Insulin-like effects of epidermal growth factor and insulin-like growth factor-I on [³H]2-deoxyglucose uptake, diacylglycerol generation and protein kinase C activation in BC3H-1 myocytes

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Epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) were found to provoke increases in [³H]2-deoxyglucose uptake, diacylglycerol (DAG) generation and membrane-bound protein kinase C activity in BC3H-1 myocytes. These effects were similar to those provoked by insulin. The increases in DAG did not appear to be derived from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) or phosphatidylinositol, but may have been derived from synthesis of phosphatidic acid *de novo*, and hydrolysis of phosphatidylcholine, as revealed by studies with [³H]glycerol and [³H]choline respectively. Accordingly, both EGF and IGF-I increased acute [³H]glycerol labelling of DAG (and other lipids) and [³H]choline labelling of phosphocholine. These labelling responses were similar in time course, suggesting that they are closely coupled. Our findings suggest that EGF and IGF-I, like insulin, increase DAG–protein kinase C signalling, apparently by activating co-ordinated lipid-synthesis and -hydrolysis responses, which are distinctly different from the PIP₂-hydrolysis response.

INTRODUCTION

Epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) are known to provoke insulin-like effects in certain tissues, by poorly understood mechanisms. In BC3H-1 myocytes, we have previously shown that (a) insulin-induced increases in 2-deoxyglucose (2-DOG) uptake are preceded by increases in diacylglycerol (DAG) content [1,2] and activation of protein kinase C [3,4] and (b) activation of protein kinase C by addition of 12-*O*-tetradecanoylphorbol 13-acetate [5], a DAG analogue, activates 2-DOG uptake. We have therefore postulated that the increase in 2-DOG uptake during insulin action may be partly or wholly mediated by enhanced DAG–protein kinase C signalling. Furthermore, we have reported that increases in DAG may be derived directly both from phosphatidic acid (PA) synthesized *de novo* and from hydrolysis of pre-existing phospholipids such as phosphatidylcholine (PC) [6,7] and the phosphatidylinositol-glycan (PI-glycan) [8–10]. In the present investigation, we examined the effects of EGF and IGF-I on 2-DOG uptake, DAG generation, PA-DAG synthesis *de novo* and PC hydrolysis.

MATERIALS AND METHODS

BC3H-1 myocytes were cultured on 35 mm (or where indicated 100 mm) diam. dishes in 3 ml (or 25) ml of Dulbecco's Modified Eagle's Medium containing 15% (v/v) controlled Process Serum replacement-I (Sigma), and grown to confluence (approx. 0.5 mg protein) over a 10–14 day period as described previously [1–6,11]. On the day of the experiment, the medium was changed to Dulbecco's phosphate-buffered saline (138 mM-NaCl/

8.1 mM-Na₂HPO₄/0.5 mM-MgCl₂/1.5 mM-KH₂PO₄/2.6 mM-KCl) containing 0.1 mM-CaCl₂, 1 mg of glucose/ml and 1 mg of bovine serum albumin/ml (DPBSGA). The cells were incubated at 37 °C for 2 h without or with 10 μCi of [³H]glycerol (or in some experiments, as described in the text, with 2 μCi of [³H]choline) to label effectively the glycerol 3-phosphate pool [6,7]. EGF, IGF-I, insulin or phenylephrine was then added, and incubation was continued as described in the text. At the end of incubation, the cells were examined for: (a) [³H]glycerol (or [³H]choline) incorporation into lipids as described previously [1–7]; (b) DAG content of lipid extracts, by using the DAG kinase method of Preiss *et al.* [12]; (c) [³H]2-DOG uptake as described previously [5,11]; or (d) protein kinase C activity (see [3,4] and below).

In some experiments, cells (in 35 mm plates) were labelled for 16 h with 1 μCi of [³H]myristic acid, or during their entire growth cycle (10 days) by adding 10 μCi of [³H]glycerol or [³H]inositol, or 1 μCi of [³H]choline, to the culture medium at the time of subculturing. On the day of the experiment, the media were replaced with radioisotope-free incubation media (as described above), and cells were equilibrated for 2 h at 37 °C before treatment with agonists. After incubation, media were removed, and extracted lipids (see [1–7]) were examined for incorporation of label into lipids and/or phospholipids, and aqueous extracts were examined for [³H]inositol labelling of inositol mono-, bis- and tris-phosphates after separation on Dowex-1 (formate form; Bio-Rad) columns (see [2]), or for [³H]choline labelling of choline, phosphocholine and CDP-choline after purification by t.l.c. (see [13]).

