Phosphatidic acid and phosphatidylinositol labelling in adipose tissue

The role of endogenously formed adenosine

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Incorporation of [32 P]P_i into phosphatidic acid and phosphatidylinositol of hamster epididymal adipocytes was partially inhibited by 3-isobutyl-1-methylxanthine. This effect of 3-isobutyl-1-methylxanthine was antagonized by isopropyl-*N*⁶-phenyladenosine but not by 2',5'-dideoxyadenosine, prostaglandin E₁ or clonidine. *N*⁶-Phenylisopropyladenosine did not affect incorporation of [32 P]P_i into phosphatidic acid or phosphatidylinositol when 3-isobutyl-1-methylxanthine was not present. In contrast with 3-isobutyl-1-methylxanthine inhibition of [32 P]P_i incorporation into phospholipids, which was blocked only by *N*⁶-phenylisopropyladenosine, accelerated lipolysis was blocked by prostaglandin E₁, clonidine and 2',5'-dideoxyadenosine as well as by *N*⁶-phenylisopropyladenosine. Phospholipid labelling was also decreased in the presence of adenosine deaminase, but not in the presence of isoprenaline (isoproterenol). The stimulatory effect of *N*⁶-phenylisopropyladenosine on [32 P]P_i incorporation into phospholipids in cells exposed to 3-isobutyl-1-methylxanthine was evident as soon as 3 min after addition of the adenosine analogue and maximum 10 min after its addition. As observed by others, [32 P]P_i incorporation into phospholipids was increased by the α_1 -selective agonist methoxamine. The stimulatory effect of methoxamine occurred with a time course similar to that of *N*⁶-phenylisopropyladenosine and was present at nearly equal magnitude in the absence or presence of 3-isobutyl-1-methylxanthine. The inhibitory effects of 3-isobutyl-1-methylxanthine and adenosine deaminase on phospholipid labelling are attributed to blockade of the action, or to the enzymic removal, of adenosine formed in and released from the fat-cells during their incubation. Supporting this view is the selective reversal of the actions of 3-isobutyl-1-methylxanthine and of adenosine deaminase by *N*⁶-phenylisopropyladenosine. These findings suggest an important role for endogenous adenosine in regulation of phospholipid turnover in adipocytes.

Many hormones, neurotransmitters and other agents have been found to accelerate the incorporation of [32 P]P_i into phospholipids, principally phosphatidylinositol and phosphatidic acid (Michell, 1975). Because the actions of many of these same hormones and neurotransmitters appear to be mediated by an increase in intracellular Ca²⁺ concentration, partly because of an increase in Ca²⁺ permeability (Michell, 1975; Michell & Kirk, 1981; Exton, 1981), the hypothesis has been enunciated that this 'phospholipid effect' is coupled to alter-

ations in cell Ca²⁺, perhaps by modifying membrane Ca²⁺ conductance (Michell, 1975). The molecular basis for the phospholipid effect is thought to involve accelerated hydrolysis of phosphatidylinositol to diacylglycerol and inositol phosphates, phosphorylation of the diacylglycerol to phosphatidic acid and subsequent resynthesis of phosphatidylinositol. Salmon & Honeyman (1980) and Putney *et al.* (1980) have proposed that the net accumulation of phosphatidic acid after breakdown of phosphatidylinositol initiates an increase in Ca²⁺ influx, thereby allowing cell Ca²⁺ to increase. In support of this view is the finding that

