Aust. J. Biol. Sci., 1987, **40**, 405–15

Adrenaline Stimulation of Phospholipid Metabolism in Adipocyte Ghosts

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Abstract

The incorporation of long-chain fatty acids into phospholipids has been detected in adipocyte ghosts that were incubated with [1-^{14} C] stearic, [1-^{14} C] linoleic or [1-^{14}C] arachidonic acid. Adrenaline and adenosine activated this incorporation within 15 s of exposure of the ghosts to the hormones and the response was dose dependent. Maximum incorporation of labelled linoleic acid occurred at 10^{-5} M adrenaline and 10^{-7} M adenosine. The α-agonist phenylephrine and the β-agonist isoproterenol were also shown to stimulate the incorporation of fatty acid in a dose dependent manner. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were each labelled preferentially with linoleic or arachidonic acid. *p*-Bromophenacylbromide, quinacrine and centrophenoxine inhibited the adrenaline-stimulated incorporation of fatty acids into ghost membrane phospholipids, and *p*-bromophenacylbromide also reduced the activation of adenylate cyclase by adrenaline. NaF, an activator of adenylate cyclase, like adrenaline, stimulated the incorporation of linoleic acid into ghost membrane phospholipids.

Extra keyword: deacylation–acylation.

Introduction

The enzymes phospholipase A_2 (EC 3.1.1.4.), long-chain-fatty acid-CoA ligase (EC 6.2.1.3.) and 1-acylglycerophosphocholine acyltransferase (EC 2.3.1.23), here termed the exchange enzymes, constitute a deacylation–acylation cycle in which the fatty acid moieties of phospholipids may be turned over independently of phosphatidate biosynthesis (Van den Bosch 1980). The restructuring of phospholipids synthesized *de novo*, by turnover of acyl chain moieties, may be necessary for maintaining the essential structural properties of membranes. It is also possible that the operation of a deacylation–acylation cycle in a specific membrane *in vivo*, for example the plasma membrane, may have significant consequences on events occuring therein. As cellular phospholipases are potentially disruptive to the membranes in which they are situated, they are under stringent control and Van den Bosch (1980) suggested that phospholipase A activation may be under hormonal control. Phospholipase A_2 has been shown to be stimulated by exogenously added cyclic 3′5′-AMP (cAMP) to homogenates of isolated adipocytes (Chiappe de Cingolani *et al.* 1972). The adipocyte is very responsive to a number of polypeptide hormones, catecholamines and adenosine, which modulate cellular levels of cAMP, and each of these hormones is a potential regulator of phospholipase A_2 activity.

However, phospholipase A_2 activity has been demonstrated in plasma membrane vesicles isolated from rat adipocytes (Wolf *et al.* 1977) and activity towards exogenous phospholipid substrates was stimulated by insulin (Bereziat *et al.* 1978). It is therefore possible that cAMP acts on phospholipase A_2 of intracellular organelles rather than the plasma membrane bound enzyme. The finding of Sauerheber *et al.* (1980) that insulin did not affect the lipid order of rat adipocyte ghosts suggests that any lysophosphatides produced in the plasma membrane from membrane phospholipids by stimulation of a phospholipase A would be reacylated rapidly.

0004-9417/87/040405$02.00

We have investigated the operation of a putative deacylation–acylation cycle, during adrenergic stimulation of adipocyte ghosts, to gain further insight into the initial events or hormonal activation of the plasma membrane bound phospholipase A_2 in its natural environment. In the presence of exogenous radiolabelled fatty acids (stearic, linoleic, or arachidonic acid), adrenaline, isoproterenol, phenylephrine and adenosine stimulated the incorporation of the fatty acids into ghosts membrane phospholipids. Fluoride ions had a stimulatory effect on linoleic acid incorporation into membrane phosopholipids in the absence of adrenergic agents. Inhibitors of phospholipase A_2, *p*-bromophenacylbromide and quinacrine, and the inhibitor of lysophosphatidylcholine acyltransferase, centrophenoxine, each inhibited the hormonally stimulated turnover of linoleic acid; *p*-bromophenacylbromide inhibited both fatty acid turnover and the production of cAMP by the ghost preparations.

Materials and Methods

Materials

The cAMP assay kit and the [1-^{14} C]-labelled stearic, linoleic and arachidonic acids were obtained from Amersham International plc. Lipid standards, *L*-α-phosphatidic acid, *L*-α-phosphatidylcholine, *L*-α-phosphatidylethanolamine, *L*-α-phosphatidylinositol, *L*-α-phosphatidyl-*L*-serine and all other chemicals were obtained from Sigma Chemical Company. Collagenase type III was supplied by Worthington and analytical reagent grade organic solvents were obtained from Ajax. Precoated (silica gel G60) aluminium backed t.l.c. plates (0.2 mm thick) were obtained from Merck and medical X-ray film from Fuji.