Abbreviations used: EGF, epidermal growth factor; IGF-I, insulin-like growth factor-I; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; 2-DOG, 2-deoxyglucose.

Cytosolic Ca^{2+} was monitored by quin 2 fluorescence as described previously [2].

To measure protein kinase C activity of cytosol and particulate fractions, after incubations cells were rapidly chilled, gently scraped from 100 mm dishes and pelleted by centrifugation (800 *g* for 10 min) in 0.5 ml of ice-cold buffer containing 20 mM-Tris, pH 7.4, 1.2 mM-EGTA, 50 mM- β -mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride. The cells were sonicated, and the resulting preparations were centrifuged at 100 000 *g* for 30 min. The cytosol (supernatant) fractions were decanted, and the pellets were resuspended by sonication in buffer containing 0.5 ml of 20 mM-Tris, pH 7.4, 5 mM-EGTA, 2 mM-EDTA, 50 μ M- β -mercaptoethanol, and 0.1% Triton X-100, and then centrifuged at 100 000 *g* for 30 min to remove undissolved debris. Protein kinase C of cytosol and solubilized membrane fraction were partially purified by applying 200 μ g of cytosolic or membrane protein to 0.5 ml DEAE-Sephacel columns, followed by stepwise elution with 2 column volumes of each buffer containing 50 mM-, 150 mM- or 500 mM-NaCl, along with 20 mM-Tris, pH 7.4, 50 μ M- β -mercaptoethanol, 1 mM-EDTA and 1 mM-EGTA. Protein kinase C activity (i.e. Ca^{2+} /phospholipid-dependent incorporation of ^{32}P into histone III) was assayed as previously described [3,4].

[2- ^3H]Glycerol (sp. radioactivity 5 Ci/mmol) was obtained from ICN. [2- ^3H]Inositol (sp. radioactivity 15 mCi/mmol) was obtained from American Radio-

Table 1. Effects of insulin, EGF and IGF-I on [^3H]2-DOG uptake in BC3H-1 myocytes

Results are means $\pm$ S.E.M. for the numbers of determinations shown in parentheses. [Note: as is apparent, there is considerable experiment-to-experiment variability in absolute values of control and stimulated [^3H]2-deoxyglucose uptake. This probably reflects variable residual stimulation of basal uptake by serum growth factors present during the culture period, along with other factors that are difficult to control, e.g. carbohydrate stores, levels of glucose transporters, efficiencies of signalling systems etc.]

Expt.	Treatment	Uptake (c.p.m./plate)
A	None (control)	1268 $\pm$ 100 (4)
	Insulin (12 ng/ml)	1702 $\pm$ 104 (4)
	Insulin (120 ng/ml)	2060 $\pm$ 110 (4)
	Insulin (1200 ng/ml)	2412 $\pm$ 118 (4)
B	None (control)	848 $\pm$ 54 (6)
	Insulin (1200 ng/ml)	2314 $\pm$ 192 (6)
	EGF (1 ng/ml)	1522 $\pm$ 150 (4)
	EGF (10 ng/ml)	1826 $\pm$ 80 (4)
	EGF (100 ng/ml)	1920 $\pm$ 58 (3)
	IGF-I (100 ng/ml)	2910 $\pm$ 146 (6)
C	None (control)	3594 $\pm$ 192 (4)
	Insulin (1200 ng/ml)	5286 $\pm$ 302 (4)
	IGF-I (10 ng/ml)	3612 $\pm$ 330 (4)
	IGF-I (100 ng/ml)	5224 $\pm$ 408 (4)
	Insulin (1200 ng/ml) + IGF-I (100 ng/ml)	5212 $\pm$ 392 (4)
D	None (control)	1432 $\pm$ 186 (4)
	Insulin (1200 ng/ml)	4750 $\pm$ 266 (2)
	IGF-I (25 ng/ml)	2968 $\pm$ 196 (3)
	IGF-I (50 ng/ml)	3666 $\pm$ 120 (3)
	IGF-I (100 ng/ml)	3920 $\pm$ 372 (3)