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phosphatidic acid behaves as a Ca^{2+} ionophore in artificial lipid bilayers (Green *et al.*, 1980). More recently, another action of phosphatidic acid has become evident. Clark *et al.* (1980) and Schimmel *et al.* (1980) reported that exposure of WI-38 fibroblasts and hamster epididymal adipocytes to phosphatidic acid inhibited hormone-stimulated cyclic AMP formation. Both groups raised the possibility that accumulation of phosphatidic acid was intimately involved in hormone attenuation of adenylate cyclase in these cells. In hamster adipocytes, adenosine, α_2 -receptor-site agonists (e.g. clonidine) and prostaglandin E_1 inhibit cyclic AMP accumulation (Schimmel *et al.*, 1980, 1981) by lowering activity of adenylate cyclase (Jakobs & Aktories, 1981), but a role of these agents in regulation of phosphatidic acid formation is uncertain. Garcia-Sainz *et al.* (1980) reported that clonidine had no detectable effect on $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipids in hamster white adipocytes, but their data were obtained under incubation conditions that allow endogenous adenosine to accumulate and act on the fat-cells during their incubation. Because accumulation of adenosine has been found to interfere with clonidine action on lipolysis and cyclic AMP accumulation in hamster fat-cells (Schimmel *et al.*, 1981), one might expect to observe clonidine stimulation of phospholipid labelling only in the absence of adenosine. It follows that a more appropriate test of clonidine action on phospholipid turnover should be made with incubation conditions that either prevent adenosine accumulation, such as by addition of adenosine deaminase, or prevent adenosine action, such as with a methylxanthine (Schimmel *et al.*, 1981). The purpose of the present studies was to determine if clonidine and other anti-lipolytic agents accelerated the turnover of phosphatidyl-inositol in hamster fat-cells incubated in the presence of 3-isobutyl-1-methylxanthine or adenosine deaminase.

Materials and methods

Preparation of adipocytes

Golden hamsters (90–100 g) were purchased from Charles River Laboratories, Lakeview, NJ, U.S.A., and fed on Purina laboratory chow and tap water *ad libitum* for at least 1 week before use. They were killed by cervical dislocation, the epididymal fat-bodies were removed and adipocytes were prepared by collagenase digestion as previously described (Schimmel *et al.*, 1981). The adipose tissue from two to four hamsters was minced and placed in plastic vessels containing 5 ml of Krebs–Ringer Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer with 5% bovine serum albumin and 1.5 mg of collagenase/ml. The composition of the

buffer used in all experiments was 115 mM-NaCl, 4.6 mM-KCl, 1.5 mM- CaCl_2 , 1.4 mM- MgSO_4 , 11 mM- NaHCO_3 and 30 mM-Hepes, adjusted to pH 7.4 after addition of the albumin. After enzymic digestion, the adipocytes were filtered through a silk screen and washed three times with albumin-containing buffer.

Incorporation of $[^{32}\text{P}]\text{P}_i$ into phospholipids

Fat-cells were incubated in 2 ml of buffer containing $10\ \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for up to 1 h. After incubation the cells were separated from the medium by flotation in silicone (Prosil-28; PCR Research Chemicals, Gainesville, FL, U.S.A.)-coated Pasteur pipettes. The media was collected for assay of glycerol as previously described (Schimmel *et al.*, 1981). The cells were washed with 2 ml of 150 mM-NaCl buffered with 10 mM-phosphate (pH 7.4) and homogenized in 3 ml of chloroform/methanol/10 M-HCl (200:200:1, by vol.). The phospholipids were extracted and isolated by two-dimensional t.l.c. on silica-gel 60 plates (E. Merck, Darmstadt, West Germany). The plates were developed in the first dimension with a basic solvent [chloroform/methanol/conc. NH_3 (sp.gr. 0.880) (65:25:4, by vol.)] and in the second dimension with an acidic solvent (butan-1-ol/acetic acid/water, 6:1:1, by vol.) (Honeyman *et al.*, 1983). Phospholipids were located by staining with iodine vapour and by radioautography with X-ray film. In most experiments, insufficient phosphatidic acid was present to be detected by iodine-vapour staining and was located solely by the radioautograph. Silica gel was scraped from the plates and radioactivity associated with each lipid determined. The phosphorus content of the phospholipids was determined by the Malachite Green method of Itaya & Ui (1968) after acid hydrolysis with 60% HClO_4 at 170°C for 60 min as described by Bowyer & King (1977).

