Stimulation of phosphoinositide metabolism in hamster brown adipocytes exposed to α_1 -adrenergic agents and its inhibition with phorbol esters

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The present experiments were undertaken to investigate the role of the phosphoinositides phosphatidylinositol 4-phosphate (PtdIns-4-*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-*P*₂) in the α_1 -adrenergic stimulation of respiration in isolated hamster brown adipocytes. Exposure of isolated brown adipocytes to the α -adrenergic-receptor agonist phenylephrine provoked a breakdown of 30–50% of the PtdIns-4-*P* and PtdIns-4,5-*P*₂ after prelabelling of the cells with [³²P]P_i. Coincident with the breakdown of phosphoinositides was an accumulation of labelled phosphatidic acid, which continued for the duration of the cell incubation. The time course of phosphoinositide breakdown was defined more precisely by pulse–chase experiments. Under these conditions, phenylephrine caused radioactivity in phosphatidylinositol, PtdIns-4-*P* and PtdIns-4,5-*P*₂ to fall by more than 50% within 30 s and to remain at the depressed value for the duration of the incubation (10 min). This phospholipid response to α -adrenergic stimulation was blocked by exposure of the cells to phorbol 12-myristate 13-acetate (PMA); likewise phenylephrine stimulation of respiration was prevented by PMA. β -Adrenergic stimulation of respiration and inhibition of respiration by 2-chloroadenosine and insulin were, however, unaffected by treatment with PMA. On the assumption that PMA is acting in these cells as an activator of protein kinase C, these results suggest the selective interruption of α -adrenergic actions in brown adipocytes by activated protein kinase C. These findings suggest that breakdown of phosphoinositides is an early event in α -adrenergic stimulation of brown adipocytes which may be important for the subsequent stimulation of respiration. The results from the pulse–chase studies also suggest, however, that phenylephrine-stimulated breakdown of inositol phospholipids is a short-lived event which does not appear to persist for the entire period of exposure to the α_1 -adrenergic ligand.

INTRODUCTION

Respiration by brown adipocytes is increased by catecholamines acting on both α - and β -adrenergic receptors (Bukowiecki, 1984; Schimmel *et al.*, 1983b). The quantitatively more prominent β -adrenergic response has been investigated extensively, and persuasive evidence implicates activation of adenylate cyclase and a cyclic-AMP-dependent activation of lipolysis as being the cause of the increased mitochondrial oxygen consumption (Bukowiecki, 1984). By contrast, the cellular mechanisms linking occupancy of α -adrenergic receptors with increased mitochondrial respiration are largely unknown. Those studies reported to date have implicated α_1 receptors (Schimmel *et al.*, 1983b; Mohell *et al.*, 1984) and suggested a requirement for extracellular Ca²⁺ for expression of this response (Schimmel *et al.*, 1983b). Although further progress in identification of those cellular–biochemical mechanisms underlying the α_1 -adrenergic regulation of brown adipocytes has been limited, investigations of other systems, particularly liver cells, have identified the hydrolysis of phosphatidylinositol (PtdIns) or the phosphoinositides, phosphatidylinositol 4-phosphate (PtdIns-4-*P*) and phosphatidylinositol 4,5-

bisphosphate (PtdIns-4,5-*P*₂), as primary events in the action of α_1 -adrenergic ligands (Exton, 1985; Williamson *et al.*, 1985). This alteration in phospholipids appears to be a fundamental membrane transducing system activated as a consequence of a wide variety of ligand–receptor interactions (Berridge, 1984). The products of PtdIns-4,5-*P*₂ hydrolysis, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, may function as intracellular second messengers of hormone action: the diacylglycerol by activating protein kinase C (Nishizuka, 1984), and the inositol 1,4,5-trisphosphate by mobilizing intracellular Ca²⁺ (Burgess *et al.*, 1984).

