Biochem. J. (1980) **186**, 781–789
Printed in Great Britain

Effect of Insulin, Catecholamines and Calcium Ions on Phospholipid Metabolism in Isolated White Fat-Cells

J. Adolfo GARCÍA-SÁINZ and John N. FAIN
Section of Physiological Chemistry, Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

(*Received 6 August* 1979)

The incorporation of [^{32}P]P_i into phosphatidylinositol by rat fat-cells was markedly increased in the presence of adrenaline. Phosphatidic acid labelling was also increased, but to a lesser extent. These effects are due to α_1-adrenergic stimulation since they were unaffected by propranolol, blocked by α-blockers in the potency order prazosin $\ll$ phentolamine $<$ yohimbine and mimicked by methoxamine. The α-adrenergic stimulation of phosphatidylinositol labelling did not require extracellular Ca^{2+}, which supports the hypothesis that an increased turnover of phosphatidylinositol is involved in α-adrenergic activation of Ca^{2+} entry. Insulin and the ionophore A23187 gave a small increase in ^{32}P labelling of phosphatidylinositol in Ca^{2+}-free medium containing 1 mM-EGTA. The increases due to insulin or ionophore A23187 were abolished if 2.5 mM-Ca^{2+} was added to medium containing EGTA. However, the increases in labelling of phosphatidylinositol due to α-adrenergic amines were still evident in medium containing EGTA and Ca^{2+}. Lipolytic agents such as corticotropin, dibutyryl cyclic AMP, adrenaline in the presence of phentolamine and isoproterenol decreased [^{32}P]P_i incorporation into phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid. This inhibitory effect may be secondary to accumulation of intracellular unesterified fatty acids, since it was decreased by incubating fewer cells in medium with 6 rather than 3% albumin and was restored by the addition of oleate to the medium. The incorporation of [^{32}P]P_i into phosphatidylcholine was unaffected by lipolytic agents. The data suggest that there is an inhibition of the synthesis of certain phospholipids in the presence of lipolytic agents, which may be secondary to intracellular accumulation of unesterified fatty acids.

In adipocytes β-adrenergic stimulators activate adenylate cyclase and lipolysis. The role of cyclic AMP as a second messenger for β-adrenergic stimulation is well established (Fain, 1979). Much less is known about α-adrenergic effects. There is little effect of α-adrenergic activation on glucose oxidation, cyclic AMP accumulation and lipolysis in rat fat-cells (Fain, 1979). However, Lawrence & Larner (1978) reported that activation of rat adipocyte phosphorylase and inactivation of glycogen synthase are produced by stimulation of α-adrenergic receptors. The inactivation of glycogen synthase by α-adrenergic catecholamines was dependent on the presence of extracellular Ca^{2+} and may be secondary to increased entry of Ca^{2+} (Lawrence & Larner, 1978).

Ca^{2+} has been postulated as a second messenger for α-adrenergic stimulation (Fain, 1979) and has also been implicated in insulin action (Fraser, 1975). In addition, an α-adrenergic stimulation of $^{42}K^+$ efflux from rat adipocytes has been seen, which may be secondary to an increase in cytosol Ca^{2+} (Perry & Hales, 1970). The mechanism by which α-adrenergic agents increase influx of extracellular Ca^{2+} is not known. Activation of α-adrenergic and muscarinic receptors is generally accompanied by increased incorporation of [^{32}P]P_i into phosphatidylinositol and it has been suggested that phosphatidylinositol turnover may be involved in the mobilization of Ca^{2+} (Michell, 1975, 1979; Jones & Michell, 1978).

Stein & Hales (1972) reported that in rat fat-cells, adrenaline stimulates the incorporation of [^{32}P]P_i into phosphatidylcholine and that incubation of fat-cells with propranolol and adrenaline decreased the formation of phosphatidylcholine, but increased incorporation into phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and cardiolipin.

0306-3283/80/030781-09 $1.50/1

De Torrontegui & Berthet (1966) found that insulin increased the incorporation of $[^{32}P]P_i$ from the medium into all fat-cell phospholipids, but especially phosphatidylinositol. The present experiments were designed to investigate the hormonal regulation of phospholipid metabolism in rat fat-cells with particular emphasis on the role of Ca^{2+}.

Materials and Methods

Chemicals

Adrenaline, isoproterenol, (±)-propranolol, insulin, glucagon, corticotropin and $N^6O^{2'}$-dibutyryl cyclic AMP were obtained from Sigma Chemical Co., yohimbine from Nutritional Biochemicals, crude collagenase (*Clostridium histolyticum*) from Worthington Biochemical Corp. (lot CLS 48A281), bovine serum albumin (fraction V) from Armour Pharmaceutical Co. (lot S11709) and $[^{32}P]P_i$ as orthophosphoric acid (carrier-free) from New England Nuclear. The following compounds were generously provided by the sources indicated: (+)-propranolol and (−)-propranolol, Ayerst Laboratories; phentolamine, CIBA Pharmaceutical Company; ionophore A23187, Eli Lilly Co.; prazosin, Pfizer; methoxamine, Burroughs Wellcome. Enzymes and coenzymes for glycerol determination were obtained from Boehringer Mannheim. Other reagents were analytical grade of the best quality available.

