0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 10
Printed in U.S.A.

The Antilipolytic Effects of Insulin and Epidermal Growth Factor in Rat Adipocytes Are Mediated by Different Mechanisms*

FRANCESC TEBAR, MARIA SOLEY, AND IGNASI RAMÍREZ

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071 Barcelona, Spain

ABSTRACT

Epidermal growth factor (EGF) and insulin induced similar effects in isolated rat adipocytes. To determine whether EGF and insulin produced similar effects through the same mechanisms, we focused on lipolysis. Insulin inhibited the lipolysis stimulated by isoproterenol, glucagon (either alone or in combination with adenosine deaminase), adenosine deaminase itself, or forskolin. In contrast, EGF did not inhibit the lipolysis stimulated by forskolin or by hormones when the cells were also incubated with adenosine deaminase. The effect of insulin, but not that of EGF, on isoproterenol-stimulated lipolysis disappeared when adipocytes were incubated with 1 μM wortmannin. These results indicate that EGF and insulin affected lipolysis through different mechanisms. We observed that EGF, but not insulin, increased cytosolic Ca^{2+}. The effect of EGF, but not that of insulin, disappeared when the cells were incubated in a Ca^{2+}-free medium. We suggest that EGF, but not insulin, mediate its antilipolytic effect through a Ca^{2+}-dependent mechanism which, however, do not involve Ca^{2+}-activated protein kinase C isoforms. This is based on the following: 1) phorbol 12-myristate 13-acetate affected lipolysis in an opposite way to that of EGF; and 2) the protein kinase C inhibitor bisindolylmaleimide GF 109203X did not affect the antilipolytic action of EGF. Our results indicate that the antilipolytic effect of EGF resembles more that of vasopressin than that of insulin. (*Endocrinology* **137:** 4181–4188, 1996)

EPIDERMAL GROWTH factor (EGF), a small polypeptide with a mol wt of about 6K, has dual effects in adipose tissue. It stimulates proliferation and blocks differentiation (lipid accretion) of adipocyte precursor cells in culture (1) and *in vivo* (2). In contrast, available data indicate that in differentiated adipocytes, EGF stimulates lipid accumulation: EGF increases acetyl-CoA carboxylase activity, stimulates lipogenesis (3), and inhibits hormone-stimulated lipolysis (4). Kurachi *et al.* (5) found that EGF is involved in the induction of obesity in ovariectomized mice. This dual effect of EGF (inhibition of preadipocyte differentiation but promotion of adipogenesis in differentiated adipocytes) was observed in cultured 3T3-L1 cells (6).

The effects of EGF in differentiated adipocytes are therefore similar to those of insulin. EGF and insulin receptors are both ligand-activated tyrosine-kinases (7–9). The autophosphorylated EGF receptor and the phosphorylated IRS-1 [the main substrate of the insulin receptor (10)] serve as docking points to several SH-2 (for Src homology) domain-containing proteins (11). As a result, both EGF and insulin activate the Ras/Raf-1/MAP kinase cascade and other signalling pathways in many cell types (for recent reviews see Refs. 12 and 13).

One of the better known effects of insulin is its interference with the lipolytic action of catecholamines in adipose tissue. This is a consequence of the phosphorylation and activation of the low K_m [cyclic GMP (cGMP) inhibited] phosphodiesterase (see Ref. 14 for review). This effect results in a decrease in the hormone-induced cAMP signal, in a lower activation of protein kinase A, and therefore, of the hormone-sensitive lipase, which ultimately leads to reduced lipolysis (15). In the last 2 years, several reports suggested that activation of phosphatidylinositol 3-kinase is necessary for the antilipolytic action of insulin (16, 17).

Our previous studies on the antilipolytic effect of EGF suggested that this is a consequence of an action at the level of the adenylylcyclase control by G proteins rather than an activation of some phosphodiesterase (4). However, EGF, like insulin, activates phosphatidylinositol 3-kinase in several cell systems (18). The aim of the studies presented here was to determine whether insulin and EGF induced their antilipolytic effects through the same mechanism. To do so, we first compared the effects of EGF and insulin on lipolysis stimulated by several mechanisms. Then, we studied the involvement of wortmannin-sensitive substrates and of calcium ions in their antilipolytic effects. The results suggest that the effect of EGF on lipolysis resembles more the action of vasopressin than that of insulin.

Material and Methods

Adipocytes were isolated from the epididymis of male Wistar rats as indicated in (4). The experimental procedures used were approved by the Committee on Animal Care of the University of Barcelona. To determine glucose oxidation, adipocytes (0.15×10^6 cells/ml) were incubated in Krebs-Henseleit buffer containing 1 mM $CaCl_2$ and 3% BSA (buffer A), supplemented with 0.1 mM [^{14}C]-U-glucose (2.48 μCi/mol). The incubation was carried out at 37 C in a shaking water bath. The cells were maintained inside capped vials with a small beaker suspended from the cap. After 2 h of incubation in the indicated conditions, 0.2 ml of 35% $HClO_4$ was injected through the cap into the cell suspension.

Received March 5, 1996.

Address all correspondence and requests for reprints to: Ignasi Ramírez, Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071-Barcelona, Spain. E-mail: sunyer@porthos.bio.ub.es.

* This work was supported by grants (PB91-0251 and PB94-0863) from D.G.I.C.Y.T. (Ministerio de Educación y Ciencia, Spain).

Another 0.2 ml of methyl benzethonium hydroxide were injected into the small beaker to trap the $^{14}CO_2$ produced during the incubation. After 45 min, the radioactivity was counted.

Incorporation of glucose into lipids was studied in isolated adipocytes incubated as indicated above in buffer A containing 0.1 mM [^{14}C]-U-glucose (2.48 μCi/mol). After 2 h of incubation, 0.8 ml of the cell suspension was placed into 2.5 ml of methanol/chloroform (2:1 by vol). Lipids were extracted and the radioactivity was counted as in (19).

Lipolysis was determined as the amount of glycerol released from the isolated adipocytes during the incubation period. Isolated adipocytes were incubated in buffer A supplemented with 5 mM glucose. At the beginning and after 30 min of incubation at 37 C, a sample was taken and immediately placed into enough $HClO_4$ to give a final concentration of 3%. Neutralized supernatants were used to determine glycerol (20).

To study the effect of insulin and EGF on lipoprotein lipase secretion, the cells (10^6 cells/ml) were incubated in buffer A supplemented with 5 mM glucose and a amino acid- and vitamin-solution (21) (buffer B), at 23 C instead of 37 C because the enzyme released to the incubation medium by the cells is rapidly inactivated at this temperature (22). After 30 min of incubation, a sample was taken, and the cells were separated from the medium by centrifugation. The cells were homogenized by sonication in (1 ml/5 × 10^6 cells) buffer containing 10 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, and 5 U heparin/ml, pH 7.4. Lipoprotein lipase activity in cells and medium was quantified as in (23). One unit of enzyme activity corresponded to the amount of enzyme that catalyzes the release of 1 μmol of oleate per min.