One concomitant of the accelerated metabolism of the inositol phospholipids is an increased labelling of phosphatidic acid and PtdIns with [³²P]P_i, and this has been determined to occur in brown adipocytes exposed to α -adrenergic ligands (Garcia-Sainz *et al.*, 1980; Mohell *et al.*, 1984). Although this observation is consistent with an α -adrenergic-promoted breakdown of the phosphoinositides in brown fat, Mohell *et al.* (1984) favoured the interpretation of an α -adrenergic-promoted synthesis of the phospholipids *de novo*. Farese *et al.* (1984) have similarly suggested synthesis of phospholipids *de novo* as the mechanism of insulin stimulation of labelling of

Abbreviations used: PtdIns, PtdIns-4-*P*, PtdIns-4,5-*P*₂, phosphatidylinositol, its 4-phosphate and 4,5-bisphosphate; PMA, 4 β -phorbol 12-myristate 13-acetate.

white-adipose-tissue phospholipids. Hence, the possible involvement of an α -adrenergic-activated hydrolysis of PtdIns-4,5- P_2 in adipose-tissue cells remains to be established. The present study was undertaken to investigate the effect of phenylephrine on the breakdown of the phosphoinositides in brown adipocytes. We also report results of experiments designed to investigate the actions of protein kinase C in brown adipocytes by determining the effects of the phorbol ester 4 β -phorbol 12-myristate 13-acetate (PMA). This agent is believed to activate protein kinase C directly by substituting for the natural activator, 1,2-diacylglycerol (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983).

METHODS

Preparation of adipocytes

Male golden hamsters (80–120 g) were purchased from Charles River, Lakeview, NJ, U.S.A., and housed in a climate-regulated vivarium for at least 1 week before use. The animals were killed either by CO₂ narcosis or with sodium pentobarbital, and the interscapular and cervical brown-fat depots were excised. The white adipose and connective tissues were removed, and the brown fat was minced with scissors for 5 min. Brown adipocytes were prepared from the minced tissues by digestion with crude bacterial collagenase (Type I, from Cooper Biologicals, Malvern, PA, U.S.A.) present at 2.0 mg/ml for 12–15 min. The buffer used was (in mM) 115 NaCl, 1.5 CaCl₂, 11 NaHCO₃, 4.6 KCl, 1.4 MgSO₄ and 30 Hepes, adjusted to pH 7.4 after addition of 10 mg of bovine serum albumin/ml. After collagenase digestion, the tissue was filtered through a nylon mesh and the adipocytes were washed with buffer of identical composition but not containing albumin. A sample of the final cell suspension was removed for assay of cell protein (Bradford, 1976), or estimation of cell number (Schimmel & McCarthy, 1985), which were then used for expression of the data. After the final wash step, the adipocytes were resuspended and incubated in buffer containing 10 or 40 mg of bovine serum albumin/ml, as indicated.

Measurement of respiration

O₂ consumption was measured polarographically at 37 °C with a YSI model 53 oxygen-meter equipped with a YSI model 5331 oxygen probe exactly as described previously (Schimmel *et al.*, 1983b; Schimmel & McCarthy, 1984). Glucose (10 mM), fructose (10 mM) and pyruvate (1 mM) were added to all media to optimize the respiratory responses (Schimmel *et al.*, 1983b). Propranolol was added at a concentration of 0.10 μ M to block the β -adrenergic activity of the phenylephrine. After introduction of the cells into the respiratory chamber, basal O₂ consumption was measured for approx. 5 min. At this time phenylephrine, isoprenaline (isoproterenol) or PMA was injected with a Hamilton syringe. Stock solutions of PMA (Sigma Chemical Co.) were prepared in ethanol and stored at –20 °C.

Incorporation of radioisotopes into phospholipids

Brown adipocytes were incubated in 3.0 ml of media containing 2–4 μ Ci of [³²P]P_i/ml (ICN, Irvine, CA, U.S.A.) at 37 °C for up to 4 h. Drug or control solutions were then added as specified in the Figure legends. In some experiments, the adipocytes were incubated under

'pulse-chase' conditions. In this case, the cells were collected by centrifugation, washed once with albumin-containing buffer, resuspended in fresh media not containing the radioisotope, and re-incubated for 45 min in media containing 2.0 mM-phosphate before addition of the drugs or control solutions. Glucose (10 mM), fructose (10 mM) and pyruvate (1.0 mM) were present during the entire incubation period.