Preparation of adipocytes and assay of glycerol

Female Sprague–Dawley rats (200–250g; Charles River CD strain) were fed laboratory chow *ad libitum*. White fat-cells were obtained by enzymic digestion of parametrial adipose tissue by the procedure of Rodbell (1964). Pooled adipose tissue (approx. 25g) from five to six rats was minced with scissors and placed in small plastic bottles. Each bottle, containing approx. 13g of tissue and 20ml of Krebs–Ringer–Tris buffer supplemented with 3% albumin and 1mg of crude collagenase/ml, was incubated for 60min at 37°C in an orbital shaker. Krebs–Ringer–Tris buffer of the following composition was used in all experiments: 120mM-NaCl; 1.4mM-$CaCl_2$; 5.2mM-KCl; 1.4mM-$MgSO_4$; 5mM-Tris. The buffer was prepared daily and adjusted to pH7.4 at 37°C, with NaOH, after addition of albumin powder. At the end of 60min digestion, cells were filtered through one layer of nylon chiffon and washed twice with albumin buffer. Glycerol was analysed as previously described (Fain *et al.*, 1973). Fat-cells were counted with a light microscope as previously described (Malbon *et al.*, 1978).

Measurement of $[^{32}P]P_i$ incorporation into phospholipids

Fat-cells were incubated in plastic bottles containing 2ml of phosphate-free medium and $[^{32}P]P_i$ at a concentration of 10μCi/ml. Usually 2ml of packed cells (about 4×10^6 cells) was added to 2ml of medium. After incubation the cells were transferred to plastic tubes and centrifuged for 15s in a clinical centrifuge. The medium was removed and a 50μl portion was used for glycerol determination. The cells were gently resuspended in 5ml of Krebs–Ringer–Tris buffer containing 1% albumin. The medium was removed and 10ml of chloroform/methanol (2:1, v/v) was added to the contents of each tube, which was then shaken and filtered through filter paper. The single-phase system obtained was separated by the addition of 5ml of 10mM-KH_2PO_4. The upper layer (aqueous phase) was discarded. The chloroform layer was washed again with 5ml of 10mM-KH_2PO_4 in water. After removal of the upper phase, 1g of silicic acid and 5ml of chloroform were added to adsorb phospholipids. The mixture was vortex-mixed for 1min and centrifuged. The supernatant was decanted and the silicic acid granules were washed twice with 2.5ml of chloroform to remove triacylglycerols. The phospholipids were removed from the silicic acid by three extractions with 2.5ml of methanol. The methanol was evaporated in a vacuum centrifuge. The phospholipids were dissolved in chloroform/methanol (2:1, v/v), separated by two-dimensional t.l.c. on glass plates coated with silica gel H (Merck) as described by Fain & Berridge (1979). The first solvent system contained chloroform/methanol/water/28% NH_3 (26:14:1:1, by vol.) and the second solvent system chloroform/methanol/water/acetic acid (75:25:3:7, by vol.).

In the studies shown in Tables 3–7 the phospholipids were separated by one-dimensional chromatography using only the first solvent system. This procedure was equally suitable for the separation of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Phosphatidic acid could not be separated from phosphatidylserine, which resulted in a lower specific radioactivity for the phosphatidic acid area. However, since the uptake of radioactivity into phosphatidylserine is less than 6% of that into phosphatidic acid; the radioactivity in this spot is assumed to be phosphatidic acid. The uptake of $[^{32}P]P_i$ into cardiolipin and sphingomyelin is only reported in Table 1 because no effects of hormones could be detected and the incorporation of radioactivity into these spots was small and quite variable. Cardiolipin and sphingomyelin were separated from the other phospholipids by one-dimensional chromatography.

Lipids were identified by using I_2-vapour staining and by radioautography with X-ray film. Phos-

phatidic acid was detected by radioautography because the amount of this phospholipid was insufficient to be seen with I_2 staining. The phosphorus content of phospholipids was determined by the micromodification of the procedure of Bartlett (1959) after acid hydrolysis of silica-gel scrapings containing each individual phospholipid. Samples of the hydrolysate were counted to determine the amount of radioactivity present in each phospholipid.

Since some variability was observed from day to day in the incorporation of $[^{32}P]P_i$ into phospholipids, the results are expressed as a percentage of control specific radioactivity of each phospholipid. Each experiment was repeated at least three times on different days and the results are expressed as means ± S.E.M.

Results

Composition and incorporation of P_i into adipocytes

Incorporation of $[^{32}P]P_i$ into phospholipids was linear for at least 2h (results not shown). A time period of 1h was selected for the incubations in this report because at this time there is substantial incorporation of radioactive phosphate into major phospholipids (Table 1). The phospholipid composition of fat-cells (Table 1) resembles that of other tissues (White, 1973). Phosphatidylcholine and phosphatidylethanolamine contribute about 70% of the phosphate present in phospholipids. The incorporation of radioactive phosphate was primarily into phosphatidylcholine, with lesser amounts in phosphatidic acid and phosphatidylinositol (Table 1). Some incorporation of $[^{32}P]P_i$ was seen in phosphatidylethanolamine, but there was very little in phosphatidylserine, cardiolipin or sphingomyelin. No clear-cut change in phospholipid composition was produced by any of the agents employed over the 1h incubation used in the present studies.

Effect of adrenergic amines on the incorporation of $[^{32}P]P_i$ into phospholipids

Adrenaline produced marked changes in the incorporation of $[^{32}P]P_i$ into phospholipids (Fig. 1).