Methods

Adipocyte ghosts were isolated from the epididymal fat pads of Sprague Dawley rats (200–300 g in weight) according to the method of Rodbell and Krishna (1974). The activity of 5′-nucleotidase (EC 3.1.3.5), was assayed by the method of Newby *et al.* (1975).

Incubations with labelled fatty acids

Labelled [1-^{14}C]stearic acid (56 mCi/mmol), [1-^{14}C]linoleic acid (56 mCi/mmol) or [1-^{14}C]arachidonic acid (53.9 mCi/mmol) were separately dissolved in toluene, and 10 μl of each solution (containing 5 nmol of fatty acid) were introduced into separate incubation tubes and dried under N_2 at room temperature. The fatty acid was then dissolved in Krebs-Ringer bicarbonate buffer, pH 7.4, by warming the tubes to 37°C. The buffer contained 1.0 mM Ca^{2+}, coenzyme A (50 nmol), ATP (500 nmol) and, where appropriate, adrenaline (0.4 nmol) in a final volume of 0.6 ml. Incubations with adenosine were identical to this, except that adrenalin was replaced with adenosine to give a final concentration ranging from 10^{-8} M to 10^{-5} M. The incubations were initiated by the addition of 0.2 ml ghosts (1.3 mg protein/ml) and performed at 37°C. At the end of the incubation, 2 ml ice-cold methanol were added to the reaction mixtures; this gave a H_2O : methanol ratio of 0.8 : 2.0. Then 2 ml chloroform were added, in preparation for the extraction of lipid by the method of Bligh and Dyer (1958).

Incubations with p-bromophenacylbromide, quinacrine and centrophenoxine

Each compound was studied separately and the conditions were identical to those of incubations with labelled fatty acid and adrenaline. When *p*-bromophenacylbromide (0.5 mM) was used, 10 μl were added directly to appropriate incubation mixtures and 10 μl methanol were added to control tubes. When quinacrine (0.5 mM) and centrophenoxine (5.0 mM) were used, 0.1 ml per incubation mixture, 0.1 ml of buffer was added to control tubes. The activity of the exchange enzymes is defined as pmol radiolabelled fatty acid incorporated per min per mg of ghost protein, for all assays with and without inhibitors, for all the conditions tested.

Adenylate cyclase [E.C. 4.6.1.1.] activity in the presence of p-bromophenacylbromide

The incubation mixture contained 5 nmol of non-labelled linoleic acid, 0.4 nmol of adrenaline, 0.4 μmol of a methanolic solution of *p*-bromophenacylbromide and theophylline (5 mM). The other components of the mixture and the conditions of incubation were identical to those employed with labelled fatty acids. The reaction was stopped by immersing the incubation flask in a boiling water bath. Aliquots of the incubation mixture were then removed for the assay of cAMP.

Analytical Procedures

Lipid analysis

Lipids were extracted from the methanolic reaction mixture by the method of Bligh and Dyer (1958). The extraction was carried out twice on each reaction mixture and the chloroform phases pooled, dried under N_2 and the residue redissolved in 40 μl of chloroform. Thin layer chromatography was performed by the method of Rouser *et al.* (1967). The entire sample was applied to t.l.c. plates and chloroform:methanol:water (65:25:4 v/v/v) was used as developing solvent. Lipids were visualized by exposure of the t.l.c. plate to iodine vapour and identified by comparison with standard, unlabelled phospholipids, *L*-α-phosphatidic acid, *L*-α-phosphatidylethanolamine, *L*-α-phosphatidylinositol, *L*-α-phosphatidyl-*L*-serine and *L*-α-phosphatidylcholine. The labelled phospholipids were located by autoradiography, then the specific areas of radioactive phospholipid on the t.l.c. plate were cut into individual squares and the radioactivity measured in 10 ml of scintillant (0.4% PPO, 0.1% POPOP in toluene) in a Beckman Scintillation Counter. Non-radiolabelled phospholipids of ghost preparations were digested by the procedure of Duck-Chong (1979) and the inorganic phosphate that was released was determined according to the method of Ames (1966).