Extraction of phospholipids and t.l.c.

Incubations were terminated by addition of trichloroacetic acid (final concn. 8%, w/v) and the resulting pellet was collected by centrifugation. The supernatant was removed and the phospholipids in the pellet were extracted into 3 ml of chloroform/methanol (2:1, v/v). Then 1.5 ml of 2 M-KCl that had been acidified to pH < 4.0 with HCl was added, and the extracts were mixed and centrifuged at 1000 g for 5 min. The upper aqueous phase and interphase were carefully removed, and the lower chloroform phase was transferred to a fresh vial and evaporated to dryness. The lipids were redissolved in chloroform, and the phospholipids were resolved by t.l.c. on oxalate-impregnated silica gel 60 high-performance plates (Merck, Darmstadt, Germany). Oxalate impregnation was performed by immersion of the plate in a solution of potassium oxalate (1%) in 50% methanol for 10 min. The plates were air-dried and then heated to 110 °C for 60 min. After application of the sample, the plate was developed in diethyl ether/acetic acid (19:1, v/v). This step served to move the neutral lipids to the top of the chromatogram without disturbing the phospholipids, which remained at the origin. The phospholipids were then resolved by a subsequent development of the chromatogram with chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8, by vol.) as described by Jolles *et al.* (1981). Appropriate standards (Sigma, St. Louis, MO, U.S.A.) were co-chromatographed for correct identification of the individual lipids. Phospholipids were detected on plates by exposure to a molybdate solution (3 g of ammonium molybdate in 50 ml of water, 5 ml of 6 M-HCl and 13 ml of 70% HClO₄) applied as an aerosol spray followed by heating to 100 °C for 10 min. Radioactivity in individual phospholipids was quantified by scraping the silica plates, followed by liquid-scintillation counting in Scinti-Verse (Fisher Scientific).

RESULTS

As has been reported by others previously (Garcia-Sainz *et al.*, 1980; Mohell *et al.*, 1984), exposure of brown adipocytes to the α -adrenergic ligand phenylephrine stimulated incorporation of [³²P]P_i into phosphatidic acid and PtdIns (results not shown). These observations are consistent with the presence of an initial phenylephrine stimulation of PtdIns-4,5- P_2 breakdown, and subsequent experiments confirmed this. As shown in Fig. 1, addition of phenylephrine to cells prelabelled with [³²P]P_i caused the radioactivity associated with PtdIns-4,5- P_2 to decrease by nearly 50% after 30 s of incubation; radioactivity associated with PtdIns-4- P likewise decreased, but by a smaller amount. The radioactivity present in both PtdIns-4,5- P_2 and PtdIns-4- P then tended to recover to that measured in unstimulated cells. Phosphatidic acid accumulation was detected at the

Fig. 1. Time course of the effect of phenylephrine on $[^{32}\text{P}]$ phospholipids in brown adipocytes prelabelled with $[^{32}\text{P}]\text{P}_i$.

Brown adipocytes (2.8–3.5 mg of cell protein) were incubated with $4 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for 3 h in the presence of 10 mM-glucose, 10 mM-fructose and 1.0 mM-pyruvate. Phenylephrine and propranolol were added at zero time to produce final concentrations of 10 μM and 0.10 μM respectively, and the cell incubation was terminated at various times thereafter. The results are expressed as means $\pm$ S.E.M. for six separate experiments.

earliest time point sampled (30 s) and continued for the duration of the incubation with phenylephrine. Phenylephrine did not modify the radioactivity in phosphatidylcholine, phosphatidylethanolamine or PtdIns (results not shown).