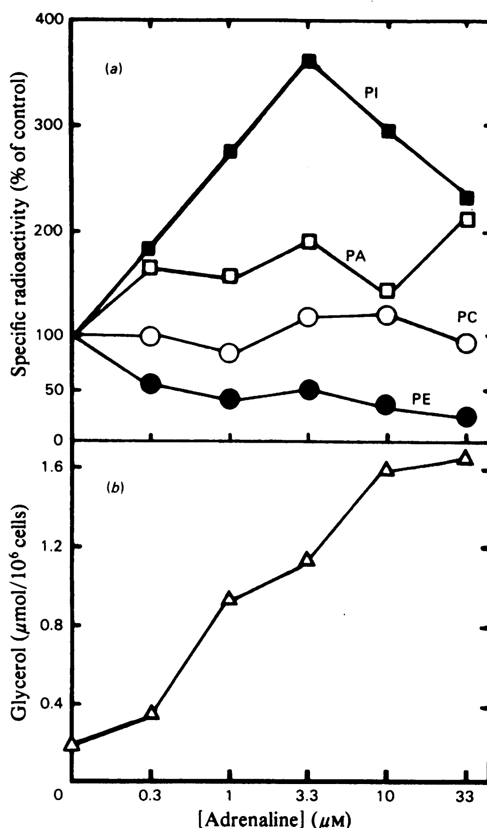

Fig. 1. *Dose–response curve for effects of adrenaline on $[^{32}P]P_i$ incorporation into phospholipids and lipolysis*
The specific radioactivities of the phospholipid (c.p.m./µg of phosphate) under control conditions were similar to those in Table 1 and taken as 100%. Values in the presence of various concentrations of adrenaline are shown as percentages of the control values without catecholamine. The values of $[^{32}P]P_i$ uptake into phospholipids are shown in (*a*). Results for phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are means for three separate experiments. The effect of adrenaline on lipolysis is shown in (*b*).

Table 1. *Incorporation of $[^{32}P]P_i$ into phospholipids of isolated fat-cells*
Fat-cells (4×10^6) were incubated for 60min in 2ml of buffer containing 3% albumin and $[^{32}P]P_i$ at a concentration of 10µCi/ml. The results are expressed as means ± S.E.M. for eight different experiments. The recovered phospholipids contained 8.1 ± 1.0µg of phosphate; the total incorporation was 19440 ± 2600c.p.m. (means ± S.E.M.).

	Phosphate (% of total)	Incorporation of $[^{32}P]P_i$ (% of total)	Specific radioactivity (c.p.m./µg of P_i)
Phosphatidylethanolamine	33.0 ± 1.9	4.7 ± 0.6	440 ± 100
Phosphatidylcholine	39.1 ± 2.0	56.6 ± 6.9	3795 ± 840
Phosphatidylinositol	8.5 ± 0.8	13.6 ± 3.2	3825 ± 580
Phosphatidic acid	2.3 ± 0.4	20.9 ± 3.9	25700 ± 3000
Phosphatidylserine	7.3 ± 0.7	1.3 ± 0.4	420 ± 100
Cardiolipin	6.2 ± 0.6	0.7 ± 0.1	325 ± 45
Sphingomyelin	3.6 ± 0.5	1.4 ± 0.2	900 ± 185

The incorporation of label into phosphatidylinositol was increased by low concentrations of adrenaline (1–3.3 μM). Higher concentration of adrenaline did not result in any further stimulation of phosphatidylinositol labelling. Phosphatidic acid labelling was only slightly stimulated by adrenaline (Fig. 1). The specific radioactivity of phosphatidylcholine was not appreciably modified by adrenaline, whereas that of phosphatidylethanolamine was markedly decreased. An inverse relationship was observed between lipolysis and the specific radioactivity of phosphatidylethanolamine (Fig. 1).

Adrenaline is both an α- and β-adrenergic agonist. To determine which actions of adrenaline were due to α-stimulation and which to β-activation, pure α- and β-adrenergic agonists and antagonists were employed. The α-adrenergic blocking agent phentolamine did not affect significantly the incorporation of label into phospholipids or lipolysis due to adrenaline (Table 2). However, the marked increase in phosphatidylinositol labelling produced by adrenaline was blocked by phentolamine. There was actually a decrease in the specific radioactivity of phosphatidylinositol and all other phospholipids in the presence of adrenaline plus phentolamine (Table 2). Similar results were seen with isoproterenol, a β-adrenergic agonist, except that phosphatidylcholine was unaltered (Table 2). Propranolol, a β-adrenergic blocking agent, alone increased the incorporation of $[^{32}P]P_i$ into phosphatidylinositol and phosphatidic acid and this effect was additive to that of adrenaline (Table 2). These results suggest that the increase in phosphatidylinositol labelling observed with adrenaline is an α-action. In support of this conclusion, the α-adrenergic agonist methoxamine also increased the specific radioactivity of phosphatidylinositol (Table 2).

Characterization of the α-receptor involved in the phosphatidylinositol effect in fat-cells.