In experiments where cAMP was to be determined, the incubations were performed at 3 × 10^5 cells/ml. At indicated times a sample (1 ml) was obtained and deproteinized as described above for lipolysis. cAMP was determined in neutralized supernatant with a radiochemical binding assay kit, following manufacturer's instructions.

Cytosolic free Ca^{2+} concentration was determined in Fura-2/AM loaded adipocytes. Isolated adipocytes were incubated (10^6 cells/ml) in buffer B for 45 min at 37 C in the presence of 5 μM Fura-2/AM. The cells were then rinsed three times in fresh Fura-2/AM-free buffer B and further incubated for 30 min at 25 C to allow deesterification of the dye, and extrusion of the nondeesterified dye. Cell were again rinsed twice in fresh buffer B before their fluorescence was monitored in a Shimadzu RF5001PC spectrofluorimeter (excitation at 340 and 380 nm and emission at 505 nm). At the end of each experiment, 0.06 ml of Triton X-100 and 0.1 ml of 100 mM EGTA were sequentially added. Cytosolic free Ca^{2+} was quantified as in Ref. 24.

EGF was obtained from Boehringer Mannheim (Mannheim, Germany); vasopressin, forskolin, and phorbol 12-myristate 13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Wortmannin was obtained from Calbiochem-Novabiochem (La Jolla, CA). Bisindolylmaleimide GF 109203X was from Calbiochem (San Diego, CA). All other chemicals were obtained as indicated elsewhere (4, 19, 23, 25). The results shown in the figures and tables correspond to a representative (one among those performed) experiment. In each experiment, every condition was incubated in triplicate. Every experiment was repeated at least three times with different cell preparations. The significance of the differences was determined by ANOVA, unless otherwise indicated. When significant F value was obtained, multiple comparisons between selected groups were made by Bonferroni test (GraphPad InStat, ISI Software, Philadelphia, PA) (26).

Results

We first compared the effects of insulin and EGF on adipocytes. Both peptides stimulated glucose oxidation (68 ± 3 nmol glucose into $^{14}CO_2$/60 min × 10^6 cells in control cells; 103 ± 6 nmol glucose into $^{14}CO_2$/60 min × 10^6 cells in insulin (2 nM)-treated cells, $P < 0.001$; 83 ± 3 nmol glucose into $^{14}CO_2$/60 min × 10^6 cells in EGF (100 nM)-treated cells, $P < 0.01$) and incorporation into lipids (43 ± 3 nmol glucose into lipids/60 min × 10^6 cells in control cells; 83 ± 5 nmol glucose into lipids/60 min × 10^6 cells in insulin (2 nM)-treated cells, $P < 0.001$; 55 ± 3 nmol glucose into lipids/60 min × 10^6 cells in EGF (100 nM)-treated cells, $P < 0.001$). These results are in keeping with previous results showing that both insulin and EGF increased acetyl-CoA carboxylase activity, and the conversion of [^{3}H]glucose into lipids (3).

Adipose tissue can also obtain fatty acids from those esterified in circulating lipoproteins. The enzyme responsible for the hydrolysis of esterified fatty acids circulating in both chylomicra and very low density lipoproteins is lipoprotein lipase (27). Insulin is the most important hormone in the regulation of lipoprotein lipase in adipose tissue (28). Here we show that EGF, like insulin, stimulated the release of lipoprotein lipase from isolated adipocytes into the incubation medium: 0.07 ± 0.04 mU/10^6 cells in control cells; 0.48 ± 0.04 mU/10^6 cells in 2 nM insulin-treated cells, $P < 0.001$; 0.21 ± 0.01 mU/10^6 cells in 100 nM EGF-treated cells, $P < 0.001$. The lipoprotein lipase activity associated to the cells was not affected by either insulin or EGF treatement (1.44 ± 0.04, 1.22 ± 0.04, and 1.45 ± 0.03 mU/10^6 cells in control, insulin-, and EGF-treated cells respectively; nonsignificant differences). The effect of insulin or EGF on the secretion of lipoprotein lipase into the incubation medium was not observed when the cells were incubated in the presence of 5 U heparin/ml (2.04 ± 0.10, 2.07 ± 0.04, and 2.16 ± 0.10 mU/10^6 cells in control, insulin-, and EGF-treated cells respectively; nonsignificant differences). The rapid, heparin-independent effect of insulin on the release of lipoprotein lipase from cultured adipocytes was already observed by Chan *et al.* (29). Adachi *et al.* (6) have recently reported that EGF, at longer exposures, increases lipoprotein lipase mRNA in cultured 3T3-L1 adipocytes.

The main purpose of our study was to compare the antilipolytic effect of EGF and insulin. The results shown in Fig. 1 indicate that maximal doses of insulin or EGF (determined in dose-response experiments; data not shown) decreased by 65% and 40% respectively, the isoproterenol-stimulated lipolysis. The addition of a maximal dose of EGF together with a maximal dose of insulin further inhibited isoproterenol-stimulated lipolysis (Fig. 1).

Next, we compared the effect of insulin and EGF on li-

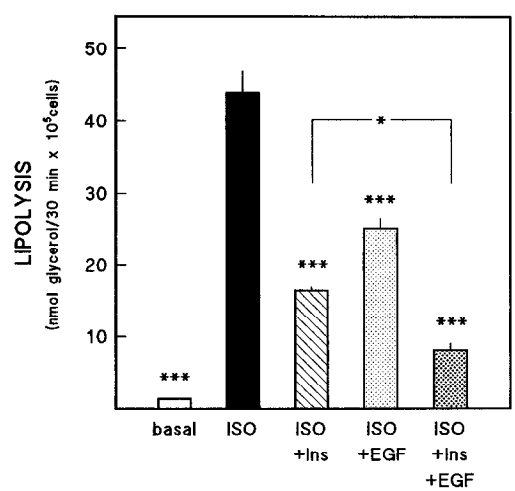

FIG. 1. Insulin and EGF decrease isoproterenol-stimulated lipolysis. Isolated adipocytes were incubated at 37 C in a medium containing 5 mM glucose at the indicated conditions. ISO, 30 nM isoproterenol; Ins, 2 nM insulin; EGF, 100 nM EGF. After 30 min of incubation a sample was processed to determine glycerol production.The results are the mean ± SE of triplicate values. *, $P < 0.05$; ***, $P < 0.001$.

polysis, which was stimulated through different mechanisms (see Fig. 2 for a schematic representation of the different mechanisms used to stimulate lipolysis in this experiment). The results are shown in Table 1. Neither insulin nor EGF affected nonstimulated lipolysis. However, insulin was able to inhibit lipolysis irrespective of the stimulating agent (except dibutyryl cAMP). EGF did not decrease forskolin-, adenosine deaminase (either alone or in combination with isoproterenol or glucagon)-, or dibutyryl cAMP-stimulated lipolysis.