Rates of glycerol production are expressed relative to the weight of dried total cell lipid extracts obtained from samples taken during the distribution of cells into the incubation vessels. Incorporation of ^{32}P into each phospholipid is expressed relative to the amount of phosphatidylcholine phosphate recovered from each plate. Phosphatidylcholine was selected for this purpose, because it is the most prevalent phospholipid and its concentration did not vary in response to hormone treatment.

Materials

Collagenase (type I) was obtained from Worthington Biochemical Corp. Isoprenaline (isoproterenol), 3-isobutyl-1-methylxanthine, adenosine deaminase, prostaglandin E_1 and bovine serum albumin (type V) were from Sigma. Methoxamine was from Burroughs–Wellcome. N^6 -Phenylisopropyladenosine was from Boehringer–Mannheim. 2',5'-Dideoxyadenosine was from P-L Bio-

chemicals. Hepes was from Calbiochem. Clonidine was generously given by Boehringer-Ingelheim. All other chemicals were of reagent grade.

Results

Of the major phospholipids found in hamster fat-cells, only phosphatidylcholine, phosphatidylinositol and phosphatidic acid became significantly

Fig. 1. $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipids in the presence of 3-isobutyl-1-methylxanthine (IBMX). Fat-cells (67–86 mg) were incubated for the times indicated in the absence (●) or presence (○) of 50 μM -IBMX. Each point represents the mean $\pm$ S.E.M. for four separate experiments. Points labelled with * are significantly different from the corresponding control value ($P < 0.05$) by Student's paired t test.

labelled with $[^{32}\text{P}]\text{P}_i$ during incubation *in vitro* for 60 min. However, in the present studies, labelling of phosphatidylcholine was not consistently altered by any treatment used (results not shown). The time course of $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidic acid and phosphatidylinositol was approximately linear for up to 60 min of incubation (Fig. 1). When 3-isobutyl-1-methylxanthine (50 μM) was present, incorporation of radioactivity into both phospholipids was significantly decreased, but the time course of labelling appeared unaltered. 3-Isobutyl-1-methylxanthine increased the rate of lipolysis, which was also nearly linear during the 60 min incubation (results not shown). Increasing the concentration of 3-isobutyl-1-methylxanthine to greater than 50 μM did not cause a greater inhibition of $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipid (Fig. 2), but the rate of lipolysis was increased when higher concentrations of the methylxanthine were present. When the α_2 -selective adrenergic agonist clonidine was present at a concentration of 10 μM , the stimulation of lipolysis in response to 50 μM -3-isobutyl-1-methylxanthine was prevented, and the stimulation caused by 100 μM -3-isobutyl-1-methylxanthine was significantly decreased ($P < 0.05$); lipolysis activated with 1 mM-3-isobutyl-1-methylxanthine was unaffected by this concentration of clonidine. In contrast with clonidine antagonism of 3-isobutyl-1-methylxanthine-stimulated lipolysis, the α_2 -adrenergic agonist did not prevent or decrease 3-isobutyl-1-methylxanthine inhibition of $[^{32}\text{P}]\text{P}_i$ incorporation into the phospholipids (Fig. 2).

The data presented in Table 1 compare the effects of 3-isobutyl-1-methylxanthine and isoprenaline on $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidic acid and phosphatidylinositol. Although both agents caused a comparable stimulation of lipolysis, only 3-isobutyl-1-methylxanthine inhibited $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipids. Isoprenaline, which increased lipolysis to an extent comparable with that in the presence of 3-isobutyl-1-methylxanthine, caused only a slight decrease in $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidic acid and phosphatidylinositol. When 3-isobutyl-1-methylxanthine and isoprenaline were present together, a larger increase in lipolysis was observed that was associated with a decrease in $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidic acid and phosphatidylinositol that exceeded the decrease in phospholipid labelling observed in the presence of 3-isobutyl-1-methylxanthine alone.