Considerable evidence indicates that α-receptors can be divided into at least two subclasses, i.e. α_1 and α_2 (Berthelsen & Pettinger, 1977; U'Prichard *et al.*, 1978; Hoffman *et al.*, 1979; Wood *et al.*, 1979). The use of selective agonists and antagonists have clarified this point. It has been shown that phentolamine is an α-adrenergic antagonist equipotent at α_1- and α_2-receptors (Hoffman *et al.*, 1979), prazosin is 3–4 orders of magnitude more potent at α_1- than at α_2-receptors (U'Prichard *et al.*, 1978; Hoffman *et al.*, 1979) and yohimbine has a 500-fold greater affinity for α_2-than for α_1-receptors (Hoffman *et al.*, 1979). On the other hand, methoxamine is considered an α_1-agonist, whereas clonidine is α_2 (Berthelson & Pettinger, 1977).

The action of α-adrenolytic agents on the adrenaline-mediated increase in phosphatidylinositol labelling was tested (Table 3). Prazosin completely blocked the effect of adrenaline (10 μM) at a concentration of 0.1 μM, whereas 100 μM-yohimbine or 10 μM-phentolamine were required to produce the same inhibition. α-Adrenergic blockers did not modify either the basal incorporation of $[^{32}P]P_i$ into phosphatidylinositol (Table 3) or adrenaline-induced lipolysis (results not shown). On the other hand, methoxamine produced a 2-fold increase in phosphatidylinositol labelling (Table 2), whereas clonidine (1–100 μM) produced no effect on this parameter (results not shown).

Effect of lipolytic agents on the incorporation of P_i into phospholipids and role of unesterified fatty acids

The effects of non-adrenergic lipolytic agents are shown in Table 4. Both corticotropin and dibutyryl

Table 2. *Effect of α- and β-adrenergic agonists and antagonists on the incorporation of $[^{32}P]P_i$ into fat-cell phospholipids*

Fat-cells (4×10^6) were incubated for 60 min in 2 ml of buffer containing 3% albumin and 10 μCi of $[^{32}P]P_i$/ml. The results are expressed as means ± S.E.M. for three experiments performed on different days. The effects of added agents are expressed as a percentage of the control value for uptake of ^{32}P and as the absolute values for glycerol release.

Treatment	Specific radioactivity: Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylinositol	Phosphatidic acid	Glycerol release (nmol/10^6 cells)
	(c.p.m./μg of P_i)				
None	335 ± 85	3635 ± 830	3505 ± 915	28745 ± 3390	150 ± 10
	(% of control)				
Adrenaline (3.3 μM)	48 ± 25	120 ± 36	281 ± 94	141 ± 14	1275 ± 163
Phentolamine (30 μM)	121 ± 24	96 ± 2	146 ± 45	106 ± 45	165 ± 35
Adrenaline (3.3 μM) + phentolamine	30 ± 15	67 ± 5	37 ± 13	68 ± 21	1325 ± 320
Isoproterenol (10 μM)	24 ± 9	100 ± 14	34 ± 16	53 ± 14	1535 ± 95
(±)-Propranolol (30 μM)	147 ± 31	98 ± 15	340 ± 146	225 ± 87	220 ± 45
Adrenaline (3.3 μM) + (±)-propranolol	193 ± 33	79 ± 3	1171 ± 241	297 ± 20	205 ± 35
Methoxamine (100 μM)	110 ± 20	84 ± 17	203 ± 17	154 ± 81	175 ± 15

cyclic AMP produced significant decreases in the specific radioactivities of phosphatidylethanolamine and phosphatidylinositol. The specific radioactivity of phosphatidic acid was decreased by corticotropin and 2.5 mM, but not 0.5 mM-dibutyryl cyclic AMP (Table 4). The incorporation of $[^{32}P]P_i$ into phosphatidylcholine was not affected by the lipolytic agents (Table 4). The effects of corticotropin and dibutyryl cyclic AMP were identical with those of isoproterenol (Table 4).

The accumulation of unesterified fatty acids intracellularly can uncouple oxidative phosphorylation (Angel *et al.*, 1971). Since large amounts of cells were employed in these studies, it is possible that the unesterified fatty acids released by adipocytes in the presence of lipolytic agents may saturate the binding sites for unesterified fatty acids on albumin and accumulate intracellularly; such accumulation of unesterified fatty acids may be responsible for the decreased incorporation of $[^{32}P]P_i$ into phospholipids. To test this point, fewer cells (2×10^6) were incubated in medium containing larger concentrations of albumin (6%). Under these conditions the decrease in phospholipid labelling produced by lipolytic agents was avoided (Table 5). The effects of lipolytic agents on $[^{32}P]P_i$ incorporation were restored if oleate was added to the medium (Table 5). Basal and hormone-stimulated lipolysis were higher if fewer cells were incubated in the medium with a higher concentration of albumin (compare Tables 2 and 4 with Table 5). The activation of lipolysis due to hormones, but not that due to dibutyryl cyclic AMP, was decreased by the addition of oleate (Table 5). Basal incorporation of $[^{32}P]P_i$ into phospholipids was not significantly affected by increasing the concentration of albumin in the medium or adding unesterified fatty acids (Table 5).

Table 3. *Effect of α-adrenergic antagonists on the action of adrenaline and methoxamine on the incorporation of radioactive phosphate into phosphatidylinositol*

Fat-cells (2×10^6) were incubated for 60 min in 2 ml of medium containing 6% albumin and 10 μCi of $[^{32}P]P_i$/ml. Basal specific radioactivity of phosphatidylinositol was 5975 ± 380 c.p.m./μg of P_i (mean ± S.E.M. for four experiments). The values are means ± S.E.M. for four experiments.