Fig. 1. Temporal effects of insulin (120 ng/ml), EGF (100 ng/ml), IGF-I (50 ng/ml) and phenylephrine (10 μ M) on DAG content of BC3H-1 myocytes

Results are means $\pm$ S.E.M. for 7–12 determinations.

labeled Chemicals. [*methyl*- ^3H]Choline (sp. radioactivity 80 mCi/mmol) and [^3H]myristic acid (sp. radioactivity 22.4 Ci/mmol) were obtained from NEN/Dupont. Insulin was obtained from Elanco. IGF-I was purchased from Bachem. EGF, phenylephrine and other biochemicals were purchased from Sigma.

RESULTS

[^3H]2-Deoxyglucose uptake

Dose-related effects of insulin, EGF and IGF-I on [^3H]2-DOG uptake are shown in Table 1. Maximal effects of EGF were observed at 10–100 ng/ml, and these effects were slightly less than those of maximally effective concentrations (20–200 nM; 120–1200 ng/ml) of insulin. IGF-I, at concentrations of 50–100 ng/ml, provoked increases in 2-DOG uptake which were comparable with, and non-additive with, those provoked by maximally effective concentrations of insulin.

DAG generation

Time-related effects of maximally or nearly maximally effective concentrations of insulin (20 nM), EGF (100 ng/ml), IGF-I (50 ng/ml) and phenylephrine (10 mM) (see [14]) on DAG content are shown in Fig. 1. Although there were slight differences in the patterns observed over the course of 20 min treatment periods, all agonists provoked rapid 2–3-fold increases in DAG content. Effects of insulin and EGF on DAG content persisted with little or no diminution over the entire 20 min period. Effects of IGF-I and phenylephrine, on the other hand,

diminished, but were nevertheless evident, over the last half of the 20 min treatment period.

[2-³H]Glycerol-labelling experiments

Findings from other studies have suggested that insulin-induced increases in DAG are derived both from

Fig. 2. Temporal effects of 1 ng of EGF/ml (a), 10 ng of EGF/ml (b) and 100 ng of EGF/ml (c) on [³H]glycerol incorporation into total phospholipids (PL), monoacylglycerol (MAG), DAG and triacylglycerol (TAG) in BC3H-1 myocytes

Cells were incubated for 2 h with [³H]glycerol, and then, over a 20 min period, EGF was present for the indicated times. Results are means $\pm$ S.E.M. for four determinations. [It should be noted that experiments conducted in each panel were not done concurrently, but at widely spaced intervals. This explains the different patterns of labelling, which, for uncertain reasons (perhaps reflecting adaptive changes in enzymes regulating carbohydrate, lipid and phospholipid metabolism), occurs with each subculturing of the myocytes.]

PA synthesis *de novo* and hydrolysis of preformed phospholipids [1–9]. To gain insight into potential sources of DAG during the actions of EGF and IGF-I, acute [³H]glycerol labelling was used to determine whether this DAG may be derived, at least partly, from PA synthesis *de novo*. As shown in Fig. 2, in cells which were preincubated for 2 h in the presence of [³H]glycerol, subsequent addition of EGF (1, 10 or 100 ng/ml) provoked increases not only in the labelling of DAG, but also in that of triacylglycerol, monoacylglycerol and total phospholipids. It may also be noted that, at 1 and 10 ng/ml, EGF effects on [³H]glycerol labelling were slower to develop, but more prolonged, than those observed with EGF at 100 ng/ml. At the higher EGF concentration (100 ng/ml), increases in [³H]glycerol incorporation were rapid and striking during the first 2–5 min, but diminished thereafter. IGF-I at a concentration of 50 ng/ml provoked a rapid, but even more transient, increase in [³H]glycerol incorporation into DAG, monoacylglycerol, triacylglycerol and total phospholipids (Fig. 3). {As might be expected from glucose-uptake studies (i.e. Table 1), IGF-I at concentrations of 1 and 10 ng/ml did not increase [³H]glycerol incorporation over a 1–20 min period (results not shown). In view of the small responses to 50 or 100 ng/ml, other doses of IGF-I were not tested.} In experiments in which rapid effects of EGF and IGF-I were directly compared with that of insulin, increases in [³H]glycerol incorporation into DAG and other lipids were greater with insulin (Table 2). As reported previously [6,7] and confirmed now (results not shown), insulin effects on acute [³H]glycerol labelling were also more prolonged than those of higher concentrations of EGF and IGF-I.