Because the mode of 3-isobutyl-1-methylxanthine action on adipocytes is thought to involve the blockade of adenosine receptors (Londos *et al.*, 1978), our next experiments sought to determine if 3-isobutyl-1-methylxanthine inhibition of phospholipid labelling could be prevented and reversed by analogues of adenosine. The data presented in Table 2 show that exposure of fat-cells to N^6 -phenyl-

Fig. 2. Clonidine does not prevent 3-isobutyl-1-methylxanthine (IBMX) inhibition of ^{32}P incorporation into phospholipids

Fat-cells (85–106 mg) were incubated for 60 min in the presence of various concentrations of IBMX, and in the absence (●) or presence (○) of clonidine (10 μM). Each point represents the mean $\pm$ S.E.M. for four separate experiments.

Table 1. Effects of isoprenaline, singly and in combination with 3-isobutyl-1-methylxanthine (IBMX), on ^{32}P labelling of phospholipids and on lipolysis

Fat-cells (74–129 mg) were incubated for 60 min in the absence or presence of isoprenaline, IBMX or both agents present simultaneously. The data are presented as the means $\pm$ S.E.M. for six separate experiments. The effects of isoprenaline and IBMX are expressed as the mean changes from the corresponding control value. * Significantly different from control ($P < 0.05$) by paired Student's t test.

	^{32}P incorporation (c.p.m./nmol of phosphate)		Glycerol production ($\mu\text{mol/h per g}$)
	Phosphatidic acid	Phosphatidylinositol	
Basal	109.9 $\pm$ 32.1	100.1 $\pm$ 21.4	2.0 $\pm$ 0.17
Change caused by			
Isoprenaline (0.5 μM)	−12.7 $\pm$ 9.1	−15.5 $\pm$ 10.6	+9.6 $\pm$ 2.1*
IBMX (0.1 mM)	−41.8 $\pm$ 13.2*	−49.0 $\pm$ 14.2*	+7.1 $\pm$ 2.3*
Isoprenaline and IBMX	−58.4 $\pm$ 12.5*	−74.8 $\pm$ 12.3*	+15.2 $\pm$ 0.17*

isopropyladenosine completely antagonized 3-isobutyl-1-methylxanthine inhibition of ^{32}P incorporation into phosphatidic acid and phosphatidylinositol. Lipolysis activated with 3-isobutyl-1-methylxanthine was similarly inhibited in the presence of this adenosine analogue. On the other hand, exposure of cells to N^6 -phenylisopropyladenosine in the absence of 3-isobutyl-1-methylxanthine had no effect on ^{32}P incorporation into phospholipids. In contrast with N^6 -phenylisopropyladenosine, 2',5'-dideoxyadenosine did not block 3-isobutyl-1-methylxanthine inhibition of phospholipid labelling, but did

inhibit activated lipolysis. Prostaglandin E_1 and clonidine similarly suppressed 3-isobutyl-1-methylxanthine-stimulated lipolysis, but did not significantly increase ^{32}P incorporation into phospholipids. As with N^6 -phenylisopropyladenosine, 2',5'-dideoxyadenosine, prostaglandin E_1 and clonidine did not affect the incorporation of ^{32}P into phospholipids in cells incubated in the absence of 3-isobutyl-1-methylxanthine.

The data in Fig. 3 illustrate the time course of N^6 -phenylisopropyladenosine stimulation of ^{32}P incorporation into phospholipids. For this experi-

Table 2. *Effect of adenosine analogues, prostaglandin E₁ and clonidine on incorporation of [³²P]P_i into phospholipids* Fat-cells (67–104 mg) were incubated for 60 min, in the absence or presence of 0.10 mM-IBMX (3-isobutyl-1-methylxanthine). *N*⁶-Phenylisopropyladenosine (PIA), 2',5'-dideoxyadenosine (DDA), prostaglandin E₁ and clonidine were present where indicated. Each value represents the mean ± s.e.m. for the numbers of separate experiments shown in parentheses. The effects of the added agents are expressed as the mean changes from the corresponding control value. *Significantly different from control (*P* < 0.05), or **significantly different from value obtained with IBMX (*P* < 0.05), by paired Student's *t* test.