The accurate definition of the time course of the breakdown of PtdIns-4,5- P_2 and PtdIns-4- P in response to phenylephrine is confounded in this experiment by the continued generation of ^{32}P -labelled phospholipids from radiolabelled ATP. Hence synthesis of PtdIns-4,5- P_2 and PtdIns-4- P from PtdIns as well as synthesis of PtdIns from phosphatidic acid would incorporate ^{32}P from ATP and thereby tend to obscure the detection of hormone-promoted breakdown of these lipids. To prevent incorporation of ^{32}P into phosphatidic acid and the phosphoinositides during phenylephrine stimulation, a similar series of experiments were conducted under 'pulse-chase' conditions. For these experiments, adipo-

Fig. 2. Time course of the effect of phenylephrine on $[^{32}\text{P}]$ -phospholipids in brown adipocytes incubated under pulse-chase conditions after prelabelling with $[^{32}\text{P}]\text{P}_i$.

Brown adipocytes (3.2–4.0 mg of cell protein) were incubated with $4 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for 4 h in media containing 10.0 mM-glucose, 10.0 mM-fructose and 1.0 mM-pyruvate. The cells were then washed and re-incubated for 45 min in fresh medium without $[^{32}\text{P}]\text{P}_i$ but containing 2 mM-phosphate. Phenylephrine and propranolol were added at zero time to produce final concentrations of 10 μM and 0.10 μM respectively. The results are expressed as means $\pm$ S.E.M. for six separate experiments.

cytes were exposed to $[^{32}\text{P}]\text{P}_i$ for 2 h (pulse), after which they were re-incubated in the absence of tracer for 45 min (chase) before addition of phenylephrine. After the chase period, radioactivity associated with PtdIns, phosphatidic acid and the phosphoinositides was found to have decreased by nearly 60%. When phenylephrine was added (Fig. 2), additional accumulation of labelled phosphatidic acid was not detected, thereby confirming the adequacy of the chase period as well as excluding the possibility of phosphatidic acid formation from an α -adrenergic-stimulated phospholipase D mechanism in brown adipocytes. This experiment confirmed the rapid breakdown of PtdIns-4,5- P_2 in response to phenylephrine and uncovered an effect of phenylephrine promoting the

Fig. 3. PMA-induced inhibition of phenylephrine-, but not isoprenaline-, stimulated respiration in brown adipocytes

Isolated brown adipocytes were incubated in 3.0 ml of buffer containing 10 mg of bovine serum albumin/ml, 10 mM-glucose, 10 mM-fructose and 1 mM-pyruvate. Respiration was monitored for 4 min (basal, □) at which time PMA was added as indicated. Respiration was monitored for an additional 1 min, after which time phenylephrine (10 μM) + propranolol (0.10 μM) (▨) or isoprenaline (0.010 μM) (▩) were added. Respiration was then measured over the next 5 min period. Each value represents the mean ± S.E.M. for 11 separate experiments.

Fig. 4. Dose-response relation for PMA inhibition of phenylephrine-stimulated respiration in brown adipocytes

Isolated brown adipocytes were incubated in 3.0 ml of buffer containing 10 mg of bovine serum albumin/ml, 10 mM-glucose, 10 mM-fructose and 1 mM-pyruvate. Respiration was monitored for 4 min (basal), at which time various concentrations of PMA were added as indicated. Respiration was monitored for an additional 1 min, after which time phenylephrine (10 μM) and propranolol (0.10 μM) were added. Respiration was then measured over the next 5 min period. Each value represents the mean ± S.E.M. for five separate experiments.

disappearance of PtdIns as well. The amounts of PtdIns-4,5- P_2 and PtdIns-4- P after phenylephrine addition in this study remained low for the duration of the incubation.