After cells had been incubated for 10 days with [³H]glycerol, subsequent addition of EGF (100 ng/ml) or IGF-I (50 ng/ml) provoked increases in [³H]DAG labelling which were more sustained (Fig. 4) than those observed with only 2 h of [³H]glycerol prelabelling. These differences may be explained by the fact that, in 10-day prelabelling experiments, increases in [³H]DAG would reflect both hydrolysis of pre-formed phospholipids and synthesis *de novo* of PA and DAG from residual label in glycerol 3-phosphate, whereas in 2 h prelabelling experiments increases in [³H]DAG are probably more reflective of PA-DAG synthesis *de novo* (see [7]). These results obtained with 10 days of [³H]glycerol prelabelling provide further confirmation of the observed increases in DAG content, as measured by the DAG kinase method (see above).

[³H]Choline-labelling experiments

Addition of EGF (100 ng/ml) or IGF-I (50 ng/ml) provoked rapid increases in [³H]phosphocholine in aqueous fractions of cells which were prelabelled for 10 days with [³H]choline (Fig. 5); the effect of IGF-I was very transient, whereas the effect of EGF was more prolonged. (The increase in cellular phosphocholine was accompanied by increased release of phosphocholine into the medium, and therefore did not occur at the expense of the latter; results not shown.) On the other hand, there were no significant changes in labelling of PC in cells which were labelled for 10 days before addition of agonist (results not shown), but in cells which were prelabelled for only 1 h with [³H]choline, stimulatory effects of EGF and IGF-I on PC labelling were observed (Table 3).

Fig. 3. Temporal effects of IGF-I (50 ng/ml) on [3 H]glycerol incorporation into total phospholipids (PL), monoacylglycerol (MAG), DAG and triacylglycerol (TAG) in BC3H-1 myocytes

Cells were incubated for 2 h in the presence of [3 H]glycerol before the treatments. See Fig. 2 for other details. Results are means $\pm$ S.E.M. for four determinations.

Table 2. Comparison of rapid effects of insulin, EGF and IGF-I on [3 H]glycerol incorporation into lipids in BC3H-1 myocytes

Myocytes in 35 mm plates were incubated for 12 min with 10 μ Ci of [3 H]glycerol in 1 ml of DPBSGA. Treatments were then added, and incubation was continued for 1 min in Group A and 2 min in Group B. Results are means $\pm$ S.E.M. for 8–9 determinations in Group A and 3–7 determinations in Group B.

Treatment	[3 H]Glycerol incorporation (c.p.m./plate)				
	DAG	Monacylglycerol	Triacylglycerol	Phospholipids	Total glycerolipids
A None (control)	2028 $\pm$ 192	1296 $\pm$ 107	3361 $\pm$ 494	3655 $\pm$ 333	10340 $\pm$ 851
Insulin (1200 ng/ml)	5579 $\pm$ 776	2356 $\pm$ 207	6195 $\pm$ 1258	5909 $\pm$ 414	19414 $\pm$ 2560
EGF (10 ng/ml)	3689 $\pm$ 592	1734 $\pm$ 174	5540 $\pm$ 988	4616 $\pm$ 362	15578 $\pm$ 171
IGF-I (100 ng/ml)	3151 $\pm$ 218	1778 $\pm$ 69	4937 $\pm$ 279	5574 $\pm$ 856	14890 $\pm$ 1015
B None (control)	272 $\pm$ 61	449 $\pm$ 61	227 $\pm$ 53	423 $\pm$ 135	1371 $\pm$ 380
EGF (100 ng/ml)	841 $\pm$ 79	1332 $\pm$ 272	788 $\pm$ 162	1427 $\pm$ 262	4388 $\pm$ 766
Insulin (1200 ng/ml)	3183 $\pm$ 165	2537 $\pm$ 706	2066 $\pm$ 209	5650 $\pm$ 752	13436 $\pm$ 1780

[3 H]Myristate-labelling experiments

Although [3 H]choline-labelled PC failed to diminish during treatment with EGF or IGF-I, these treatments provoked substantial decreases in [3 H]myristate-labelled PC (Fig. 6). As reported previously [18], EGF and IGF-I also provoked decreases in [3 H]myristate-labelled PI-glycan, but the latter labelling was less than 5% of that of PC. These changes in myristate-labelled PC may reflect changes in a PC pool which serves to replenish PI and PI-glycan pools during hydrolysis of the latter.