	³² P incorporation into phospholipid (c.p.m./nmol of phosphate)					
	Phosphatidic acid		Phosphatidylinositol		Glycerol production (μmol/h per g)	
	Control	IBMX	Control	IBMX	Control	IBMX
Basal (15)	149 ± 22	81 ± 39*	121 ± 16	51 ± 8*	1.48 ± 17	8.72 ± 1.65*
Change by						
PIA (0.1 μM) (9)	–25 ± 34	48 ± 19**	–34 ± 36	41 ± 21		–5.38 ± 0.81**
PIA (1 μM) (10)	–31 ± 34	56 ± 11**	–39 ± 37	49 ± 12**		–5.79 ± 58**
DDA (30 μM) (6)	–13 ± 24	29 ± 21	9 ± 21	8 ± 12		–5.47 ± 0.59**
Prostaglandin E ₁ (1 μg/ml) (8)	–2 ± 0	21 ± 30	9 ± 26	35 ± 26		–6.74 ± 96**
Clonidine (10 μM) (6)	29 ± 21	–4 ± 36	–6 ± 7	–7 ± 9		–3.87 ± 0.41**

Fig. 3. *Time course of *N*⁶-phenylisopropyladenosine stimulation of [³²P]P_i incorporation into phospholipids* Fat-cells (119–133 mg) were incubated for 40 min in media containing [³²P]P_i and 3-isobutyl-1-methylxanthine (IBMX) at a concentration of 50 μM. After 40 min of incubation, *N*⁶-phenylisopropyladenosine (PIA) was added to some incubations, which were terminated after 3, 6, 10 or 20 min (●). Cells not exposed to *N*⁶-phenylisopropyladenosine are designated as control (○). Incorporation of [³²P]P_i into phosphatidic acid and phosphatidylinositol in cells not exposed to IBMX was 249 and 182 c.p.m./nmol of P_i respectively. Each point represents the mean ± s.e.m. for six separate experiments.

ment, fat-cells were incubated with [³²P]P_i and 3-isobutyl-1-methylxanthine for 40 min. *N*⁶-Phenylisopropyladenosine was then added and the

Table 3. *Methoxamine stimulates [³²P]P_i incorporation into phospholipids in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX)*

Fat-cells (119–144 mg) were incubated for 60 min in the absence, designated as basal, or in the presence of 0.10 mM-IBMX. Methoxamine was present where indicated. Each value represents the mean ± s.e.m. for five separate experiments. *Significantly different from appropriate control (*P* < 0.05) by paired Student's *t* test.

	³² P incorporation into phospholipid (c.p.m./nmol of P _i)	
	Phosphatidic acid	Phosphatidylinositol
Basal	123 ± 29	115 ± 28
Methoxamine (10 μM)	254 ± 56	315 ± 81
Δ	131 ± 36*	200 ± 71*
IBMX (0.10 mM)	80 ± 19	54 ± 19
IBMX + Methoxamine (10 μM)	168 ± 41	196 ± 56
Δ	88 ± 12*	142 ± 41*

incubation terminated 3, 6, 10 or 20 min later. As soon as 3 min after addition of *N*⁶-phenylisopropyladenosine, [³²P]P_i incorporation into phosphatidic acid and phosphatidylinositol was increased, and between 6 and 10 min after addition of *N*⁶-phenylisopropyladenosine phospholipid labelling was maximum. The addition of the adenosine analogue under these conditions did not fully reverse the inhibition of [³²P]P_i incorporation into phospholipids caused by 3-isobutyl-1-methylxanthine.

Fain and his colleagues (Garcia-Sainz *et al.*, 1980; Garcia-Sainz & Fain, 1980) have demonstrated stimulation of phospholipid turnover in fat-cells exposed to α₁-selective adrenergic agents. The results presented in Table 3 show stimulation of

Table 4. *Inhibition of [32 P] P_i incorporation into phospholipids by adenosine deaminase*

Fat-cells (96–138 mg) were incubated for 60 min in the absence or presence of adenosine deaminase. N^6 -Phenylisopropyladenosine (PIA) was present where indicated. The data are presented as means $\pm$ S.E.M. for five separate experiments. *Significantly different from basal ($P < 0.05$), or **significantly different from adenosine deaminase ($P < 0.05$), by paired Student's t test.

