	700·8 ± 31·4 (5)			405·7 ± 39·4 (5)*	
		ATP concn	190 + 6 (4)	123 + 7 (4)			67 ± 4 (4)*	
6	100	Specific radioactivity	1088·3 ± 51·3 (5)				453·3 ± 36·5 (5)*	1049·5 ± 25·3 (5)
		ATP concn	198 ± 14 (4)				140 ± 11 (4)‡	208 ± 13 (4)
7	77	Specific radioactivity	870 ± 20·7 (4)				760·6 ± 50·3 (5)	856·2 ± 42 (5)
		ATP concn	172 ± 9 (4)				102 ± 2 (4)*	165 ± 3 (4)

Fat cells were incubated in Expts 1 4 for 1 hr with or without adrenaline (5·4 × 10^{-6} M) and propranolol (3·4 × 10^{-5} M). In Expts 5–7, fat cells were preincubated 20 min with or without phenoxybenzamine (10^{-4} M), then adrenaline (5·4 × 10^{-6} M) added, and incubated 1 hr. Controls, and fat cells with phenoxybenzamine alone were incubated 80 min. Incubation medium as in Materials and Methods. Specific radioactivity (cpm/μmole ATP × 10^{-4}) and ATP concn (nmoles ATP/g fat cells) expressed as mean ± S.E.M. followed by No. of incubations in parentheses.

Significance (P) of difference from control values were estimated by Student's t-test. *$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$.

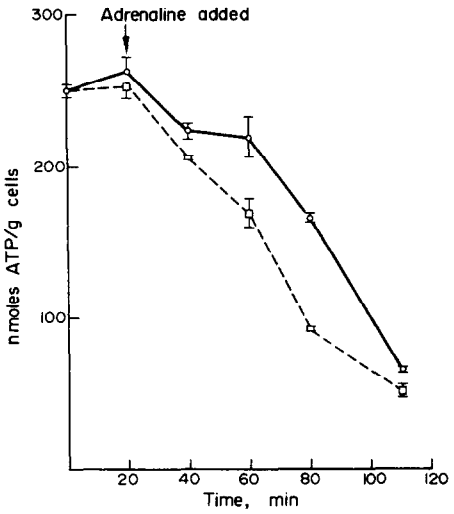

Fig. 1. Time-course of the effect of adrenaline and phenoxybenzamine on fat cell ATP concentration. ATP was assayed in incubated fat cells (111 mg/ml). ----, cells incubated with phenoxybenzamine (1×10^{-4} M) and adrenaline ($5{\cdot}4 \times 10^{-6}$ M); ——, cells incubated with adrenaline alone ($5{\cdot}4 \times 10^{-6}$ M). Each point is mean ± S.E.M. of four incubations.

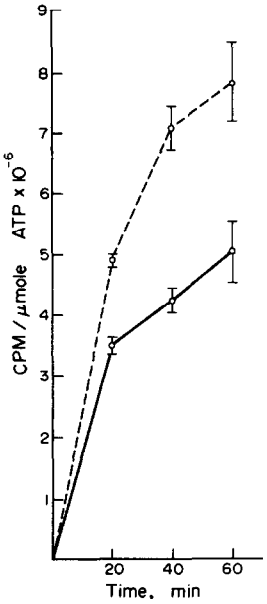

Fig. 2. Time-course of the effect of adrenaline and propranolol on the specific radioactivity of ATP in fat cells. The incubation medium contained [^{32}P]P_i (see Materials and Methods section). The specific radioactivity of ATP was measured in incubated fat cells (130 mg/ml). ----, cells incubated with propranolol ($3{\cdot}4 \times 10^{-5}$ M) and adrenaline ($5{\cdot}4 \times 10^{-6}$ M); ——, incubated control cells. Each point is mean ± S.E.M. of four incubations. Specific radioactivity of medium from cells incubated 1 hr with adrenaline and propranolol was $6{\cdot}8 \times 10^{6}$ cpm/μatom P, and from control cells at 1 hr was $7{\cdot}6 \times 10^{6}$ cpm/μatom P.

ing agent, phenoxybenzamine (10^{-4} M) and adrenaline ($5{\cdot}4 \times 10^{-6}$ M) resulted in a decrease in both specific radioactivity and ATP concentration compared with controls (Table 1, Expts. 5–7). Incubation with either propranolol or phenoxybenzamine alone, in the absence of adrenaline caused no significant changes (Table 1, Expts. 2–4, 6–7). When adrenaline ($5{\cdot}4 \times 10^{-6}$ M) in the absence of adrenergic blocking agents was incubated with fat cells there was no change in the specific radioactivity of ATP, although there was a fall in the concentration of ATP (Table 1, Expts. 1, 3–5). The fall in ATP concentration was time-dependent, but was less than that resulting from incubation with adrenaline together with phenoxybenzamine (Fig. 1).

The increased incorporation of [^{32}P]P_i into ATP resulting from incubation of fat cells with adrenaline and β-adrenergic blocking agent (Table 1, Expts 1–3) was compared with control cells in a time-course experiment (Fig. 2). At each time-point, the specific radioactivity of ATP of the β-blocked adrenaline-stimulated fat cells was significantly higher than the controls ($P < 0{\cdot}001$ at 20 and 40 min; $P < 0{\cdot}05$ at 60 min). Since this effect might be a result of increased oxidative phosphorylation, oxygen uptake by fat cells was measured under the various conditions of hormonal stimulation (Table 2). Fat cells incubated with adrenaline ($5{\cdot}4 \times 10^{-6}$ M) together with propranolol ($3{\cdot}4 \times 10^{-5}$ M) showed no increased oxygen uptake compared with controls. Fat cells incubated with adrenaline ($5{\cdot}4 \times 10^{-6}$ M) alone, or with adrenaline ($5{\cdot}4 \times 10^{-6}$ M) together with phenoxybenzamine (10^{-4} M) showed a greater oxygen uptake than incubated controls. Incubation with adrenergic blocking agents alone caused no change in oxygen uptake.