Changes in inositide metabolism

In cells prelabelled with [3 H]inositol during their entire 10-day life span, subsequent addition of EGF (100 ng/

ml) provoked rapid increases in the labelling of PI and polyphosphoinositides (PPI: PIP and PIP₂) (Fig. 7), but did not increase the production of inositol phosphates (results not shown). These effects of EGF were similar to those of insulin (Table 4). IGF-I, on the other hand, failed to increase [3 H]inositol labelling of inositol lipids (Table 4) or inositol phosphates (results not shown).

Changes in cytosolic Ca²⁺

We have previously reported that phenylephrine, but not insulin, increases cytosolic Ca²⁺, as measured by quin 2 fluorescence [2,14]. In the present work, we observed no changes in cytosolic Ca²⁺ during treatment with EGF (100 ng/ml) or IGF-I (100 ng/ml) (results not shown).

Fig. 4. Temporal effects of insulin (1200 ng/ml), EGF (100 ng/ml) and IGF-I (50 ng/ml) on $[^3\text{H}]$ glycerol incorporation into DAG in BC3H-1 myocytes which were prelabelled for 10 days with $[^3\text{H}]$ glycerol

Results are means $\pm$ S.E.M. for four determinations.

Activation of protein kinase C

We have previously shown that insulin provokes increases in both particulate and cytosolic protein kinase C activity [3,4], and phenylephrine provokes increases in particulate, and decreases in cytosolic, protein kinase C activity [14]. These effects of insulin were documented in the present work, and we also found that particulate protein kinase C activity was increased by both EGF and IGF-I, and cytosolic protein kinase C activity was diminished by EGF, and mildly, but insignificantly, increased by IGF-I treatment (Table 5). [More recently, we have found that insulin decreases immunoassayable protein kinase C, especially in the cytosol fraction, despite increasing enzymic activity. These results suggest that insulin provokes two changes in protein kinase C, namely translocation with consequent proteolytic degradation, and an increase in intrinsic enzyme activity (perhaps by DAG, which increases the V_{max} of protein kinase C).]

DISCUSSION

The present results indicate that the growth factors EGF and IGF-I provoke insulin-like effects on $[^3\text{H}]$ -2-DOG uptake, DAG generation and activation of particulate protein kinase C in BC3H-1 myocytes. The association of these three phenomena has been observed previously in studies of both insulin [1-7] and phenylephrine [14] action in BC3H-1 myocytes. Although

Fig. 5. Temporal effects of EGF (100 ng/ml) and IGF-I (50 ng/ml) on $[^3\text{H}]$ choline labelling of phosphocholine in BC3H-1 myocytes which were prelabelled for 10 days with $[^3\text{H}]$ choline

Results are means $\pm$ S.E.M. for four determinations.

Table 3. Effects of EGF and IGF-I on acute labelling of PC with $[^3\text{H}]$ choline in BC3H-1 myocytes

Cells were incubated for 60 min with $2 \mu\text{Ci}$ of $[^3\text{H}]$ choline, and then treated for 10 min as described in the text. Comparable results were obtained in repeat experiments, and at earlier times of treatment. Results are means $\pm$ S.E.M. for four determinations (separate culture plates) from a representative experiment repeated on three occasions.

Treatment	$[^3\text{H}]$ Choline incorporation into PC (c.p.m./plate)
None (control)	3993 ± 192
EGF (100 ng/ml)	6281 ± 296
IGF-I (50 ng/ml)	5014 ± 99

more studies are required, these findings collectively provide further support for the hypothesis that DAG-protein kinase C signalling may be important in the activation of certain metabolic processes, such as glucose transport, in BC3H-1 myocytes.

Fig. 6. Effects of EGF (100 ng/ml) and IGF-I (50 ng/ml) on [3 H]myristate-labelled PC

Cells were labelled for 16 h with [3 H]myristate before treatment with agonist. Results are means $\pm$ S.E.M. for four determinations.