	32 P incorporation into phospholipids (c.p.m./nmol of phosphate)		Glycerol production (μ mol/h per g)
	Phosphatidic acid	Phosphatidylinositol	
Basal	166 $\pm$ 19	127 $\pm$ 17	0.63 $\pm$ 0.06
Adenosine deaminase (10 μ g/ml)	131 $\pm$ 23	81 $\pm$ 18	
Δ	33 $\pm$ 11*	46 $\pm$ 10*	1.23 $\pm$ 0.29
Adenosine deaminase (10 μ g/ml) and PIA (1 μ M)	159 $\pm$ 31	114 $\pm$ 29	
Δ caused by PIA	28 $\pm$ 9**	33 $\pm$ 11**	

[32 P] P_i incorporation into phosphatidic acid and phosphatidylinositol in fat-cells exposed to the α_1 -site-selective agent methoxamine. This stimulatory effect of methoxamine was present and of nearly equal magnitude in cells exposed to 3-isobutyl-1-methylxanthine (Table 3). It should be noted that the extent of [32 P] P_i incorporation into phospholipids in cells exposed to both 3-isobutyl-1-methylxanthine and methoxamine was greater than that in cells not exposed to either 3-isobutyl-1-methylxanthine or methoxamine. In contrast, N^6 -phenylisopropyladenosine antagonized the inhibitory effect of 3-isobutyl-1-methylxanthine, but did not cause [32 P] P_i incorporation into phospholipids to exceed that detected under basal unstimulated conditions (Table 2). We have found the time course of the methoxamine effect to be similar to that of N^6 -phenylisopropyladenosine (results not shown).

The data presented in Table 4 show that exposure of fat-cells to adenosine deaminase causes a significant decrease in [32 P] P_i incorporation into both phospholipids. As with 3-isobutyl-1-methylxanthine, this effect of adenosine deaminase was antagonized by N^6 -phenylisopropyladenosine. The stimulation of lipolysis caused by adenosine deaminase, although statistically significant ($P < 0.05$), was very much less than that observed in cells exposed to 3-isobutyl-1-methylxanthine.

Discussion

The observation, made first by Clark *et al.* (1980) and then by Schimmel *et al.* (1980), that phosphatidic acid can decrease cell cyclic AMP concentrations led to the hypothesis that accelerated formation of this lipid by hydrolysis of phosphatidylinositol may be involved in hormonal attenuation of cyclic AMP generation. The results presented herein and in the preceding paper (Honey-

man *et al.*, 1983), however, are enigmatic on this point. On one hand, our data indicate stimulation of phosphatidate formation by adenosine, which occurs concomitantly with inhibition of lipolysis and of cyclic AMP accumulation (Schimmel *et al.*, 1980), and are therefore in accord with a role of phosphatidate in regulation of cyclic AMP formation. In addition, increased accumulation of phosphatidate in cells exposed to phospholipase C (Honeyman *et al.*, 1983) is perhaps the simplest explanation for its well-documented inhibitory effects on lipolysis and accumulation of cyclic AMP (Rodbell & Jones, 1966; Rosenthal & Fain, 1971; Fredholm *et al.*, 1978). By similar reasoning, accumulation of phosphatidic acid may be implicated in the cyclic AMP-lowering and anti-lipolytic effects of insulin and oxytocin (T. W. Honeyman, W. Strohsnitter, C. R. Scheid & R. J. Schimmel, unpublished work). On the other hand, a role of phosphatidic acid in the actions of clonidine and prostaglandin E_1 is not supported by the present results documenting a lack of effect of these agents on incorporation of [32 P] P_i into phosphatidic acid and phosphatidylinositol. A role of phosphatidic acid in mediation of the effects of clonidine and prostaglandin E_1 would appear to require postulating formation of this lipid by a reaction not involving phosphorylation of diacylglycerol, such as through a phospholipase D (Kanfer, 1972).