Protein determinations

Protein concentrations were assayed according to the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978). This modification involved the addition of sodium dodecyl sulfate in the alkali reagent and an increase in the amount of copper tartrate reagent. This modification allowed a more exact estimation of protein content in samples containing relatively high concentrations of lipids.

cAMP determinations

The protocol supplied by Amersham was followed. The assay was achieved by the use of a protein which has a high specificity and affinity for cAMP. The protein binds both unlabelled and added tritium-labelled cAMP. Thus, measurement of the protein bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated. This assay procedure is based on that of Gilman and Murat (1974).

Results

In the presence of [1-^{14}C]-labelled stearic acid, linoleic acid or its biologically active metabolite arachidonic acid, there was a slow basal incorporation of radioactivity into the total phospholipid constituents of the adipocyte ghosts (Fig. 1). Adrenaline (0.5 μM) increased both the rate and the extent of turnover, inducing maximum incorporation of fatty acid into phospholipids 15 s after exposure of the ghost to the hormone (Fig. 1). Almost complete turnover of the radioactively labelled molecules had occurred by 60 s and a steady state similar to that for the basal level of turnover continued for the remainder of the incubation time of 10 min. In the absence of adrenaline, linoleic and stearic acids produced a small stimulatory effect on their own incorporation into membrane lipid. This may have been related to a particular interaction between the fatty acid and the membrane lipid but was not investigated further. Significantly, however, both the basal and hormonally stimulated incorporation of labelled fatty acid into phospholipid was completely dependent on ATP and CoA, since no incorporation was observed in the absence of either ATP or CoA (results not shown). Therefore, the activation of the fatty acid to acyl-CoA was necessary for the incorporation to occur. The basal incorporation of linoleic acid into phospholipid remained unchanged when 2 mM cAMP was added to the reaction mixture in the absence of adrenaline.

Results obtained from the estimation of individual phospholipids in the ghost preparations (Table 1), together with results expressing the amount of radiolabelled fatty acid incorporated into the phospholopids (Fig. 1) were used to calculate the percentage of total phospholipids involved in turnover. Maximum amounts of 0.099% and 0.067% were obtained when linoleic acid and arachidonic acid, respectively, were incorporated into total phospholipid (Table 2).

Using *p*-bromophenacylbromide, an inhibitor of phospholipase A_2 (Volwerck *et al.* 1974), the fatty acid exchange process was disrupted. In the presence of *p*-bromophenacylbromide (0.5 mM), the adrenaline-stimulated enzyme activities were reduced to 12% of the control (Fig. 2). The effect of *p*-bromophenacylbromide on adenylate cyclase activity was determined in the presence of theophylline (5.0 mM). cAMP accumulation was reduced by 50% as shown in Fig. 3.

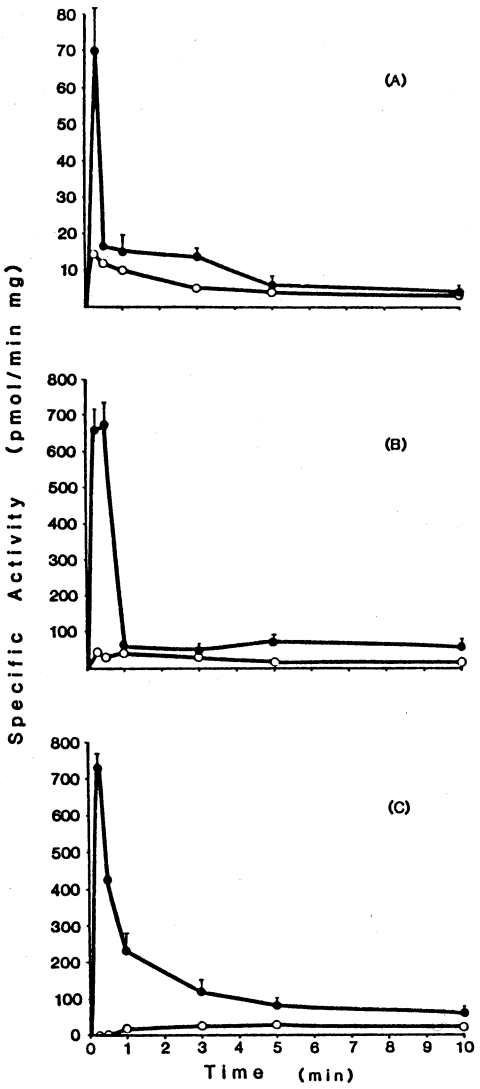

Fig. 1. Fatty acid incorporation in the presence (●) and in the absence (○) of adrenaline (0.5 μM) for (*A*) stearic acid, (*B*) linoleic acid and (*C*) arachidonic acid. Incubations were performed as described in the Methods. Tubes contained ghost protein (0.26 mg), labelled fatty acid (5 nmol), coenzyme A (50 nmol) and ATP (500 nmol), with or without adrenaline (0.5 μM), in a total volume of 0.8 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, at 37°C. Results are expressed as the mean ±s.e.m. of four separate experiments in which exchange enzyme activity has been defined as the pmol or radioactive fatty acid incorporated into ghost phospholipids per min per mg ghost protein.