The use of phosphatidylinositol 3-kinase inhibitors has revealed that this enzyme is involved in many actions of insulin in adipocytes, such as the stimulation of glucose uptake (16), the stimulation of glycogen synthase (30), and also the inhibition of hormone-stimulated lipolysis (17). Therefore, to determine whether the differences in the antilipolytic action of insulin and EGF are the consequence of differences in the signaling mechanism, we incubated isolated adipocytes in the absence and in the presence of wortmannin (1 μM) (an inhibitor of phosphatidylinositol 3-kinase (31)) for 30 min before the addition of hormones. The results are shown in Fig. 3. Wortmannin completely abolished the antilipolytic effect of insulin. However, the effect of EGF was only moderately reduced (30% inhibition without wortmannin, and 25% inhibition in the presence of wortmannin).

In search of differences in EGF and insulin signaling, we focused on Ca^{2+}. First, we studied the effect of EGF on cytosolic free Ca^{2+} in Fura-2-loaded adipocytes. As shown in Fig. 4, EGF increased cytosolic Ca^{2+} concentration. We performed eleven experiments like that shown in Fig. 4. The mean basal Ca^{2+} concentration was 155 ± 3 nM (similar basal cytosolic Ca^{2+} concentration was reported by others (32)) and increased to 184 ± 5 nM after the addition of EGF ($P < 0.001$, determined by the paired Student's t test). This effect was, however, quite moderate. As shown in the same Fig. 4,

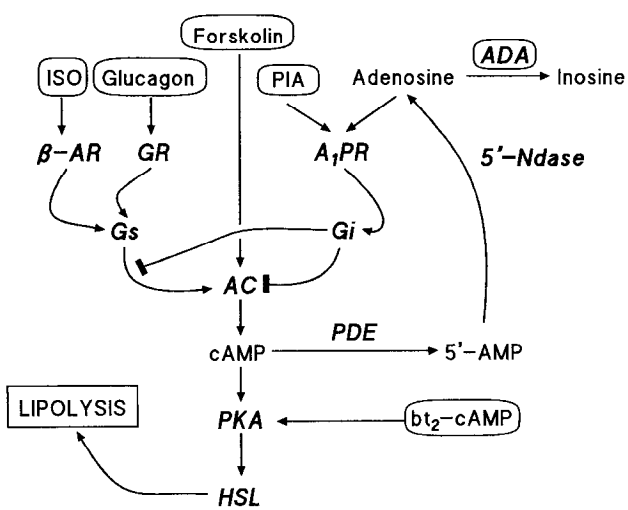

FIG. 2. Schematic representation of the different mechanisms used to stimulate lipolysis in experiments shown in Table 2. A_1PR, A_1-purinergic receptor; AC, adenylylcyclase; ADA, adenosine deaminase; βAR, β-adrenergic receptor; bt_2cAMP, dibutyryl cAMP; Gi, G_i-protein; GR, glucagon receptor; Gs, G_s-protein; HSL: hormone-sensitive lipase; ISO: isoproterenol; 5′-Ndase: 5′-Nucleotidase; PDE, phosphodiesterase; PIA, N^6-(phenylisopropil)adenosine; PKA, protein kinase A (cAMP-dependent protein kinase).

TABLE 1. Insulin and EGF affect differently lipolysis stimulated through different mechanisms

	Control	Insulin	EGF
Stimulation conditions			
Nonstimulated	5 ± 1	6 ± 4 NS	5 ± 3 NS
ADA (2 μg/ml)	9 ± 1	5 ± 1[a]	9 ± 1 NS
ISO (100 nM)	33 ± 2	17 ± 2[b]	21 ± 2[b]
ISO + ADA	71 ± 4	31 ± 2[b]	73 ± 5 NS
Glucagon (50 nM)	45 ± 3	36 ± 2[a]	39 ± 2[c]
Glucagon + ADA	73 ± 5	58 ± 2[a]	77 ± 5 NS
Forskolin (5 μM)	57 ± 2	38 ± 2[a]	56 ± 3 NS
bt_2-cAMP (350 μM)	43 ± 2	43 ± 6 NS	43 ± 1 NS

Isolated adipocytes were incubated at 37 C in a medium containing 5 mM glucose with the indicated agents, and in the absence (control) or in the presence of either insulin (2 nM) or EGF (100 nM). After 30 min of incubation, a sample was processed to determine glycerol production. The results are the mean ± SE of triplicate values. Comparisons were made versus control value.

[a] $P < 0.01$; [b] $P < 0.001$; [c] $P < 0.05$. NS, nonsignificant differences. The production of glycerol in all stimulation conditions was significantly higher than in non-stimulated (basal) condition (comparisons not shown). ISO, Isoproterenol; ADA, adenosine deaminase; bt_2-cAMP, dibutyryl cAMP.

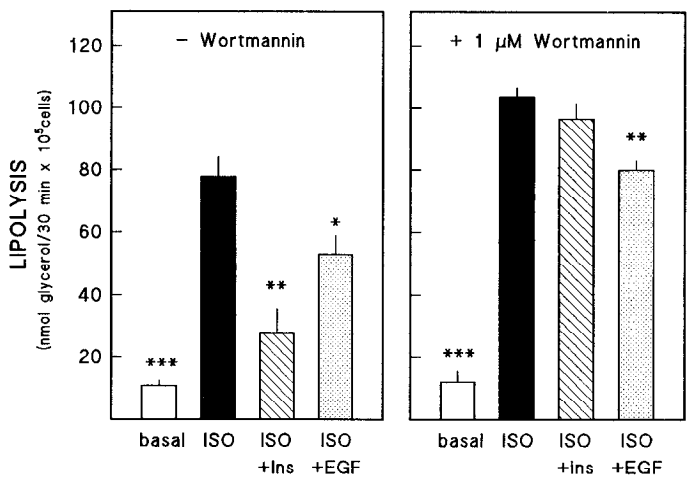

FIG. 3. Wortmannin abolishes the effect of insulin, but not of EGF, on isoproterenol-stimulated lipolysis. Isolated adipocytes were incubated for 30 min without or with 1 μM wortmannin before the addition of hormones (zero time). At zero time or 30 min after hormone additions (100 nM isoproterenol (ISO) alone or in combination with either 2 nM insulin or 100 nM EGF), a sample was taken to determine glycerol production. The results are the mean ± SE of triplicate values. *, $P < 0.05$; **, $P < 0.01$.

vasopressin raised intracellular Ca^{2+} concentration to a much higher level. When adipocytes were incubated in a Ca^{2+}-free medium, the nonstimulated intracellular Ca^{2+} concentration decreased. In this condition, EGF did not increase cytosolic Ca^{2+} concentration (Fig. 4), and vasopressin produced a more moderate and transient effect. In agreement with previous reports (33), we did not observe any effect of insulin on intracellular Ca^{2+} concentration (data not shown).