The conclusion that adenosine can accelerate phosphatidylinositol labelling in hamster adipocytes derives from the observations that 3-isobutyl-1-methylxanthine and adenosine deaminase decrease [32 P] P_i incorporation into phosphatidic acid and phosphatidylinositol and that this inhibition is reversed selectively by the adenosine analogue N^6 -phenylisopropyladenosine. We interpret the inhibition of [32 P] P_i incorporation into phospholipids by 3-isobutyl-1-methylxanthine to be a direct consequence of blockade of adenosine R-type (Daly

et al., 1981) receptors on fat-cells and suggest that endogenously formed adenosine constitutes a stimulus for phospholipid turnover. Evidence presented by several laboratories indicates that fat-cells incubated *in vitro* release adenosine into the media (Schimmel *et al.*, 1981; Schwabe *et al.*, 1973), which alters the metabolism of the cells by interacting with specific membrane receptors (Trost & Schwabe, 1981). 3-Isobutyl-1-methylxanthine and other methylxanthines interfere with adenosine action by antagonizing adenosine R-type receptors (Londos *et al.*, 1978; Trost & Schwabe, 1981). We therefore propose that the degree of [32 P]P_i incorporation into phospholipids in 'unstimulated' adipocytes depends on the accumulation of adenosine in the media. Thus the blockade of adenosine action by 3-isobutyl-1-methylxanthine or removal of adenosine with adenosine deaminase decreases phospholipid labelling by removing an endogenous stimulus to phospholipid turnover. *N*⁶-Phenylisopropyladenosine is without effect in the absence of 3-isobutyl-1-methylxanthine, because, according to our view, the cells are already maximally stimulated by endogenous adenosine in their cell preparations. Garcia-Sainz & Fain (1980) also reported that neither adenosine nor *N*⁶-phenylisopropyladenosine modified incorporation of [32 P]P_i into phospholipids in white fat-cells. In their studies adipocytes were incubated at high density, and it is likely that effects of exogenously added adenosine were not observed because the cells were already maximally stimulated by endogenously formed nucleotide.

An alternative explanation for our results is that the decreased incorporation of [32 P]P_i into phospholipids in cells exposed to 3-isobutyl-1-methylxanthine results from the intracellular accumulation of non-esterified fatty acids and the lowered ATP concentrations that occur concomitant with accelerated lipolysis in adipocytes (Heindel *et al.*, 1974). Indeed, Garcia-Sainz & Fain (1980) reported decreased [32 P]P_i incorporation onto phospholipids of rat white fat-cells exposed to the lipolytic stimuli corticotropin, dibutyl cyclic AMP and isoprenaline. Because the effect of these lipolytic agents on [32 P]P_i incorporation into phospholipids was prevented or decreased by increasing the albumin concentration in the medium, they proposed that lipolytic stimuli lower phospholipid labelling through a mechanism involving increased lipolysis and accumulation of non-esterified fatty acids within fat-cells. Unfortunately, Garcia-Sainz & Fain (1980) did not include methylxanthines in their study.

The present results do not diminish the importance of intracellular non-esterified fatty acids and ATP as potential mediators of the effects of lipolytic stimuli on phosphatidylinositol and phosphatidic acid labelling. However, a role for non-esterified fatty acids and ATP in 3-isobutyl-1-methylxanthine