Table 1. Estimation of phospholipids in adipocyte ghosts

Adipocyte ghost lipids were extracted according to the method described. The phospholipid spots were scraped from the t.l.c. plates and digested and the phosphate was estimated. Results are expressed as mean ± s.e.m. for four separate experiments

Phospholipid	Content (nmol phosphate/ mg ghost protein)	% of total phospholipid
Total phospholipids	343.3 ± 27.0	100.0
Phosphatidylcholine	111.5 ± 5.0	32.5
Phosphatidylethanolamine	76.9 ± 3.6	22.4
Phosphatidylserine + phosphatidylinositol	61.5 ± 3.7	17.9

Quinacrine, another phospholipase A_2 inhibitor, inhibits the incorporation of all classes of fatty acids into phosphatidylcholine, phosphatidylethanolamine and triglycerides. However, quinacrine also increases the incorporation of fatty acids into phosphatidylinositol (Erman *et al.* 1983). The results presented in Fig. 4*A* demonstrate that, at 0.5 mM, quinacrine reduced the hormonally stimulated activity of the exchange enzymes by 40%, at 15 s and 30 s after treatment with the inhibitor, thus indicating that fatty acid turnover was occurring when the incorporation into phosphatidylcholine and phosphatidylenthanolamine was likely to have been inhibited, as with other phospholipase A_2 enzymes.

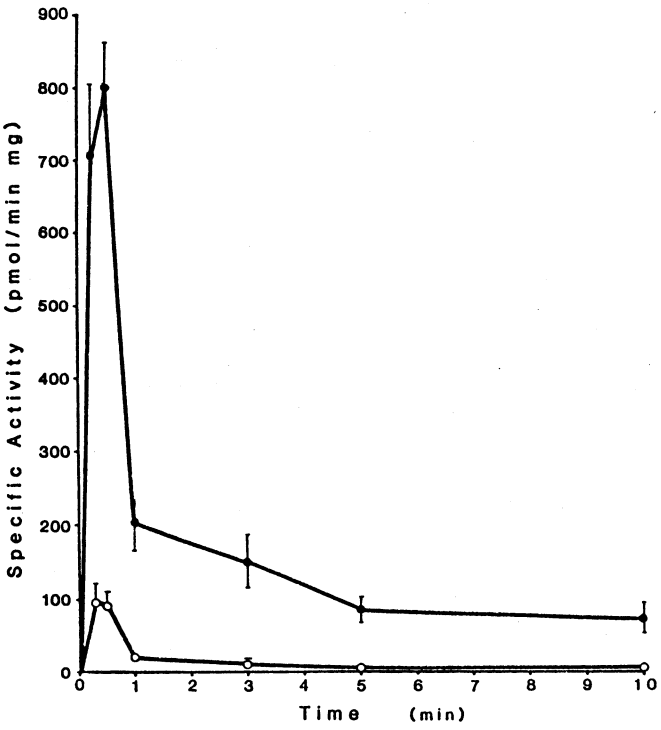

Fig. 2. Effect of *p*-bromophenacylbromide on the adrenaline stimulated incorporation of linoleic acid into phospholipids in the presence (●) of adrenaline (0.5 μM) and methanol (10 μl) and in the presence (○) of adrenaline (0.5 μM) and *p*-bromophenacylbromide (0.5 mM) in methanol. Incubation conditions were identical to those in Fig. 1. Results are expressed as the mean ±s.e.m. of four separate experiments.

Centrophenoxine (5.0 mM), an inhibitor of the lysophosphatidylcholine acyltransferase (Parthasarathy *et al.* 1981), reduced the adrenaline (0.5 μM) stimulated incorporation of labelled linoleic acid by 40% (Fig. 4*B*.)