	Specific radioactivity (% of control)	
Antagonist	Basal	Adrenaline (10 μM)
None	100	490 ± 50
Prazosin (0.01 μM)		355 ± 64
Prazosin (0.1 μM)	105 ± 10	108 ± 10
Prazosin (1 μM)	102 ± 1	73 ± 5
Yohimbine (1 μM)		437 ± 66
Yohimbine (10 μM)	106 ± 12	313 ± 71
Yohimbine (100 μM)	104 ± 14	114 ± 17
Phentolamine (1 μM)		335 ± 86
Phentolamine (10 μM)	94 ± 5	83 ± 9

Effect of propranolol on the incorporation of P_i into phospholipids

In an effort to clarify the action of propranolol on the incorporation of phosphate into phosphatidylinositol, the action of the optical isomers of propranolol was tested. The (+)-isomer does not block the lipolytic response due to adrenaline (Table 6). However, (+)-propranolol increased phosphatidylinositol labelling and this action was additive to that of adrenaline (Table 6). The (−)-isomer of propranolol produced the same effects as the racemic mixture on lipolysis and incorporation of $[^{32}P]P_i$ into phosphatidylinositol (cf. Tables 2 and 6).

Effect of insulin, adenosine, and ionophore A23187 on the incorporation of P_i into phospholipids

Adenosine, phenylisopropyladenosine and carbamoylcholine did not modify the incorporation of

Table 4. *Effect of corticotropin and dibutyryl cyclic AMP on the incorporation of $[^{32}P]P_i$ into fat-cell phospholipids*

Fat-cells (4×10^6) were incubated for 60 min in 2 ml of buffer containing 3% albumin and 10 μCi of $[^{32}P]P_i$/ml. The values are means ± S.E.M. for three separate experiments.

	Specific radioactivity				
Treatment	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidic acid + phosphatidylserine	Glycerol release (nmol/10⁶ cells)
	(c.p.m./μg of P_i)				
None	790 ± 170	3990 ± 1110	4175 ± 385	6255 ± 1610	150 ± 15
	(% of control)				
Corticotropin (10 μg/ml)	41 ± 16	108 ± 6	63 ± 13	136 ± 55	705 ± 145
$N^6O^{2'}$-Dibutyryl cyclic AMP (0.5 mM)	99 ± 3	111 ± 9	86 ± 10	94 ± 26	395 ± 100
N^6O-Dibutyryl cyclic AMP (2.5 mM)	23 ± 6	124 ± 30	13 ± 2	22 ± 9	1920 ± 25

[^{32}P]P$_i$ into any phospholipid (results not shown). In three experiments the phosphatidylinositol specific radioactivity of cells incubated in regular buffer containing Ca^{2+} as a percentage of the control value was 196, 254, and 358 in the presence of insulin and 121, 156, and 234 in the presence of ionophore

Table 5. *Effect of 6% albumin and unesterified fatty acids on the changes in incorporation of [^{32}P]P$_i$ into fat-cell phospholipids by lipolytic agents*

Fat-cells (2×10^6) were incubated for 60min in 3ml of buffer containing 6% albumin either without or with added oleate. Unesterified-fatty acid-to-albumin molar ratio was 0.8 for 6% albumin without and 2.6 for 6% albumin with added oleate. The values are means ± S.E.M. for three experiments.

Treatment	Added oleate	Specific radioactivity: Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidic acid + phosphatidylserine	Glycerol release (nmol/10^6 cells)
		(c.p.m./μg of P$_i$)				
None	–	770 ± 230	4990 ± 930	4500 ± 800	8210 ± 1260	265 ± 30
		(% of control)				
Adrenaline (3.3 μM)	–	147 ± 20	174 ± 20	517 ± 73	188 ± 7	2665 ± 280
Adrenaline (3.3 μM) + phentolamine (30 μM)	–	77 ± 20	140 ± 28	131 ± 21	102 ± 7	2770 ± 300
Isoproterenol (10 μM)	–	136 ± 31	183 ± 40	117 ± 33	96 ± 6	2870 ± 290
Corticotropin (10 μg/ml)	–	94 ± 12	122 ± 23	107 ± 31	86 ± 12	980 ± 40
$N^6O^{2'}$-Dibutyryl cyclic AMP (2.5 μM)	–	30 ± 9	145 ± 31	38 ± 12	43 ± 8	3530 ± 30
		(c.p.m./μg of P$_i$)				
None	+	1020 ± 20	5080 ± 990	3820 ± 540	7270 ± 1320	225 ± 5
		(% of control)				
Adrenaline (3.3 μM)	+	77 ± 1	102 ± 3	479 ± 74	141 ± 22	1565 ± 30
Adrenaline (3.3 μM) + phentolamine (30 μM)	+	51 ± 6	88 ± 12	41 ± 3	78 ± 11	1705 ± 100
Isoproterenol (10 μM)	+	59 ± 8	126 ± 8	66 ± 4	75 ± 13	1990 ± 25
Corticotropin (10 μg/ml)	+	60 ± 2	84 ± 9	50 ± 1	55 ± 4	850 ± 40
$N^6O^{2'}$-Dibutyryl cyclic AMP (2.5 μM)	+	21 ± 6	72 ± 5	16 ± 1	14 ± 2	3175 ± 50

Table 6. *Effect of adrenaline and propranolol on the incorporation of [^{32}P]P$_i$ into fat-cell phospholipids*

Fat-cells (4×10^6) were incubated for 60min in 2ml of buffer containing 3% albumin and 10 μCi of [^{32}P]P$_i$/ml. The values are means ± S.E.M. for three paired experiments.