inhibition of [32 P]P_i incorporation into phospholipids seems unlikely for several reasons. Firstly, the inhibitory effect of 3-isobutyl-1-methylxanthine on phospholipid labelling was evident after only 20 min of incubation (Fig. 1), but cellular accumulation of non-esterified fatty acids would be expected to be minimal at this time (Heindel *et al.*, 1974). Secondly, the incorporation of [32 P]P_i into phosphatidic acid was decreased maximally by 50 μ M-3-isobutyl-1-methylxanthine, but lipolysis was increased only slightly at this concentration of the methylxanthine (Fig. 2). Thirdly, 3-isobutyl-1-methylxanthine-accelerated lipolysis was antagonized by clonidine (Fig. 2), prostaglandin E₁ (Table 2), 2',5'-dideoxyadenosine (Table 2) and *N*⁶-phenylisopropyladenosine, but 3-isobutyl-1-methylxanthine inhibition of [32 P]P_i incorporation into phospholipids was reversed only by *N*⁶-phenylisopropyladenosine. Fourthly, when the stimulus for lipolysis was provided by isoprenaline rather than 3-isobutyl-1-methylxanthine, phospholipid labelling was not decreased (Table 1), even though the rates of lipolysis in the presence of each agent were nearly identical. Lastly, adenosine deaminase decreased phospholipid labelling while causing only a slight increase in lipolysis (Table 4). Taken together, these observations show no correlation between the rate of lipolysis and the degree of phospholipid labelling, and therefore argue strongly against the possibility that the effects of 3-isobutyl-1-methylxanthine and *N*⁶-phenylisopropyladenosine on [32 P]P_i incorporation into phosphatidylinositol and phosphatidic acid result from changes in lipolysis and the resultant changes in cellular concentrations of non-esterified fatty acids.

As reported first by Garcia-Sainz & Fain (1980), α_1 -selective agonists increase [32 P]P_i labelling of phosphatidic acid and phosphatidylinositol. This effect, as well as those of insulin, oxytocin and phospholipase C (Honeyman *et al.*, 1983), is readily detected under incubation conditions that allow endogenously formed adenosine to accumulate. In contrast, *N*⁶-phenylisopropyladenosine stimulation of [32 P]P_i incorporation into phospholipids is seen only in the absence of endogenous adenosine (Table 2). Comparing these two observations leads to two conclusions. Firstly, we can exclude the possibility that the effects of α_1 -catecholamines and those of insulin, oxytocin and phospholipase C on phospholipid labelling (T. W. Honeyman, W. Strohsnitter, C. R. Scheid & R. J. Schimmel, unpublished work) result from increased production of adenosine. Secondly, the changes in phospholipid labelling caused by these agents may resemble each other only superficially and may not arise through common biochemical sequelae. For example, increased incorporation of [32 P]P_i into phosphatidic acid and phosphatidylinositol by insulin, oxytocin, α_1 -catecholamines, phospholipase C

and adenosine may reflect accelerated turnover of different molecular species of phospholipid.

In recent years, numerous studies of adenosine action on a variety of cells have appeared (reviewed by Daly *et al.*, 1981). Most of these studies have focused on adenosine stimulation and/or inhibition of adenylate cyclase (Londos *et al.*, 1978; Daly *et al.*, 1981). Along these lines, several laboratories (Londos *et al.*, 1978; Trost & Stock, 1977) have shown adenosine inhibition of adipocyte adenylate cyclase activity. However, adenosine exerts effects on fat-cells that appear to be unrelated to its effects on the adenylate cyclase-cyclic AMP system. For example, adenosine or *N*⁶-phenylisopropyl-adenosine has been shown to accelerate glucose transport and oxidation by fat-cells (Schwabe *et al.*, 1974), to activate cyclic AMP phosphodiesterase (Teo *et al.*, 1981) and to activate pyruvate dehydrogenase (Honeyman *et al.*, 1983). The present paper documents yet another action of adenosine on fat-cells that appears to be independent of cyclic AMP, namely acceleration of ³²P labelling of phosphatidylinositol and phosphatidic acid. The possibility that the stimulation by adenosine of glucose metabolism, pyruvate dehydrogenase activity and cyclic AMP phosphodiesterase result from stimulated phospholipid turnover is an attractive yet unproven idea. It should be noted, however, that a similar conclusion has been reached in regard to the effects of insulin and other insulin-like agents (Farese *et al.*, 1982; Honeyman *et al.*, 1983).

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