Next, we compared the effects of EGF and vasopressin on cAMP concentration and on lipolysis in isolated adipocytes. Neither EGF nor vasopressin affected basal cAMP concentration (1.7 ± 0.2, 1.8 ± 0.2, and 1.7 ± 0.1 pmol/10^5 cells in control, 100 nM EGF-, and 300 nM vasopressin-treated cells respectively; nonsignificant differences) or lipolysis (5.2 ±

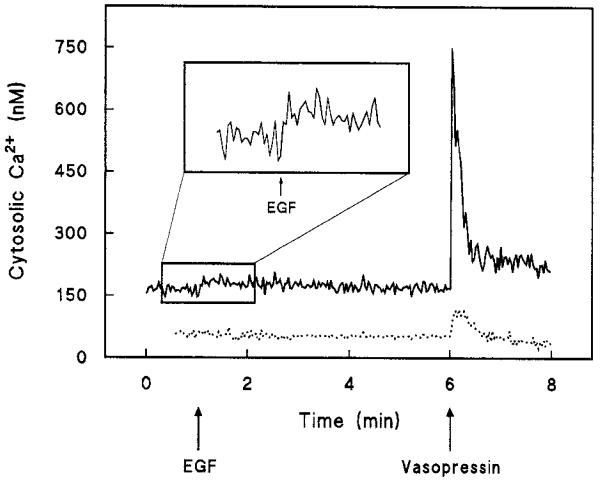

FIG. 4. EGF and vasopressin increase cytosolic Ca2+ in isolated adipocytes. Isolated adipocytes were loaded with Fura-2/AM and used to determine the effect of 100 nM EGF and 30 nM vasopressin on cytosolic Ca^{2+}. At the indicated time (*arrows*) EGF or vasopressin were added. Continuous line, cells incubated in medium containing 1 mM $CaCl_2$. *Dotted line*, Cells incubated in medium lacking $CaCl_2$ and supplemented with 0.5 mM EGTA.

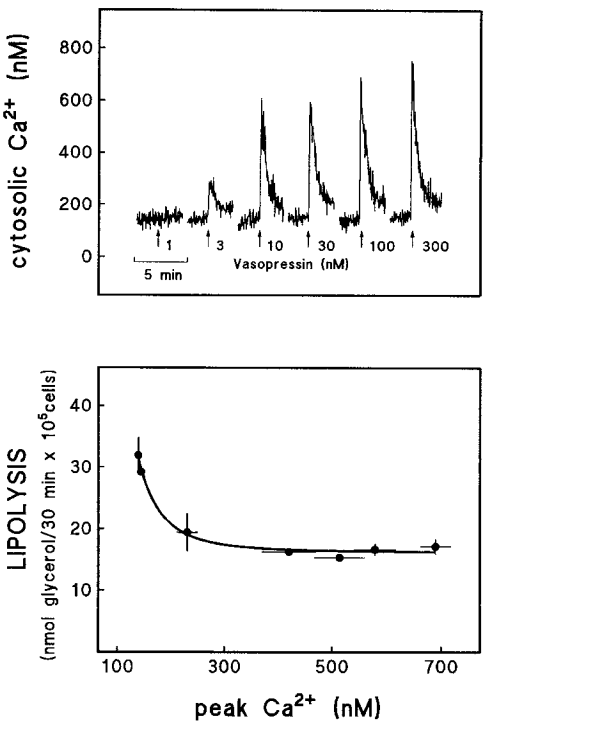

FIG. 5. Relationship between increase in cytosolic Ca^{2+} and the inhibition of isoproterenol-stimulated lipolysis by vasopressin. Fura-2/AM-loaded adipocytes were exposed to increasing concentrations of vasopressin and the increase in cytosolic Ca^{2+} was monitored (*upper panel*). Simultaneously, other samples of the same cell batch were incubated for 30 min in the presence of 50 nM isoproterenol and increasing concentrations of vasopressin to determine the effect on lipolysis. The lower panel shows the relationship between the peak cytosolic Ca^{2+} concentration (mean ± SE of the next 20 sec. after vasopressin addition), and the lipolytic response to isoproterenol (mean ± SE of triplicate values).

0.3, 5.0 ± 0.2, and 4.9 ± 0.2 nmol glycerol/30 min × 10^5 cells in control, EGF-, and vasopressin-treated cells respectively; nonsignificant differences). EGF or vasopressin decreased both parameters in isoproterenol-stimulated cells (cAMP in pmol/10^5 cells: 4.0 ± 0.2, 3.2 ± 0.1, $P < 0.01$, and 2.8 ± 0.1, $P < 0.001$ in 30 nM isoproterenol-, (isoproterenol+EGF)-, and (isoproterenol+vasopressin)-treated cells, respectively; lipolysis in nmol glycerol/30 min × 10^5 cells: 19.5 ± 0.7, 11.8 ± 0.9, $P < 0.01$, and 13.0 ± 0.3, $P < 0.001$ in isoproterenol-, (isoproterenol+EGF)-, and (isoproterenol+vasopressin)-treated cells respectively). However, none of these parameters were affected by EGF or vasopressin in forskolin-stimulated cells (cAMP in pmol/10^5 cells: 17.4 ± 0.7, 18.1 ± 1.7, and 16.8 ± 0.6 in 5 μM forskolin-, (forskolin+EGF)-, and (forskolin+vasopressin)-treated cells respectively, nonsignificant differences; lipolysis in nmol glycerol/30 min × 10^5 cells: 57 ± 2, 56 ± 1, and 58 ± 1 in forskolin-, (forskolin+EGF)-, and (forskolin+vasopressin)-treated cells, respectively, nonsignificant differences).

The antilipolytic effect of EGF and vasopressin were of similar magnitude, as shown above, but the increase in cytosolic Ca^{2+} was much higher when the cells were stimulated with vasopressin than with EGF (see Fig. 4). Therefore, we studied the relationship between the increase in cytosolic Ca^{2+} and the antilipolytic effect of vasopressin in a dose-response experiment. Vasopressin increased cytosolic Ca^{2+} in a dose-dependent manner (Fig. 5, *upper panel*). When we plotted the antilipolytic effect in isoproterenol-stimulated cells as a function of the peak Ca^{2+} concentration achieved by increasing vasopressin concentration (Fig. 5, *lower panel*), we observed that lipolysis was sensitive to a small increase in cytosolic Ca^{2+}, and that a larger increase in Ca^{2+} did not further decrease lipolysis.

We have shown above that maximal doses of EGF and insulin induced an almost additive antilipolytic effect. Therefore, we studied whether saturating concentrations of EGF (100 nM) (4) and vasopressin (300 nM) (see Fig. 5) induced additive antilipolytic effects. In this experiment, 100 nM isoproterenol increased lipolysis from a basal 7 ± 1 nmol glycerol/30 min × 10^5 cells to 38 ± 2 nmol glycerol/30 min × 10^5 cells ($P < 0.001$). When EGF or vasopressin were added in combination with isoproterenol, lipolysis was reduced to 28 ± 1 and to 28 ± 2 nmol glycerol/30 min × 10^5 cells, respectively ($P < 0.05$ *vs.* isoproterenol value). When both EGF and vasopressin were added simultaneously, the isoproterenol-stimulated lipolysis was not further reduced (26 ± 1 nmol glycerol/30 min × 10^5 cells (differences were nonsignificant either *vs.* ISO + EGF or ISO + VP).