The above data suggest that α_1 -adrenergic activation of brown adipocytes causes accumulation of 1,2-diacylglycerol from hydrolysis of phosphoinositides. In other systems, 1,2-diacylglycerol activates protein kinase C (Nishizuka, 1984), and this effect can be mimicked pharmacologically with phorbol esters, such as PMA (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983). To investigate the actions of protein kinase C in brown adipocytes, the effects of phorbol esters on isolated hamster brown adipocytes were determined. Exposure of adipocytes to PMA (10 nM–1 μM) for up to 15 min had no effect on basal respiration. After exposure to PMA, however, cells were significantly less responsive to subsequent stimulation by phenylephrine (Fig. 3). This effect of PMA was evident after brief exposures to the phorbol (60 s in the experiment depicted in Fig. 3). The minimum effective concentration of PMA was 10 nM, which inhibited phenylephrine-stimulated respiration by approx. 20% (Fig. 4). At the highest concentration of PMA tested (1.0 μM), phenylephrine-stimulated respiration was inhibited by nearly 80%. When PMA was added to cells after they had been stimulated with phenylephrine, partial inhibition of stimulated respiration occurred which was manifest between 30 and 60 s after addition of

the phorbol (Fig. 5). In contrast, when the stimulus for adipocyte respiration was provided by isoprenaline, PMA added 1 min before addition of the β -adrenergic agonist (Fig. 3) or several minutes after the β -agonist (Fig. 5) had no significant inhibitory effect. In other experiments, however (see Fig. 6), exposure to PMA for 15 min or 30 min did partially inhibit isoprenaline-stimulated respiration. Two inactive phorbol esters (4 β -phorbol and 4 α -phorbol 12,13-didecanoate) did not block phenylephrine-stimulated respiration.

Our next study investigated the effect of PMA on phenylephrine-stimulated breakdown of PtdIns-4,5- P_2 . For this study, cells were labelled with [32 P]P_i and then treated with PMA before exposure to phenylephrine. The results depicted in Table 1 show that treatment of the adipocytes with PMA significantly decreased phenylephrine-stimulated breakdown of PtdIns-4,5- P_2 . By itself, however, PMA had no marked effect on incorporation of [32 P]P_i into the phosphoinositides.

PMA has been found to promote the phosphorylation of a number of membrane receptors (Davis & Czech, 1984; Moon *et al.*, 1984; Iwashita & Fox, 1984), including the receptor for insulin (Jacobs *et al.*, 1983) and the β -adrenergic receptor (Kelleher *et al.*, 1984). PMA has also been found to promote phosphorylation and inactivation of N_i (guanine nucleotide regulatory protein

Fig. 5. Inhibition of phenylephrine-stimulated respiration by addition of PMA

Isolated brown adipocytes were incubated in 3.0 ml of buffer containing 10 mg of bovine serum albumin/ml, 10 mM-glucose, 10 mM-fructose and 1 mM-pyruvate. Respiration was monitored for 5 min, at which time phenylephrine (10 μ M)+propranolol (0.10 μ M) or isoprenaline (0.01 μ M) were added as indicated. Respiration was monitored for an additional 4 min, after which time PMA (1.0 μ M) was added. Respiration was then measured over the next 6 min period. Each value represents the mean $\pm$ S.E.M. for eight separate experiments.

that couples inhibitory receptors to adenylate cyclase) and thereby interrupt ligand inhibition of adenylate cyclase (Katada *et al.*, 1985; Jacobs *et al.*, 1985). However, little is currently known about the possible consequences of these phosphorylation reactions to the physiological responsiveness of intact cells. Our final series of

Fig. 6. Effects of PMA on isoprenaline-stimulated respiration and on inhibition by insulin and 2-chloroadenosine of stimulated respiration in brown adipocytes

Isolated brown adipocytes were incubated in 3.0 ml of buffer containing 10 mg of bovine serum albumin/ml, 10 mM-glucose, 10 mM-fructose and 1 mM-pyruvate without and with 1 μ M-PMA for various periods of time. Unstimulated respiration was then monitored for 5 min (Basal), after which time isoprenaline was then added (final concn. 0.02 μ M). Respiration was monitored for an additional 4 min, after which time 2-chloroadenosine (2CIA; 0.1 μ M) or insulin (INS; 25 ng/ml) was added. Respiration was then measured over the next 7 min. Each value represents the mean $\pm$ S.E.M. for five separate experiments.