Treatment	Specific radioactivity: Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidic acid + phosphatidylserine	Glycerol release (nmol/10^6 cells)
	(c.p.m./μg of P$_i$)				
None	805 ± 225	3640 ± 1145	4685 ± 725	6520 ± 1390	160 ± 9
	(% of control)				
Adrenaline (3.3 μM)	66 ± 21	134 ± 35	302 ± 118	238 ± 65	984 ± 146
(+)-Propranolol (10 μM)	104 ± 9	68 ± 7	151 ± 25	127 ± 13	150 ± 5
Adrenaline (3.3 μM) + (+)-propranolol (10 μM)	99 ± 19	123 ± 23	605 ± 124	192 ± 19	1155 ± 205
(+)-Propranolol (30 μM)	133 ± 34	92 ± 20	263 ± 51	170 ± 41	185 ± 30
Adrenaline (3.3 μM) + (+)-propranolol (30 μM)	89 ± 21	96 ± 21	636 ± 157	262 ± 96	1445 ± 25
(−)-Propranolol (10 μM)	110 ± 30	139 ± 13	312 ± 89	171 ± 34	205 ± 25
Adrenaline (3.3 μM) + (−)-propranolol (10 μM)	151 ± 19	130 ± 11	616 ± 228	261 ± 64	155 ± 15
(−)-Propranolol (30 μM)	137 ± 17	96 ± 4	302 ± 72	168 ± 38	190 ± 35
Adrenaline (3.3 μM) + (−)-propranolol (30 μM)	135 ± 23	104 ± 15	840 ± 117	362 ± 117	200 ± 40

A23187. These results are similar to those shown in Table 7 for cells incubated in Ca^{2+}-free buffer containing 1 mM-EGTA. However, insulin increased slightly the labelling of all phospholipids in regular buffer (results not shown).

Role of Ca^{2+} in the labelling of phospholipids with P_i

The role of extracellular Ca^{2+} in the action of α-adrenergic agents, propranolol, insulin and ionophore A23187 on phospholipid labelling was tested by incubating the cells in medium with or without added Ca^{2+} in which 1 mM-EGTA was present. The specific radioactivity of phosphatidylinositol was lower in cells incubated in Ca^{2+}-free buffer containing 1 mM-EGTA (the values for cells incubated in the absence of Ca^{2+} were 44, 72, and 82% of those in the presence of Ca^{2+} for the three experiments shown in Table 7). Incubation of fat-cells in the absence of Ca^{2+} and presence of 1 mM-EGTA did not decrease but actually enhanced the increases due to insulin and ionophore A23187 of [^{32}P]P_i incorporation into phosphatidylinositol (Table 7). The values for cells incubated with insulin as a percentage of the control value were 367, 197 and 208 in the absence of Ca^{2+} and 66, 83 and 92 in the presence of Ca^{2+} for the three experiments shown in Table 7. Similarly the values for cells incubated with ionophore A23187 as a percentage of the control value were 236, 201 and 195 in the absence of Ca^{2+} and 74, 60 and 79 in the presence of Ca^{2+}. These data clearly indicate that if fat-cells are incubated with 1 mM-EGTA and Ca^{2+}, the stimulation by both insulin and ionophore A23187 of phosphatidylinositol labelling that is seen in regular buffer or Ca^{2+}-free buffer plus EGTA is lost.

The effects of α-catecholamines and propranolol were not dependent on extracellular Ca^{2+}. The increases in specific radioactivity of phosphatidylinositol due to 30 μM-propranolol as a percentage of the control values were 217, 282, and 204 for cells incubated in the absence of Ca^{2+} and 125, 120 and 217 for cells incubated with Ca^{2+} for the studies shown in Table 7. The increases due to methoxamine or adrenaline in the presence of propranolol were unaffected by EGTA or EGTA plus Ca^{2+} (Table 7).

Recently, Hirata *et al.* (1979) reported that β-adrenergic agonists increase the methylation of phospholipids in rat reticulocyte 'ghosts'. The possibility that the decrease in the amount of labelled phosphatidylethanolamine might be due in part to its conversion into phosphatidylcholine was studied by incubating cells in medium containing L-[*Me*-^{3}H]methionine. No increase in the incorporation of ^{3}H was observed with β-adrenergic amines. In fact, in two of the three experiments performed a small decrease was produced (results not shown).

Table 7. *Effect of Ca^{2+} on the actions of α-adrenergic agents, insulin and ionophore A23187 on the incorporation of [^{32}P]P_i into fat-cell phospholipids*

Fat-cells (4×10^6) were incubated for 60 min in 2 ml of buffer containing 3% albumin, 1 mM-EGTA and 10 μCi of [^{32}P]P_i/ml either without or with 2.5 mM-Ca^{2+}. The values are means ± S.E.M. for three paired experiments.