Insulin requires the presence of Ca^{2+} ions in the incubation medium to produce maximal effects (34). Therefore, we studied the requirement of extracellular Ca^{2+} for the antilipolytic effect of insulin, EGF, and vasopressin. As shown in Fig. 6, isoproterenol-stimulated lipolysis was somewhat lower in the absence of Ca^{2+} ions. The antilipolytic effect of insulin was also reduced by the lack of Ca^{2+} (71% inhibition in the presence of Ca^{2+}, 58% in its absence). The effects of EGF or vasopressin were completely abolished in the absence of extracellular Ca^{2+}. In this condition, neither EGF nor vasopressin interfered with the cAMP signal generated by isoproterenol (data not shown).

To study the involvement of some of the Ca^{2+}-dependent

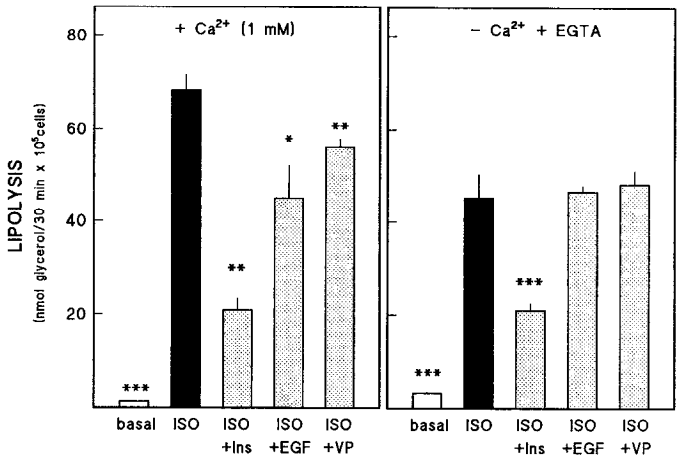

FIG. 6. The effects of EGF and vasopressin on isoproterenol-stimulated lipolysis are Ca^{2+}-dependent. Isolated adipocytes were incubated in a medium containing Ca^{2+} ions (1 mM $CaCl_2$) or in medium without Ca^{2+} ions (no $CaCl_2$ and 0.5 mM EGTA). In each condition, the cells were incubated without additions (basal) or in the presence of 100 nM isoproterenol alone (ISO), or in combination with 2 nM insulin (ISO + Ins), with 100 nM EGF (ISO + EGF), or with 30 nM vasopressin (ISO + VP). Results are the mean ± SE of triplicate values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2. EGF and PMA have different effects on lipolysis

	cAMP (pmol/10^5 cells)	Lipolysis (nmol glycerol/30 min × 10^5 cells)
basal	1.2 ± 0.3	3 ± 1
+ PMA (100 nM)	1.2 ± 0.2	3 ± 1
+ EGF (100 nM)	1.2 ± 0.2	2 ± 1
ISO	4.2 ± 0.2	108 ± 1
+ PMA (100 nM)	5.4 ± 0.1[a]	130 ± 3[a]
+ EGF (100 nM)	3.6 ± 0.1[b]	65 ± 5[a]
ADA	2.4 ± 0.1	41 ± 1
+ PMA (100 nM)	2.8 ± 0.1[a]	80 ± 7[a]
+ EGF (100 nM)	2.4 ± 0.1	42 ± 2
ISO + ADA	230 ± 6	130 ± 3
+ PMA (100 nM)	316 ± 22[b]	134 ± 2
+ EGF (100 nM)	229 ± 3	130 ± 2
ISO + ADA + PIA	3.2 ± 0.4	60 ± 3
+ PMA (100 nM)	4.6 ± 0.2[b]	104 ± 9[b]
+ EGF (100 nM)	2.4 ± 0.2[b]	48 ± 3[a]

Isolated adipocytes were incubated in the following conditions: ISO: 100 nM isoproterenol; ADA: 2 μg adenosine deaminase/ml, ISO + ADA: combination of isoproterenol and adenosine deaminase; ISO + ADA + PIA: combination of isoproterenol, adenosine deaminase and 10 nM N^6-(phenylisopropyl)adenosine. At 10 and 30 min of incubation, a sample was taken to determine cyclic AMP and lipolysis, respectively. The results are the mean ± SE of triplicate values.
[a] $P < 0.01$; [b] $P < 0.05$.

protein kinase C isoforms in the antilipolytic effect of EGF, we compared first the effects of EGF and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) on cAMP and lipolysis. The results are shown in Table 2. EGF decreased isoproterenol-stimulated lipolysis and cAMP only when adipocytes were incubated without adenosine deaminase or when, besides adenosine deaminase, the nondegradable analogue N^6-(phenylisopropyl)adenosine (PIA) was also added. PMA increased cAMP under any stimulatory condition. Lipolysis was also synergistically increased by PMA except when it was stimulated by the combination of isoproterenol and adenosine deaminase. In this condition, lipolysis exceeded 125 nmol glycerol/30 min × 10^5 cells. As shown in Fig. 7, at this value, an additional increase in cAMP did not further increase lipolysis. Honnor *et al.* (35) also reported saturation of lipolysis when studied as a function of protein kinase A activity ratio. Figure 7 plots data shown in Table 2. Note that control-, PMA-, and EGF-values all fit to the same curve. This indicates that the opposite effects of PMA and EGF on lipolysis can be explained by their effects on cAMP levels.

We have reported that EGF enhances the sensitivity of the G_s-mediated activation of adenylylcyclase to the inhibitory (G_i-mediated) effect of adenosine (4). Therefore, we also compared the effects of EGF and PMA on the inhibition of isoproterenol-stimulated lipolysis by PIA. As shown in Fig. 8, EGF decreased IC_{50} from control value of 30 ± 2 nM to 11 ± 1 nM ($P < 0.001$). EGF does not affect however the maximal inhibitory effect of PIA. PMA not only did not decrease IC_{50}, but actually increased this parameter to 39 ± 2 nM ($P < 0.01$). PMA also reduced the maximal inhibitory effect of PIA.

To rule out any role of some of the Ca^{2+}-dependent protein kinase C isoforms in EGF inhibition of hormone-stimulated lipolysis, we studied the effect of the protein kinase C-inhibitor bisindolylmaleimide GF 109203X (36). The results are shown in Table 3. The incubation of adipocytes for 30 min with 2 μM GF 109203X completely prevented the effect of PMA on isoproterenol-stimulated lipolysis. The effect of EGF was, however, insensitive to this protein kinase C inhibitor.

Discussion

There are few studies on the effect of EGF in mature adipocytes (3–6). The data available suggest that, like insulin, EGF may promote lipid accumulation. Here we show that EGF and insulin stimulate glucose metabolism (oxidation and incorporation into lipids); they also stimulate the release of lipoprotein lipase and inhibit catecholamine-stimulated

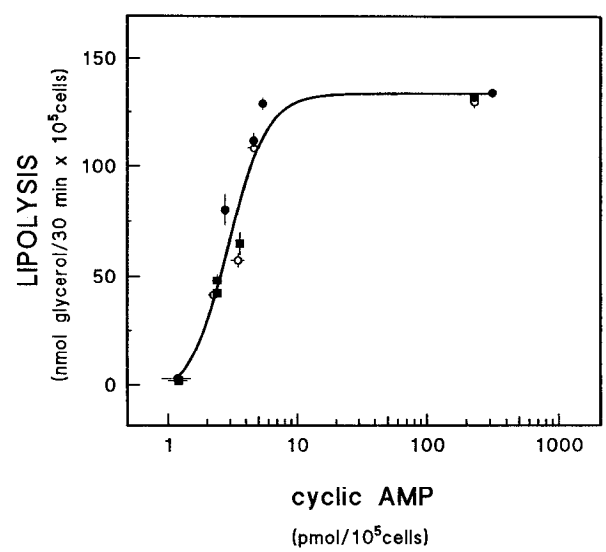

FIG. 7. EGF and PMA do not affect the relationship between cAMP and lipolysis. Data in Table are plotted in this figure to show the relationship between cAMP and lipolysis. ●, Cells incubated in the presence of PMA; n, cells incubated in the presence of EGF; ○, control cells.