Table 2. Effect of fatty acid incorporation on ghost phospholipids

The percentage of the phospholipids in the adipocyte ghost preparations altered as a result of fatty acid incorporation was calculated from results of fatty acid incorporation experiments (Fig. 1) expressed as pmol fatty acid incorporated into adipocyte ghost phospholipids per mg adipocyte ghost protein and the estimation of total phospholipid in adipocyte ghost preparations expressed as nmol phosphate per mg ghost protein (Table 1)

Fatty acid incorporated	% of total phospholipid containing incorporated fatty acids after:		
	15 s	30 s	60 s
Stearic	0.005	0.002	0.005
Linoleic	0.047	0.099	0.012
Arachidonic	0.052	0.062	0.067

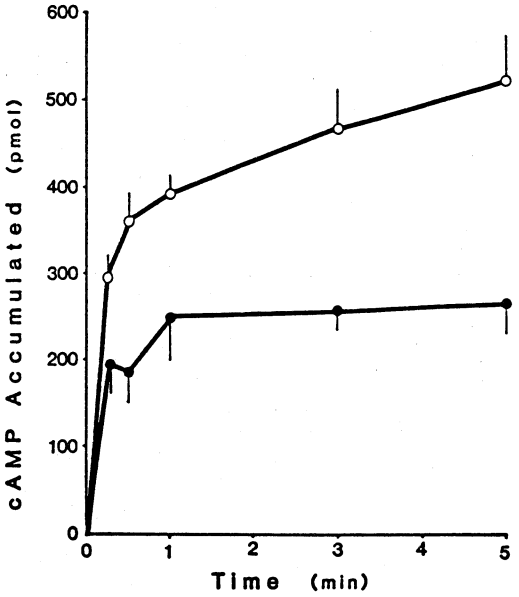

Fig. 3. Effects of *p*-bromophenacylbromide on the adrenaline stimulated activity of adenylate cyclase in adipocyte ghosts. Results are expressed as the amount of cAMP accumulated in the presence (○) of adrenaline (0.5 μM) and in the presence (●) of adrenaline (0.5 μM) and *p*-bromophenacylbromide (0.5 mM). All experimental and control tubes contained theophylline (5.0 mM). Results are expressed as the mean ±s.e.m. of four separate experiments.

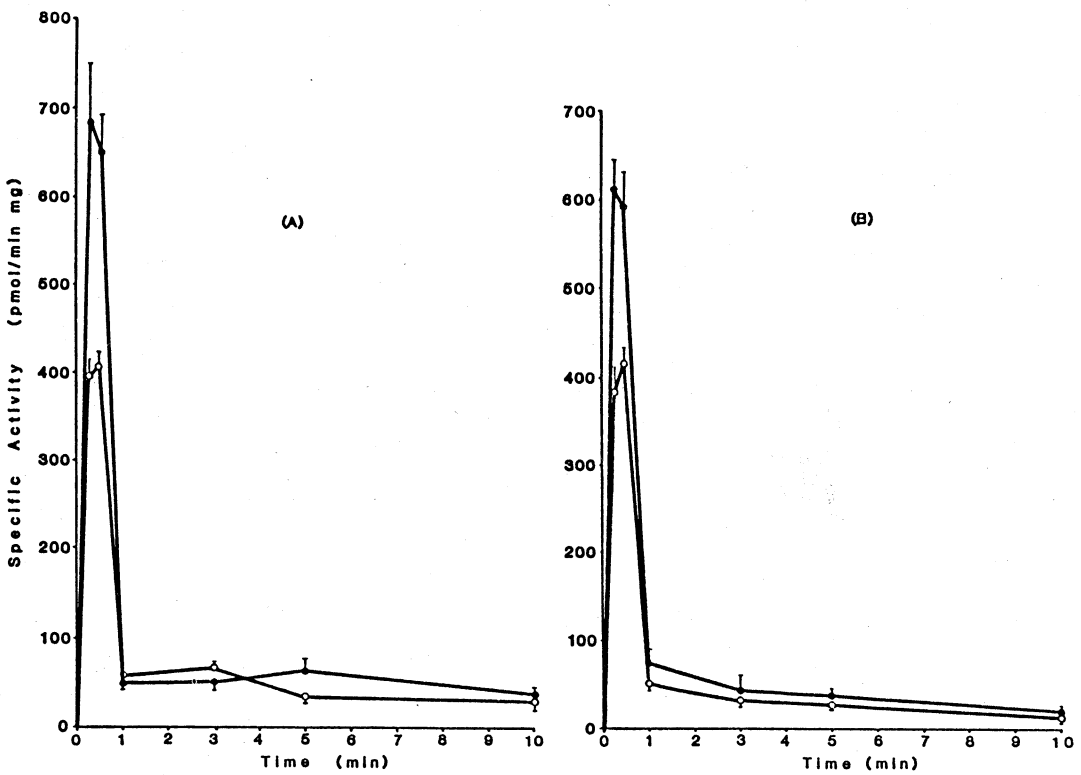

Fig. 4. Effects of quinacrine (*A*) and centrophenoxine (*B*) on the incorporation of [1-^{14}C]linoleic acid into adipocyte ghost phospholipids. Results are expressed as the rate of incorporation of linoleic acid in the presence of (●) adrenaline (0.5 μM) and in the presence of (○) adrenaline (0.5 μM) and quinacrine (0.5 mM) or centrophenoxine (5.0 mM). Incubation conditions were identical to those in Fig. 1. Results are expressed as the mean ±s.e.m. of four separate experiments.