Treatment	Added $CaCl_2$ (mM)	Specific radioactivity: Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylinositol	Phosphatidic acid + phosphatidylserine
		(c.p.m./μg of P_i)			
None	0	680 ± 240	4240 ± 1160	4450 ± 1150	8375 ± 3645
		(% of control)			
(±)-Propranolol (30 μM)	0	121 ± 21	77 ± 17	234 ± 24	157 ± 7
Adrenaline (3.3 μM) + (±)-propranolol (30 μM)	0	135 ± 33	113 ± 24	404 ± 66	181 ± 36
Methoxamine (100 μM)	0	161 ± 21	117 ± 21	239 ± 26	175 ± 29
Insulin (200 μ-units/ml)	0	149 ± 30	135 ± 6	257 ± 55	207 ± 7
Ionophore A23187 (10 μM)	0	132 ± 16	70 ± 27	210 ± 13	200 ± 47
		(c.p.m./μg of P_i)			
None	2.5	680 ± 85	4760 ± 830	6620 ± 775	8350 ± 2490
		(% of control)			
(±)-Propranolol (30 μM)	2.5	108 ± 17	76 ± 6	154 ± 31	118 ± 19
Adrenaline (3.3 μM) + (±)-propranolol (30 μM)	2.5	145 ± 31	79 ± 3	440 ± 43	192 ± 48
Methoxamine (100 μM)	2.5	74 ± 13	77 ± 18	169 ± 11	121 ± 30
Insulin (200 μ-units/ml)	2.5	85 ± 7	100 ± 14	80 ± 7	72 ± 7
Ionophore A23187 (10 μM)	2.5	77 ± 15	80 ± 14	71 ± 5	85 ± 11

Discussion

The present results indicate that the incorporation of [^{32}P]P_i into phosphatidylinositol is markedly increased by adrenaline. This is an α-adrenergic effect, since it was not abolished by propranolol, but was blocked by prazosin, phentolamine and yohimbine and mimicked by methoxamine. In addition the order of potency of adrenolytic antagonists (prazosin $\gg$ phentolamine $>$ yohimbine) and the fact that this effect is mimicked by methoxamine, but not by clonidine, clearly indicate that the catecholamine receptor involved is an α_1-receptor. Similar results have been seen in rat pineal glands, where prazosin was a potent inhibitor of the catecholamine-induced increases in phosphatidylinositol synthesis (Smith *et al.*, 1979).

The α-adrenergic increase in phosphatidylinositol labelling was also observed in Ca^{2+}-free medium containing EGTA; this indicates that it is independent of extracellular Ca^{2+}. Lawrence & Larner (1978) reported that methoxamine was unable to inactivate adipocyte glycogen synthase in Ca^{2+}-free medium containing EGTA. In addition, the cation ionophore A23187 mimicked this effect of methoxamine on glycogen synthase. Our data are consistent with the hypothesis that phosphatidylinositol metabolism plays an important role in Ca^{2+} gating (Michell, 1975; Berridge & Fain, 1979; Fain & Berridge, 1979). Alternatively, catecholamine effects on Ca^{2+} gating and phosphatidylinositol metabolism may be independent. However, there is a close association between α-adrenergic regulation of phosphatidylinositol turnover and Ca^{2+} entry in many systems (Michell, 1975, 1979; Jones & Michell, 1978).

Stein & Hales (1974) found that insulin increased the incorporation of phosphate into rat fat-cell phospholipids, which was attributed to an increase in the specific radioactivity of ATP. The increase due to insulin in the labelling of all phospholipids in regular buffer could be due to changes in the specific radioactivity of ATP, as already shown by Stein & Hales (1974). However, the increases in phosphatidylinositol labelling due to α-adrenergic catecholamines are apparently unrelated to changes in the specific radioactivity of ATP, since similar increases were not seen in labelling of other phospholipids. The same argument applies to the inhibition of [^{32}P]P_i incorporation into certain phospholipids by agents that activate lipolysis including β-catecholamines. The only way to explain the effects of either α- or β-catecholamines on phospholipid labelling through changes in the specific radioactivity of ATP is to postulate separate ATP pools involved in the synthesis of each phospholipid.

In medium containing 1mM-EGTA plus 2.5mM-Ca^{2+} insulin did not increase the labelling of phosphatidylinositol. However, we observed that under these conditions the ability of insulin to stimulate [1-^{14}C]glucose oxidation and inhibit lipolysis was unaltered (results not shown). The explanation for these results is not apparent, but does indicate that the effects of α-adrenergic agonists on phosphatidylinositol labelling occur by mechanisms separate from those involved in any effect of insulin or ionophore A23187 on phospholipid labelling.

The action of propranolol on phosphatidylinositol labelling seems to be independent of its ability to block β-adrenergic receptors since the (+)-isomer that is inactive in this respect produced nearly the same action as the active isomer on phosphatidylinositol labelling. This effect is independent of the presence of Ca^{2+} in the medium and is probably related to its local-anaesthetic properties. In fact local anaesthetics and propranolol enhanced the incorporation of labelled phosphate into phosphatidylinositol and phosphatidic acid in rat pineal glands (Eichberg *et al.*, 1978). These lipid-soluble cationic drugs exert their effects on phospholipid metabolism by redirection of synthesis towards phosphatidylinositol probably owing to inhibition of phosphatidate phosphohydrolase (Allan & Michell, 1975; Brindley & Bowley, 1975; Eichberg *et al.*, 1978).