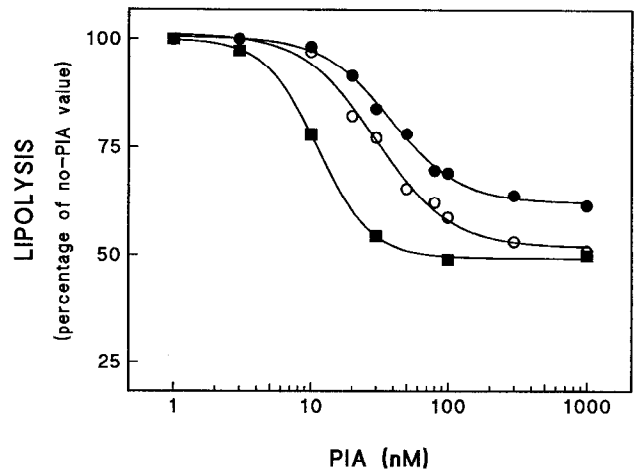

FIG. 8. EGF increases, but PMA decreases, the sensitivity of isoproterenol-stimulated lipolysis to inhibition by N^6-(phenylisopropyl)adenosine. The effect of increasing concentrations of PIA on lipolysis stimulated by 100 nM isoproterenol plus 2 μg adenosine deaminase/ml was determined in the absence of further additions (control: ○) or in the presence of either 100 nM EGF (■) or 100 nM PMA (●). Results are the mean of duplicate values.

lipolysis. Therefore, both promote all the actions required for the efficient accumulation of triacylglycerols in adipocytes. Our findings on the short-term effects of EGF are in keeping with those on the long-term effect reported by Adachi *et al.* (6). They showed that EGF actually increased the triacylglycerol content in 3T3-L1 adipocytes, when the peptide was added for 10 days on and after the sixth day after the induction of differentiation.

Because antilipolysis is the best known effect among those of insulin in adipocytes, we centered our studies on this pathway to determine whether EGF and insulin produced a similar effect through the same or different mechanism.

Insulin stimulates the phosphorylation, which results in the activation, of one of the phosphodiesterase isoforms: the low K_m (cGMP inhibited) phosphodiesterase (37). Recent studies indicate that this effect is sufficient to account for the antilipolytic effect of insulin (38). Our data concerning the insulin effect (see Table 1) are compatible with this mode of action. Insulin did not decrease dibutyryl cAMP-stimulated lipolysis because dibutyryl cAMP is resistant to hydrolysis by phosphodiesterase. Insulin inhibited the lipolytic action of isoproterenol, glucagon, adenosine deaminase (alone or in combination with lipolytic hormones), and forskolin. In these conditions, adenylylcyclase is stimulated through different mechanisms (see Fig. 2). Because the action of insulin is downstream of adenylylcyclase, it can inhibit the lipolytic action of this variety of stimulating agents.

Our previous studies indicated that the antilipolytic effect of EGF involves the regulation of G protein interaction in the control of adenylylcyclase (4). Therefore, it was important to compare EGF and insulin antilipolytic effects directly. The results obtained indicate that the effects are mediated by different mechanisms. If EGF were activating a phosphodiesterase, then its antilipolytic effect 1) would not disappear when adenosine deaminase was added to the cell suspension, and 2) would be observed in forskolin-stimulated cells.

TABLE 3. Bisindolylmaleimide GF 109203X does not interfere with the effect of EGF on Isoproterenol-stimulated lipolysis

	Lipolysis (nmol glycerol/30 min × 10^5 cells)	
	−GF 109203X	+GF 109203X (2 μM)
Control	103 ± 2	88 ± 2
+ PMA (100 nM)	114 ± 2[a]	88 ± 1
+ EGF (100 nM)	97 ± 1[a]	78 ± 2[a]

Isolated adipocytes were incubated at 37 C with or without the bisindoylmaleimide GF 109203X (2 μM) for 30 min before the addition of isoproterenol (final concentration: 100 nM; control), isoproterenol with PMA (+ PMA), or isoproterenol with EGF (+ EGF) in equal volumes. Then, a sample was taken (zero time) and another sample was taken 30 min afterwards to determine lipolysis. Results are the mean ± SE of triplicate values.

[a] $P < 0.05$.

The conclusion that the antilipolytic effect of EGF is mediated by a different mechanisms to that of insulin is further supported by an additional observation reported herein (and shown in Fig. 1): EGF potentiated the effect of a maximal dose of insulin.

The use of the phosphatidylinositol 3-kinase inhibitor wortmannin (31) revealed that phosphatidylinositol 3-kinase is required for the antilipolytic action of insulin (16), and for the phosphorylation and activation of the low Km (cGMP-inhibited) phosphodiesterase (17). It have been recently shown that wortmannin and its structural analogue demethoxyviridin can also inhibit phospholipase A_2 in Swiss 3T3 cells (39). Therefore, the involvement of phosphatidylinositol 3-kinase in insulin antilipolysis might be reevaluated. The activation of phosphatidylinositol 3-kinase is a common feature for many growth factors in many cells (13). However, because wortmannin affected very moderately the effect of EGF on isoproterenol-stimulated lipolysis, we conclude that phosphatidylinositol 3-kinase, and other wortmannin sensitive substrates, do not contribute (or contribute very modestly) to EGF antilipolysis.

In search of differences in EGF and insulin signalling, we focused on Ca^{2+} because insulin does not increase intracellular Ca^{2+} in adipocytes (33) and, although it was mentioned that EGF had no effect on cytosolic Ca^{2+} in mature adipocytes (33), it is known that the EGF receptor phosphorylates the γ-isoform of phosphoinositide-specific phospholipase C (40). This effect of EGF results in the increase in cytosolic free Ca^{2+} concentration in many cells types including NIH 3T3 cells (41). We found that EGF, but not insulin, increased this messenger in isolated adipocytes (see Fig. 4). Indeed, this effect was much milder than that of vasopressin. The results obtained in a Ca^{2+}-free medium indicate that the increase in cytosolic free Ca^{2+}, induced by both EGF and vasopressin, depends on extracellular Ca^{2+} either because they stimulate Ca^{2+} inflow, or because extracellular Ca^{2+} is required for maintenance of the internal stores.