Table 1. PMA-induced inhibition of phenylephrine-stimulated breakdown of PtdIns-4,5- P_2 in brown adipocytes

Brown adipocytes were incubated with 4 μ Ci of [32 P]P_i/ml for 2 h in media containing 10 mg of bovine serum albumin/ml, 10 mM-glucose, 10 mM-fructose and 1 mM-pyruvate. PMA (1 μ M) or vehicle was then added as indicated. At 1 min after addition of PMA (zero time), phenylephrine (10 μ M) and propranolol (0.10 μ M) were added and the cell incubations terminated at various times thereafter. The results are means $\pm$ S.E.M. for ten separate experiments. The data are presented as the measured radioactivity associated with PtdIns-4,5- P_2 , expressed as c.p.m. ($\times 10^{-3}$) present per 10^6 cells, as the mean decrease in PtdIns-4,5- P_2 radioactivity caused by phenylephrine and as the percentage decrease caused by phenylephrine.

Time after phenylephrine (min) . . .	10 ⁻³ $\times$ Radioactivity in PtdIns-4,5- P_2			
	0	1	3	10
Control	20.5 $\pm$ 4.2	11.8 $\pm$ 3.8	15.1 $\pm$ 2.9	16.2 $\pm$ 2.7
Decrease by phenylephrine		8.7 $\pm$ 1.2*	5.4 $\pm$ 1.8*	4.3 $\pm$ 1.7*
Decrease (%)		42.4 $\pm$ 6.6*	26.3 $\pm$ 3.9*	20.9 $\pm$ 5.1*
PMA	19.1 $\pm$ 1.8	15.9 $\pm$ 3.1	15.7 $\pm$ 2.7	17.5 $\pm$ 3.4
Decrease by phenylephrine		3.2 $\pm$ 1.8	3.4 $\pm$ 1.7	1.6 $\pm$ 2.2
Decrease (%)		16.8 $\pm$ 6.9	17.9 $\pm$ 7.5	8.3 $\pm$ 5.1

* Difference significantly greater than zero ($P < 0.05$) by paired Student's t test.

experiments investigated if PMA treatment of brown adipocytes modified the actions of insulin or adenosine, both of which inhibit stimulated respiration, and if longer exposures to the phorbol ester would interfere with isoprenaline-stimulated respiration. PMA treatment for up to 15 min did not diminish the inhibitory effects of insulin or 2-chloroadenosine on isoprenaline-stimulated respiration (Fig. 6). This stimulated respiration was decreased slightly after treatment with PMA for 15 min, and markedly after 30 min.

DISCUSSION

Ligand-promoted incorporation of [32 P] P_i into PtdIns and phosphatidic acid has been widely observed in systems in which cellular activation is coupled to or dependent on Ca^{2+} translocation (Michell, 1975). Although a role of Ca^{2+} as a second messenger of hormone action has yet to be established for adipocytes, evidence consistent with the existence of a PtdIns cycle in this tissue has been presented. Thus increased incorporation of ^{32}P into PtdIns and phosphatidic acid has been found in white adipocytes exposed to insulin (Stein & Hales, 1974; Honeyman *et al.*, 1983), oxytocin (Honeyman *et al.*, 1983), adenosine (Schimmel *et al.*, 1983a) or α -adrenergic agents (Garcia-Sainz & Fain, 1980; Schimmel *et al.*, 1983a) and in brown adipocytes exposed to α -adrenergic ligands (Garcia-Sainz *et al.*, 1980; Mohell *et al.*, 1984). The studies described herein extend these previous findings by demonstrating a rapid decrease in radioactivity associated with the phosphoinositides in brown adipocytes exposed to phenylephrine and provide evidence, which has been previously lacking for these cells, for a ligand-activated hydrolysis of the phosphoinositides.