The incorporation of [1-^{14}C]linoleic acid into phospholipids was increased as the concentration of adrenaline was increased from 10^{-8} to 10^{-5} M for a maximum incorporation time of 15 s. The specific β-adrenergic agonist isoproterenol also stimulated an increase in the incorporation of linoleic acid (Fig. 5*A*). The response to adenosine was dose dependent over the range 10^{-8} to 10^{-7} M, but decreased to a lower saturation level between 10^{-7} and 10^{-5} M. The specific α-adrenergic agonist phenylephrine, however, produced a dose dependent response between 10^{-8} and 10^{-5} M (Fig. 5*B*).

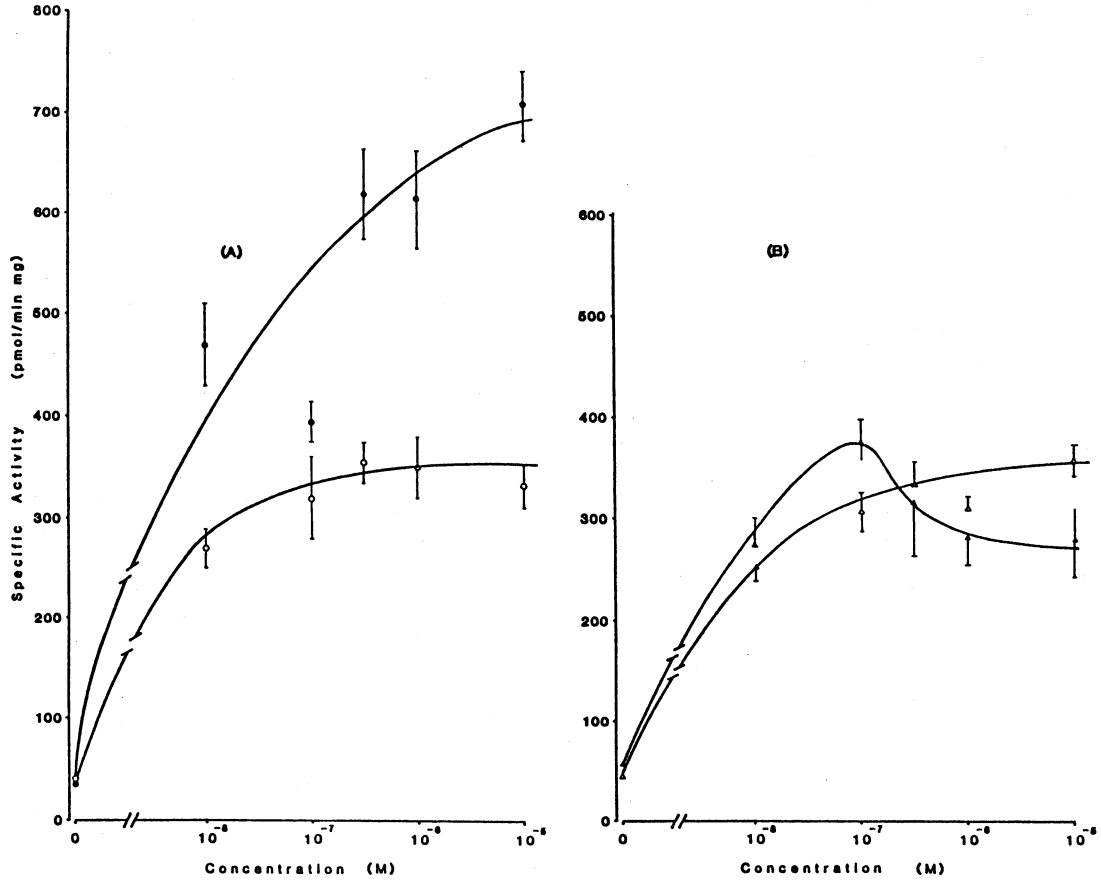

Fig. 5. Incorporation of [1-^{14}C]linoleic acid into adipocyte ghost phospholipids under increasing concentrations of (*A*) adrenaline (●) and isoproterenol (○) and (*B*) adenosine (▲) and phenylephrine (△). Incubation conditions were identical to those in Fig. 1 except that the incubation time was 15 s. Results are expressed as the mean ±s.e.m. of six separate experiments.