Vasopressin infusion to fasted rats has a rapid antilipolytic effect (42). These authors did not observe such an effect in isolated adipocytes (43). We report here that vasopressin, like EGF, decreases the cAMP signal and the lipolytic response of adipocytes to isoproterenol. Our results further suggest that EGF and vasopressin decrease the lipolytic response of isolated adipocytes through similar mechanisms. This is based on the following: 1) both had similar antilipolytic potencies;

2) both decreased isoproterenol- but not forskolin-stimulated lipolysis and cAMP levels; 3) both antilipolytic effects were completely dependent on the presence of free Ca^{2+} in the incubation medium; and 4) maximal doses of each peptide did not induce additive effects. It could be argued that although both induced similar antilipolytic effects, vasopressin increased intracellular Ca^{2+} concentration more than 5-fold whereas EGF did so only 1.2-fold. However, in dose-response experiments comparing the increase in cytosolic Ca^{2+} and the ability of vasopressin to decrease isoproterenol-stimulated lipolysis, we observed that such a large increase in Ca^{2+} is not required to reach maximal antilipolysis (see Fig. 5). In fact, lipolysis was very sensitive to moderate increases in Ca^{2+}. Therefore, we propose that both EGF and vasopressin decrease hormone-stimulated lipolysis through a similar Ca^{2+}-dependent mechanism.

Among the mediators of Ca^{2+}, we studied the involvement of protein kinase C in the antilipolytic effect of EGF. Protein kinase C is involved in some of the effects of EGF in several cell types, including the late phase activation of $p70^{s6k}$ (44, 45) and the stimulation of phospholipase D (46) in 3T3 cells. Other effects of EGF are, however, independent of protein kinase C (47, 48). Our results suggest that protein kinase C induces an effect on cAMP and lipolysis opposed tot that of EGF. This conclusion is based in the studies involving PMA and the bisindolylmaleimide GF 109203X. It should be noted that PMA only activate diacylglycerol-stimulated protein kinase C isoforms (49). Among them, those which are also Ca^{2+}-dependent: α, $\beta I/\beta II$, and γ (50). Those isoforms are also the most sensitive to GF 109203X (51). Therefore, our results, although do not exclude the involvement of all protein kinase C isoforms (specially ζ, which is completely insensitive to PMA (50) and rather insensitive to the GF 109203X (51)), do exclude the involvement of the classical protein kinase C isoforms which are sensitive to both PMA and GF 109203X.

The effect of protein kinase C on the cAMP generating system is complex and appears to be cell-specific (52). Naghshineh *et al.* (53) found that purified protein kinase C activates adenylylcyclase from adipocyte membranes. In addition to this effect, it is conceivable that protein kinase C could phosphorylate and inactivate the function of some G_i protein in adipocytes, as it was observed in intact hepatocytes (54). Our results showing that PMA decreases the sensitivity of isoproterenol-stimulated lipolysis to the inhibition by PIA (Fig. 8), and those of Chambaut-Guerin and Thomopoulos (55) (who observed that the effect of PMA on cAMP was prevented by the pretreatment of the cells with pertussis toxin), are in keeping with this latter hypothesis. The results reported herein (Fig. 8) indicate that EGF produces an opposite effect to that of Ca^{2+}-sensitive protein kinase C isoforms on the sensitivity of adenylylcyclase to G_i proteins: EGF increase the sensitivity of isoproterenol-stimulated adenylylcyclase to the inhibitory effect of PIA. In a previous report (4), we showed that the effect of EGF on PIA-inhibited lipolysis was only observed if adenylylcyclase was stimulated by G_s-coupled receptors.

In conclusion, we observed that EGF and insulin promote similar effects in mature adipocytes. Although both EGF and insulin receptors share common capabilities in their respective mechanisms of action, our results demonstrate that, at least one of these effects, the antilipolysis, is mediated by different mechanisms. In particular, the effect of EGF requires of Ca^{2+}, although Ca^{2+}-activated protein kinase C isoforms are not involved.

Acknowledgment

We thank Robin Rycroft for editorial help.

References

1. **Serrero G** 1987 EGF inhibits the differentiation of adipocyte precursors in primary cultures. Biochem Biophys Res Commun 146:194–202
2. **Serrero G, Mills D** 1991 Physiological role of epidermal growth factor on adipose tissue development *in vivo*. Proc Natl Acad Sci USA 88:3912–3916
3. **Haystead TAJ, Hardie DG** 1986 Both insulin and epidermal growth factor stimulate lipogenesis and acetyl-CoA carboxylase activity in isolated adipocytes. Importance of homogeneization procedure in avoiding artefacts in acetyl-CoA carboxylase assay. Biochem J 234:279–284
4. **Tebar F, Ramírez I, Soley M** 1993 Epidermal growth factor modulates the lipolytic action of catecholamines in rat adipocytes. Involvement of a Gi protein. J Biol Chem 268:17199–17204
5. **Kurachi H, Adachi H, Ohtsuka S, Morishige K, Amemiya K, Keno Y, Shimomura I, Tokunaga K, Miyake A, Matsuzawa Y, Tanizawa O** 1993 Involvement of epidermal growth factor in inducing obesity in ovariectomized mice. Am J Physiol 265:E323–E331
6. **Adachi H, Kurachi H, Homma H, Adachi K, Imai T, Morishige K-I, Matsuzawa Y, Miyake A** 1994 Epidermal growth factor promotes adipogenesis of 3T3–L1 cell *in vitro*. Endocrinology 135:1824–1830
7. **Cohen S, Carpenter G, King Jr L** 1980 Epidermal growth factor-receptor-kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. J Biol Chem 255:4834–4842
8. **Kasuga M, Karlsson FA, Kahn CR** 1982 Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science 215:185–187
9. **Kasuga M, Zick Y, Blithe DL, Crettaz M, Kahn CR** 1982 Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature 298:667–669
10. **White MF, Maron R, Kahn CR** 1985 Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. Nature 318:183–186
11. **Pawson T** 1995 Protein modules and signalling networks. Nature 373:573–580
12. **Fantl WJ, Johnson DE, Williams LT** 1993 Signalling by receptor tyrosine kinases. Annu Rev Biochem 62:453–481
13. **Malarkey K, Belham CM, Paul A, Graham A, McLees A, Scott PH, Plevin R** 1995 The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. Biochem J 309:361–375
14. **Manganiello VC, Degerman E, Smith CJ, Vasta V, Tornqvist H, Belfrage P** 1992 Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin. In: Strada SJ, Hidaka H (eds) Advances in Second Messenger and Phosphoprotein Research. Raven Press, New York, vol 25:147–164
15. **Belfrage P, Fredrickson G, Stralfors P, Tornqvist H** 1984 Adipose tissue lipases. In: Borgström B, Brockman HL (eds) Lipases. Elsevier, Amsterdam, pp 365–416
16. **Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M** 1994 Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J Biol Chem 269:3568–3573
17. **Rahn T, Ridderstråle M, Tornqvist H, Manganiello V, Fredrikson G, Belfrage P, Degerman E** 1994 Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes: studies using the selective inhibitor wortmannin. FEBS Lett 350:314–318
18. **Miller ES, Ascoli M** 1990 Anti-phosphotyrosine immunoprecipitation of phosphatidylinositol-3′ kinase activity in different cell types after exposure to epidermal growth factor. Biochem Biophys Res Commun 173:289–295
19. **Zorzano A, Soley M, Herrera E** 1989 Rapid effect of insulin and glucose on the hepatic incorporation of gluconeogenic substrates into glyceride glycerol and glycogen. Int J Biochem 21:1071–1075
20. **Wieland OH** 1984 Glycerol: UV-method. In: Bergmeyer HV, Bergmeyer J, Grabl M (eds) Methods in Enzymatic Analysis. Verlag Chemie, Weinheim, pp 504–510
21. **Burgaya F, Peinado J, Vilaró S, Llobera M, Ramírez I** 1989 Lipoprotein lipase activity in neonatal-rat liver cell types. Biochem J 259:159–166
22. **Peinado-Onsurbe J, Soler C, Soley M, Llobera M, Ramírez I** 1992 Lipoprotein lipase and hepatic lipase activities are differentially regulated in isolated hepatocytes from neonatal rats. Biochim Biophys Acta 1125:82–89
23. **Ramírez I, Kryski AJ, Ben-Zeev O, Schotz MC, Severson DL** 1985 Characterization of triacylglycerol hydrolase activities in isolated myocardial cells from rat heart. Biochem J 232:229–236