DISCUSSION

There is evidence that both α- and β-adrenergic receptors occur in adipose tissue of several species, including man [13], rat [1, 7, 14, 15] and hamster [16], although it seems likely that the proportion of α- to β-receptors may vary from one species to another [13]. The present work, in which adrenergic effects were obtained during β-receptor blockade, is further evidence for the existence of α-receptors in rat adipose tissue. Although the receptors in fat cells cannot unequivocally be shown to correspond to α-adrenergic receptors in other tissues, for the purposes

Table 2. Effect of adrenaline and of α- and β-adrenergic blocking agents on oxygen uptake by fat cells

Control	Adrenaline	Adrenaline + propranolol	Propranolol	Adrenaline + phenoxybenzamine	Phenoxybenzamine
100	132·1 ± 3·0 (7)	104·0 ± 4·1 (8)	105·7 ± 5·1 (8)	139·5 ± 11·6 (4)	107·5 ± 7·7 (4)

Fat cells were incubated with adrenaline ($5{\cdot}4 \times 10^{-6}$ M), propranolol ($3{\cdot}4 \times 10^{-5}$ M), and phenoxybenzamine (10^{-4} M) as indicated. Oxygen uptake for each treatment was determined and is expressed as a percentage of the corresponding control. Values are mean ± S.E.M. with No. of estimations in parentheses. Oxygen uptake of incubated control cells in the four separate experiments summarised were 276, 191, 432 and 414 μl/O_2 per g fat cells per hr.

of this discussion the term 'α-adrenergic stimulation' will be used for experiments in which fat cells were incubated with adrenaline in the presence of the β-receptor blocking drug, propranolol. Similarly, 'β-adrenergic stimulation' will be used to describe the effect of adrenaline in the presence of the α-receptor blocking agent, phenoxybenzamine.

These results show that α-adrenergic stimulation of fat-cells incubated in medium containing [^{32}P]P_i and in the absence of glucose results in an increase in the specific radioactivity of ATP. The mechanism of this effect does not appear to be increased synthesis of ATP, since there was no stimulation of oxygen uptake (Table 2, column 3) and furthermore there was no concomitent fall in ATP concentration and hence increased concentration of ADP (Table 1, Expts 1–3). However, the possibility cannot be excluded that there may be increased synthesis of a very small amount of ATP, from a separate, highly labelled pool of phosphate—e.g., by stimulation of glycolysis and thus of substrate-level phosphorylation.

An alternative explanation may be that transport of phosphate into the cytoplasmic or intramitochondrial pool may be relatively slow, and that it is this that limits the rate of labelling of ATP, rather than the rate of ATP synthesis; the effect of α-adrenergic stimulation will then be to increase the rate of phosphate transport, and so to increase the specific radioactivity of ATP. The data available support this, since it can be calculated from the ATP concentration (Table 1) and oxygen uptake (Table 2) that the ATP turnover time for fat cells incubated without hormonal stimulation was 0·1–0·2 min, whereas the labelling of fat cell ATP with [^{32}P]P_i was comparatively slow (Fig. 2). Similar relatively slow labelling of fat cell ATP has previously been described [3, 12], and whole fat cells incubated under identical conditions also accumulated [^{32}P]P_i fairly slowly [17].

Oxygen uptake by fat cells may give only an approximate measure of ATP synthesis, since there is evidence that respiration is not completely coupled to phosphorylation, and that the increased oxygen uptake caused by adrenaline results from an increase in non-phosphorylating respiration [18]. It has been suggested that accumulation of lipolytic products causes impairment of oxidative phosphorylation [19] and the results of this work are consistent with this.

Under all hormonal conditions investigated, ATP concentrations in incubated fat cells were inversely related to lipolysis. When lipolysis was stimulated with adrenaline the fat cell ATP concentration fell, and when lipolysis was enhanced by β-adrenergic stimulation [1, 7] the fall in ATP concentration was more pronounced and also incorporation of [^{32}P]P_i into ATP was markedly impaired (Table 1, Expts 5–7 and Fig. 1). Conversely, blockade of β-adrenergic receptors resulted in abolition of the lipolytic response to adrenaline, and there was no decrease in ATP concentration.

This work gives evidence of an adrenergic response that is not mediated by the β-receptors and the adenyl cyclase system, and can now be related to a similar stimulation of incorporation of [^{32}P]P_i into fat cell phospholipids [1]. In addition, it may be observed that the effects of α-adrenergic stimulation of fat cells show many similarities to fat cells stimulated with insulin [12]. In both systems ATP concentrations were not changed, incorporation of [^{32}P]P_i into ATP was increased, and the specific radioactivities of phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and cardiolipin were increased. A single difference was that in the insulin-stimulated cells the specific radioactivity of phosphatidylcholine was increased and this did not occur in the α-receptor stimulated system. These investigations have shown that adrenaline and adrenergic blocking agents have well-marked effects on ATP in whole fat cells, and these may be correlated with the effects on changes in phospholipid metabolism. This will enable us to distinguish between effects on phospholipids which are merely simple reflections of changes in ATP, and those which are true expressions of hormonal stimulation of phospholipid synthesis.

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