In the absence of hormones, fluoride ions stimulate adenylate cyclase activity. The presence of fluoride (10.0 mM) stimulated the incorporation of [1-^{14}C]linoleic acid into adipocyte ghost phospholipids in the absence of adrenaline; however, the response in the presence of fluoride was only 60% of the maximal response observed when adrenaline was used. Although the stimulated increase in [1-^{14}C]linoleic acid incorporation occurred within 15 s, it was not maintained at 30 s (Fig. 6), as it was in the presence of adrenaline.

Discussion

The incorporation of fatty acids into the phospholipids of adipocyte ghosts, in the presence of adrenaline (0.5 μM) was obtained at a Ca^{2+} ion concentration of 1.0 mM and at pH 7.4. Most phospholipase A_1 A_2 activity is dependent on Ca^{2+} but the pH optimum varies according to the cellular location of the enzyme, the plasma membrane bound enzyme of the adipocyte being active at neutral to alkaline pH (Wolf *et al.* 1977). The low basal level of fatty acid turnover in the adipocyte ghost membrane indicated that some triggering action was necessary to stimulate phospholipase A activity at pH 7.4 and 1.0 mM Ca^{2+} ion. These conditions were more physiological than those of Wolf *et al.* (1977). Furthermore, as *p*-bromophenacylbromide specifically binds to the histidine residue in the active site of phospholipase A_2 (Volwerck *et al.* 1974), the inhibition of fatty acid turnover by *p*-bromophenacylbromide implicates phospholipase A_2.

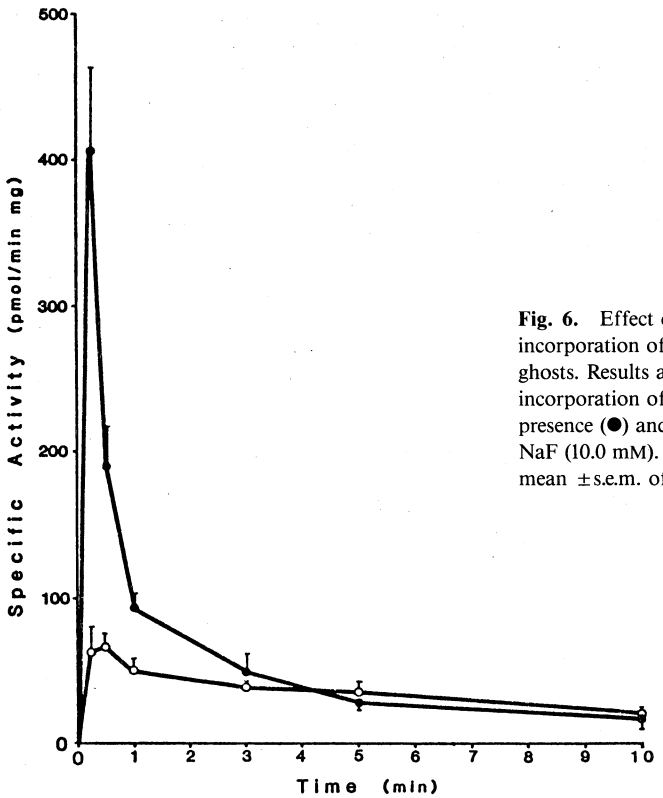

Fig. 6. Effect of NaF on the rate of incorporation of linoleic acid into adipocyte ghosts. Results are expressed as the rate of incorporation of [1-^{14}C]linoleic acid in the presence (●) and in the absence (○) of NaF (10.0 mM). Results are expressed as the mean ±s.e.m. of four separate experiments.