The ability of lipolytic agents to decrease the labelling of all phospholipids may be related to an intracellular accumulation of unesterified fatty acids, since it was prevented by increasing the concentration of albumin in the medium and the presence of medium to which fatty acids were added restored it. The ability of adrenaline to decrease the incorporation of [^{32}P]P_i into phosphatidylethanolamine was not observed in the presence of (+)-propranolol. No clear explanation exists to this finding, but it may be related to the local-anaesthetic properties of this compound.

Stein & Hales (1972) reported that adrenaline markedly increased the incorporation of [^{32}P]P_i into phosphatidylcholine and this effect was blocked by propranolol. Stein (1975, 1977) subsequently reported that adrenaline had no effect on the specific radioactivity of ATP, markedly decreased ATP content and increased the incorporation of [^{32}P]P_i into CDP-choline, but not into CDP-ethanolamine. The decrease in ATP due to adrenaline in the experiments of Stein (1975, 1977) and Stein & Hales (1972) may have activated cholinephosphotransferase, which is known to be activated in liver by a decrease in ATP (Sribney *et al.*, 1976). Our results are different, since we saw no increase in the incorporation of [^{32}P]P_i into phosphatidylcholine in the presence of β-catecholamines or other lipolytic agents. However, it was interesting that under conditions in which the uptake of [^{32}P]P_i into phosphatidic acid, phosphatidylethanolamine and phos-

phatidylinositol was markedly decreased by lipolytic agents there was little effect on phosphatidylcholine (Table 5). This suggests that the formation of this phospholipid is not regulated by the same factors that affect the other phospholipids.

J. A. G.-S. is an International Fellow sponsored by the N.I.H. (Fellowship no. 1FO 5 TWO2691-01). This work was supported by United States Public Health Service Research grants AM 10149 and AM 21470 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health. We thank Dr. Margaret E. M. Tolbert and Dr. Alice C. White for reading and criticizing the manuscript and Mrs. Gerlinde Celona for secretarial assistance.

References

Allan, D. & Michell, R. H. (1975) *Biochem. J.* **148**, 471–478

Angel, A., Desai, K. S. Halperin, M. L. (1971) *J. Lipid Res.* **12**, 203–212

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468

Berridge, M. J. & Fain, J. N. (1979) *Biochem. J.* **178**, 59–69

Berthelsen, S. & Pettinger, W. A. (1977) *Life Sci.* **21**, 595–606

Brindley, D. N. & Bowley, M. (1975) *Biochem. J.* **148**, 461–469

De Torrontegui, G. & Berthet, J. (1966) *Biochim. Biophys. Acta* **116**, 477–481

Eichberg, J., Killion, J., Guerrant, G. & Hauser, G. (1978) in *Cyclitols and Phosphoinositides* (Wells, W. W. & Eisenberg, F., eds.), pp. 183–199, Academic Press, New York

Fain, J. N. (1979) *Biochem. Actions Horm.* **7**, in the press

Fain, J. N. & Berridge, M. J. (1979) *Biochem. J.* **178**, 45–58

Fain, J. N., Czech, M. P. & Saperstein, R. (1973) in *Methods in Investigative and Diagnostic Endocrinology, Peptide Hormones* (Berson, S. A. & Yalow, R. S., eds.), pp. 267–273, North Holland Publishing Co., Amsterdam

Fraser, T. R. (1975) *Proc. R. Soc. Med.* **68**, 785–791

Hirata, F., Strittmatter, W. J. & Axelrod, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 368–372

Hoffman, B. B., DeLean, A., Wood, C. L., Schocken, D. D. & Lefkowitz, R. F. (1979) *Life Sci.* **24**, 1739–1746

Jones, L. M. & Michell, R. H. (1978) *Biochem. Soc. Trans.* **6**, 673–688

Lawrence, J. C. & Larner, J. (1978) *Mol. Pharmacol.* **14**, 1079–1091

Malbon, C. C., Moreno, F. J., Cabelli, R. J. & Fain, J. N. (1978) *J. Biol. Chem.* **253**, 671–678

Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147

Michell, R. H. (1979) *Trends Biochem. Sci.* **4**, 128–131

Perry, M. C. & Hales, C. N. (1970) *Biochem. J.* **117**, 615–621

Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380

Smith, T. L., Eichberg, J. & Hauser, G. (1979) *Life Sci.* **24**, 2179–2184

Sribney, M., Knowles, C. L. & Lyman, E. (1976) *Biochem. J.* **156**, 507–511

Stein, J. M. (1975) *Biochem. Pharmacol.* **24**, 1659–1662

Stein, J. M. (1977) *Biochem. Pharmacol.* **26**, 531–541

Stein, J. M. & Hales, C. N. (1972) *Biochem. J.* **128**, 531–541

Stein, J. M. & Hales, C. N. (1974) *Biochim. Biophys. Acta* **337**, 41–49

U'Prichard, D., Charness, M. E., Robertson, D. & Snyder, S. H. (1978) *Eur. J. Pharmacol.* **50**, 87–89

White, D. A. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 441–448, Elsevier Scientific Publishing Co., Amsterdam

Wood, C. L., Arnett, C. D., Clarke, W. R., Tasai, B. S. & Lefkowitz, R. J. (1979) *Biochem. Pharmacol.* **28**, 1277–1282