24. **Grynkiewicz G, Poenie M, Tsien RY** 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
25. **Quintana I, Grau M, Moreno F, Soler C, Ramírez I, Soley M** 1995 The early stimulation of glycolysis by epidermal growth factor in isolated rat hepatocytes is secondary to the glycogenolytic effect. Biochem J 308:889–894
26. **Kullback S, Keegel JC** 1991 Categorical data problems using information theoretic approch. In: Krisbnaiah PR, San PK (eds) Handbook of Statistics. North-Holland, Amsterdam, vol 4:831–871
27. **Robinson DS** 1970 The function of triglycerides in fatty acid transport. Compr Biochem 18:51–116
28. **Eckel RH** 1987 Adipose tissue lipoprotein lipase. In: Borensztajn J (ed) Lipoprotein Lipase. Evener, Chicago, pp 79–132
29. **Chan BL, Lisanti MP, Rodriguez-Boulan E, Saltiel AR** 1988 Insulin-stimulated release of lipoprotein lipase by metabolism of its phosphatidylinositol anchor. Science 241:1670–1672
30. **Standaert ML, Bandyopadhyay G, Farese RV** 1995 Studies with wortmannin suggest a role for phosphatidylinositol 3-kinase in the activation of glycogen synthase and mitogen-activated protein kinase by insulin in rat adipocytes: comparison of insulin and protein kinase C modulators. Biochem Biophys Res Commun 209:1082–1088
31. **Ui M, Okada T, Hazeki K, Hazeki O** 1995 Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. Trends Biochem Sci 20:303–307
32. **Kelly KL, Deeney JT, Corkey BE** 1989 Cytosolic free calcium in adipocytes. Distinct mechanisms of regulation and effects on insulin action. J Biol Chem 264:12754–12757
33. **Blackmore PF, Augert G** 1989 Effect of hormones on cytosolic free calcium in adipocytes. Cell Calcium 10:561–567
34. **Draznin B, Sussman K, Kao M, Lewis D, Sherman N** 1987 The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. J Biol Chem 262:14385–14388
35. **Honnor RC, Dhillon GS, Londos C** 1985 cAMP-dependent protein kinase and lipolysis in rat adipocytes. II. Definition of steady-state relationship with lipolytic and antilipolytic modulators. J Biol Chem 260:15130–15138
36. **Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J** 1991 The bisindoylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781
37. **Degerman E, Smith CJ, Tornqvist H, Vasta V, Belfrage P, Manganiello VC** 1990 Evidence that insulin and isoprenaline activate cGMP inhibited low Km cAMP phosphodiesterase in rat fat cells by phosphorylation. Proc Natl Acad Sci USA 874:533–537
38. **Eriksson H, Ridderstråle M, Degerman E, Ekholm D, Smith CJ, Manganiello VC, Belfrage P, Tornqvist H** 1995 Evidence for the key role of the adipocyte cGMP-inhibited cAMP phosphodiesterase in the antilipolytic action of insulin. Biochim Biophys Acta 1266:101–107
39. **Cross MJ, Stewart A, Hodgkin MN, Kerr DJ, Wakelam MJO** 1995 Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A_2 activity in Swiss 3T3 cells - wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. J Biol Chem 270:25352–25355
40. **Carpenter G** 1992 Receptor tyrosine kinase substrates - src homology domains and signal transduction. FASEB J 6:3283–3289
41. **Pandiella A, Beguinot L, Velu TJ, Meldolesi J** 1988 Transmembrane signalling at epidermal growth factors overexpressed in NIH 3T3 cells. Phosphoinositide hydrolysis, cytosolic Ca2+ increase and alkalinization correlate with epidermal-growth-factor-induced proliferation. Biochem J 254:223–228
42. **Rofe AM, Williamson DH** 1983 Metabolic effects of vasopressin infusion in the starved rat. Reversal of ketonaemia. Biochem J 212:231–239
43. **Rofe AM, Williamson DH** 1983 Mechanism for the 'anti-lipolytic' action of vasopressin in the starved rat. Biochem J 212:899–902
44. **Susa M, Olivier AR, Fabbro D, Thomas G** 1989 EGF induces biphasic S6 kinase activation: late phase is protein kinase C-dependent and conrtibutes to mitogenicity. Cell 57:817–824
45. **Susa M, Vulevic D, Lane HA, Thomas G** 1992 Inhibition or down-regulation of protein kinase-C attenuates late phase p70(s6k) activation induced by epidermal growth factor but not by platelet-derived growth factor or insulin. J Biol Chem 267:6905–6909
46. **Yeo E-J, Exton JH** 1995 Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. J Biol Chem 270:3980–3988
47. **Franklin CC, Kraft AS** 1992 Protein kinase-C-independent activation of c-jun and c-fos transcription by epidermal growth factor. Biochim Biophys Acta 1134:137–142
48. **Boniece IR, Wagner JA** 1993 Growth factors protect PC12 cells against ischemia by a mechanism that is independent of PKA, PKC, and protein synthesis. J Neurosci 13:4220–4228
49. **Nishizuka Y** 1988 The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665
50. **Nishizuka Y** 1995 Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484–496
51. **Martiny-Baron G, Kananietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marmé D, Schächtele C** 1993 Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J Biol Chem 268:9194–9197
52. **Houslay MD** 1991 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur J Biochem 195:9–27
53. **Naghshineh S, Noguchi M, Huang K-P, Londos C** 1986 Activation of adipocyte adenylate cyclase by protein kinase C. J Biol Chem 261:14534–14538
54. **Pyne NJ, Murphy GJ, Milligan G, Houslay MD** 1989 Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein G_i. FEBS Lett 243:77–82
55. **Chambaut-Guerin A-M, Thomopoulos P** 1987 Protein kinase C potentiates isoproterenol-mediated cycli AMP production without modifying the homologous desensitization process in J774 cells. Eur J Biochem 170:381–387