The inhibitory effect of quinacrine on adrenaline-stimulated incorporation of linoleic acid into phospholipid can be considered a specific effect on phospholipase A_2 activity because kinetic studies, performed on model bilayer membranes, with quinacrine (Jain and Jahagudar 1985) have shown that the substrate interface is altered by the inhibitor, which prevents the phospholipase A_2 from binding with its substrate. Thus quinacrine interacts with phosphatidylcholine and phosphatidylethanolamine and shields these lipids, whereas other phospholipids such as phosphatidylserine and phosphatidylinositol are not affected and may still be hydrolysed by phospholipase A_2. The adipocyte ghost contains 32.5% phosphatidylcholine, 24% phosphatidylethanolamine and 17.9% phosphatidylserine and phosphatidylinositol combined. The 40% reduction of adrenaline-stimulated incorporation of linoleic acid into the phospholipids of the adipocyte ghost, in the presence of 0.5 mM quinacrine, has shown that both major

and minor phospholipid components could be involved in the exchange process. Reacylation of lysophosphatides by lysophosphatidylcholine acyltransferase was indicated by the inhibitory effect of centrophenoxine on the incorporation of linoleic acid into ghost membrane phospholipid. Parathasarathy *et al.* (1984) showed that centrophenoxine was a specific inhibitor of lysophosphatidylcholine acyltransferase and that it did not have any effect on membrane bound phospholipase A_2 activity. As the adipocyte ghost membrane contains 32.5% phosphatidylcholine, the 30% inhibition of adrenaline-stimulated incorporation of linoleic acid by 5.0 mM centrophenoxine indicates almost complete inhibition of a membrane-bound lysophosphatidylcholine acyltransferase. The results with the inhibitors quinacrine and centrophenoxine have also shown that a variety of phospholipid substrates could be involved in the exchange process. Lysosomal phospholipases exhibit pH optima between 4 and 5 and are inhibited by Ca^{2+} ions (Franson *et al.* 1971); thus it is unlikely that contaminating lysosomal material in the ghost preparations contributed to the turnover of fatty acids.

The incorporation of linoleic acid into phospholipids, exhibited by adipocyte ghost preparations in the presence of adrenaline, isoproterenol, phenylephrine or adenosine was dose dependent. This suggests that stimulation of phospholipase and lysophosphatidyl acyltransferase activity was receptor mediated by agonists which elicit opposing responses by the adenylate cyclase. The adipocyte is particularly responsive to β-adrenergic agents which stimulate the adenylate cyclase and to adenosine which inhibits it (Londos *et al.* 1978). As deacylation-acylation was evident within 15 s, the enzymes responded very rapidly to the stimulus, but only 0.099% of total phospholipid was turned over, indicating that only localized regions of the plasma membrane were affected. The production of small amounts of endogenous lysophosphatides and free fatty acids in membrane domains would be likely to induce changes in the organization of membrane components in these domains. Lysophosphatides increase membrane fluidity and permeability; free fatty acids associate with specific domains of membrane lipid according to the nature of the fatty acid (Klausner *et al.* 1980).

p-Bromophenacylbromide inhibited both the exchange process and cAMP production. It is apparent that the activation of the exchange process was independent of cAMP production, because adenosine stimulated the exchange process but does not stimulate adenylate cyclase (Londos *et al.* 1978) and exogenous cAMP did not increase the basal exchange process. Therefore, even if *p*-bromophenacylbromide were to inhibit adenylate cyclase activity directly, and not by inhibition of the exchange process, the effect of reduced cAMP production would be minimized by the direct effect of *p*-bromophenacylbromide on the phospholipase A_2. However, the sensitivity of the adenylate cyclase to its lipid environment in other cell types, particularly with respect to both lysophosphatides (Houslay and Palmer 1979; Lad *et al.* 1979) and fatty acids (Orly and Schramm 1975; Rimon *et al.* 1978) has been clearly demonstrated. In addition to an optimum lipid environment the catecholamine sensitive adenylate cyclase requires activation by both catecholamine and a GTP-dependent guanine nucleotide regulatory protein, but catecholamine stimulation of adenylate cyclase can be replaced by fluoride. Dependence of fluoride stimulation of erythrocyte adenylate cyclase on the regulatory protein has been demonstrated (Downs *et al.* 1980); and direct evidence that fluoride activates the guanine nucleotide regulatory protein has been obtained with extracts from S_{49} lymphoma cells of regulatory protein in Lubrol 12A9 (Howlett *et al.* 1979). In the case of the adipocyte, our results have shown that fluoride replaced the adrenergic agents and stimulated the activity of the exchange enzymes in the same short time interval as that obtained with these agents.

The presence of exchange enzymes in the plasma membrane of the adipocyte and the stimulation of their activity by α and β agonists have given indications of transient but physiologically regulated alterations to plasma membrane lipid. The activation of the exchange enzymes occurred prior to maximal production of cAMP and a correlation exists between the two events as indicated by disruption of deacylation-acylation and inhibition of cAMP production in the presence of adrenaline and *p*-bromophenacylbromide.

Acknowledgment

This work was supported by a University of Sydney Research Grant.

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Manuscript received 8 December 1986, accepted 19 June 1987