On the basis of results gathered from a variety of other systems (Berridge, 1984), the disappearance of PtdIns-4,5- P_2 described in the present experiments most probably reflects its hydrolysis to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. However, the present results point to a conclusion that differs from that derived from other systems. According to current thinking, phosphoinositides are continuously hydrolysed and resynthesized (Michell *et al.*, 1981; Agranoff *et al.*, 1983; Berridge, 1984; Downes & Wusteman, 1983) many times over during cell stimulation. In this way, elevated concentrations of inositol 1,4,5-trisphosphate can be sustained during cell activation despite a limited storage of PtdIns-4,5- P_2 for hydrolysis. Results from the pulse-chase experiments, however, are not compatible with the notion that hydrolysis and resynthesis of PtdIns-4,5- P_2 occur continually during the entire period of phenylephrine stimulation. In these studies, phenylephrine caused the radioactivity in the phosphoinositides to decrease rapidly to a new value, which then persisted unchanged for the duration of the incubation. If breakdown and re-formation of PtdIns-4,5- P_2 continued during the entire period of cell incubation, a continuous decline of radioactivity associated with this lipid would have occurred from its synthesis from non-radioactive ATP. The stability of the labelled phosphoinositides during phenylephrine treatment in this study points to the conclusion that breakdown of PtdIns-4,5- P_2 must be short-lived, terminating 30–60 s after its initiation, in spite of the continued presence of the activating ligand.

In contrast with the transient nature of phenylephrine-

promoted breakdown of phosphoinositides, formation of phosphatidic acid occurs for as long as the α -adrenergic ligand is present. This temporal dissociation between disappearance of PtdIns-4,5- P_2 and formation of phosphatidic acid indicates that formation of phosphatidic acid and hence 1,2-diacylglycerol is largely unrelated to breakdown of PtdIns-4,5- P_2 ; only phosphatidic acid formed within the first 1 min of phenylephrine stimulation can be derived from PtdIns-4,5- P_2 . A similar conclusion has been reached by others (Allan & Cockcroft, 1983; Hughes *et al.*, 1984; Bocckino *et al.*, 1985) from analysis of the acyl composition of the 1,2-diacylglycerols formed in stimulated human neutrophils or isolated liver cells. Alternatively, our data may still be consistent with a metabolic flux through the phosphoinositides if that flux occurred only through a small pool of the cell's PtdIns-4,5- P_2 that did not mix with the remainder of the phosphoinositides in the cells. Along these lines, Billah & Lapetina (1982) and Monaco & Woods (1983) have presented data suggesting the presence of hormone-sensitive and hormone-insensitive pools of PtdIns in platelets and fibroblasts.

The present results suggest that α_1 -adrenergic receptors on brown adipocytes are linked to the same membrane-transducing system, hydrolysis of phosphoinositides, as are α_1 -adrenergic receptors present on liver and other tissues. In other tissues, an apparent negative feedback control of phosphoinositide hydrolysis has been described in which protein kinase C, activated either with 1,2-diacylglycerol derived from hydrolysis of phospholipids, or pharmacologically with phorbol esters, partially uncouples receptors controlling hydrolysis of inositol phospholipids and inhibits associated cell responses (Corvera & Garcia-Sainz, 1984; Garcia-Sainz *et al.*, 1985; Cooper *et al.*, 1985; Danthuri & Deth, 1984; Orellana *et al.*, 1985; Rittenhouse & Sasson, 1985; Watson & Lapetina, 1985; Zavoico *et al.*, 1985; Whiteley *et al.*, 1984). The present data show that exposure to PMA for only 1 min blocks phenylephrine-promoted breakdown of PtdIns-4,5- P_2 and stimulated respiration, findings consistent with a protein-kinase-C-mediated uncoupling of α_1 -adrenergic receptors in brown adipocytes. The concomitant loss of phenylephrine-stimulated PtdIns-4,5- P_2 breakdown and O_2 consumption with PMA is consistent with a causal relationship between these responses.

In addition to preventing phenylephrine stimulation of respiration, PMA treatment of brown adipocytes also decreased the ability of β -adrenergic receptor mechanisms to stimulate respiration. Data from other systems suggest that both effects could result from a common mechanism, namely enhanced receptor phosphorylation. PMA-induced phosphorylation and concomitant inactivation of α_1 receptors on cultured smooth-muscle cells has been reported (Leeb-Lundberg *et al.*, 1985), as has PMA-induced phosphorylation of β -adrenergic receptors and concomitant functional uncoupling from adenylate cyclase (Kassis *et al.*, 1985; Kelleher *et al.*, 1984). On the other hand, the temporal dissociation between PMA-induced inactivation of α - and β -adrenergic mechanisms observed in the present study raises the possibility that receptor phosphorylation is not the underlying mechanism of both responses.

The more rapid inactivation of α -adrenergic mechanisms in brown adipocytes exposed to PMA suggests modification of a component of this transduction

receptor system not shared by the β -adrenergic system. This component is not likely to be phospholipase C, since vasopressin activation of phosphoinositide breakdown in liver cells is not interrupted by phorbol ester treatment (Cooper *et al.*, 1985). The possibility that as yet unidentified coupling proteins are phosphorylated and inactivated by protein kinase C is similarly argued against by the apparent selectivity of PMA action observed in the liver cell system (Cooper *et al.*, 1985). Interestingly, such selectivity of PMA action was not observed in cultured astrocytoma cells, in which PMA blocked both carbachol- and histamine-stimulated inositol phosphate formation, observations which led Orellana *et al.* (1985) to suggest an action of the phorbol ester on some component of the transduction systems other than the membrane receptors. The reports by Katada *et al.* (1985) and Jacobs *et al.* (1985) of PMA-induced phosphorylation of N_i in platelet membranes, coupled with other studies (Bokoch & Gilman, 1984; Backlund *et al.*, 1985; Okajima & Ui, 1984) suggesting that N_i may couple receptors to phospholipase C, lend credence to the possibility that phosphorylation by protein kinase C of brown-adipocyte N_i is the mechanism underlying the rapid PMA-induced inactivation of α_1 -adrenergic responses. The present experiments, however, disclosed no effect of PMA on the inhibitory effects of 2-chloroadenosine on isoprenaline-stimulated respiration. Since 2-chloroadenosine inhibition of stimulated respiration depends on the functional activity of N_i (Schimmel *et al.*, 1985), the failure of PMA to affect 2-chloroadenosine action suggests that this protein remains functional after treatment of the cells with PMA.

The present study found insulin inhibition of stimulated respiration to be unaffected by treatment with PMA. On the assumption that phosphorylation of the insulin receptor on brown adipocytes occurs during PMA treatment in our studies, our findings would indicate that this modification of the receptor does not interfere with its inhibition of respiration. O'Brien (1982) similarly did not detect inhibition of insulin action with PMA treatment, but others (Van de Werve *et al.*, 1985) have observed some attenuation of insulin stimulation of lipogenesis in white adipocytes exposed to phorbol esters.

A major unresolved question in brown adipocytes is the nature of signals linking α -receptor activation to mitochondrial respiration. A role for protein kinase C in triggering mitochondrial respiration is not supported by the present findings, since exposure of cells to PMA by itself did not promote O_2 consumption. Since α -adrenergic stimulation of respiration appears to require Ca^{2+} (Schimmel *et al.*, 1983b), it would seem logical to focus on the possibility that changes in cell Ca^{2+} are involved in α -adrenergic actions. Other support for this possibility comes from the findings by Connolly *et al.* (1984) of an α -adrenergic-induced efflux of intracellular Ca^{2+} in hamster brown adipocytes. However, their suggestion of mitochondria as the locus of the mobilizable Ca^{2+} pool is at odds with evidence from other systems against mitochondria being a source of intracellular Ca^{2+} mobilized by inositol 1,4,5-trisphosphate (Williamson *et al.*, 1985). Koepfer-Hobelsberger & Wieland (1984) reported stimulation of pyruvate dehydrogenase activity in detergent-permeabilized white adipocytes exposed to inositol trisphosphate and in isolated mitochondria exposed to 1,2-dioleoylglycerol, suggesting that this mitochondrial enzyme can be regulated by protein kinase

C or by Ca^{2+} released in response to inositol trisphosphate. Taken together, these findings are consistent with the notion that mitochondrial functions can be modulated by one or both breakdown products of $PtdIns-4,5-P_2$, although clarification of the precise mechanisms involved will require additional experimentation.

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