Table 4. Effects of EGF, IGF-I and insulin on [3 H]inositol labelling of PI and polyphosphoinositides (PPI) in BC3H-1 myocytes

Myocytes were cultured for 10 days in the presence of [3 H]inositol as described in the Materials and methods section. After replacement of the media and equilibration for 120 min, treatments were added, and after incubation for 30 min cells were harvested and examined for 3 H labelling of PI and PPI. Results are means $\pm$ S.E.M. for the numbers of determinations shown in parentheses, from a representative experiment repeated on at least three occasions.

Treatment	[3 H]Inositol labelling (c.p.m./plate)	
	PI	PPI
None (control)	10461 $\pm$ 2007 (7)	1315 $\pm$ 247 (7)
EGF (100 ng/ml)	30672 $\pm$ 4177 (4)	1911 $\pm$ 74 (4)
IGF-I (100 ng/ml)	8191 $\pm$ 3076 (4)	1111 $\pm$ 251 (4)
Insulin (1200 ng/ml)	28513 $\pm$ 1843 (4)	2830 $\pm$ 232 (4)

In comparison with insulin, EGF and, in particular, IGF-I provoked lesser and more transient increases in synthesis *de novo* of DAG and other glycerolipids. We have recently concluded that the pathway of PA synthesis *de novo* appears to account for only 15–30% of the

Fig. 7. Temporal effects of EGF (100 ng/ml) on [3 H]inositol incorporation into PI and polyphosphoinositides (PPI) in BC3H-1 myocytes which were prelabelled for 10 days with [3 H]inositol

Results are means $\pm$ S.E.M. for four determinations.

Table 5. Comparison of insulin, EGF and IGF-I treatment on protein kinase C activity

Cells were treated for 20 min with or without the indicated treatments as described in the Materials and methods section. Cytosolic and particulate proteins were isolated and examined for protein kinase C activity. Mean control protein kinase C activity was 300 pmol/min per 200 μ g of cytosolic protein and 100 pmol/min per 200 μ g of membrane-associated protein. See the Materials and methods section for other details. Data, expressed as percentage change from the control, are means $\pm$ S.E.M. for the numbers of separate experiments shown in parentheses.

Treatment	Protein kinase C activity (% change from control)	
	Cytosolic fraction	Particulate fraction
Insulin (1200 ng/ml)	+54 $\pm$ 20 (4)	+109 $\pm$ 28 (4)
EGF (10 ng/ml)	-51 $\pm$ 11 (6)	+70 $\pm$ 20 (6)
IGF-I (50 ng/ml)	+21 $\pm$ 9 (5)	+108 $\pm$ 35 (5)

observed increases in DAG content during insulin action in BC3H-1 myocytes [7], and that phospholipid hydrolysis is probably an important source of DAG during insulin action. The latter conclusion seems even more

clear during the actions of both EGF and IGF-I, as time-related increases in DAG content did not correlate well with increases in [^3H]glycerol incorporation into DAG in acute 2 h prelabelling experiments.

The rapidity of turnover of EGF- and IGF-I induced increases in [^3H]glycerol-labelled lipids is striking and noteworthy for several reasons. First, at higher agonist concentrations, peak increases in [^3H]glycerol-labelled lipids were readily apparent at early time points of EGF or IGF-I treatment, but were markedly diminished or no longer apparent several minutes later. It is therefore clear that there is an extremely rapid turnover and lability of these [^3H]glycerol-labelled lipids, including DAG. Thus these labelled lipids do not appear to be programmed simply for storage purposes, and could serve in a signalling capacity. Second, the transient nature of the synthesis *de novo* of PA (and other glycerolipids) further suggests that the factor which is responsible for its provocation is short-lived and rapidly depleted, especially during IGF-I action. Third, the different responses of glycerolipid synthesis *de novo* to insulin, EGF and IGF-I indicate that these hormones are acting through their own specific receptors. This postulation is in accord with previous findings in BC3H-1 myocytes [15], which indicate that: (a) IGF-I does not compete effectively with insulin for binding to the insulin receptor, or vice versa; and (b) the effects of IGF-I observed here appear to occur within the range of IGF-I concentrations that are associated with apparent IGF-I receptor occupancy. Fourth, the increases in [^3H]glycerol incorporation into DAG in the 2 h prelabelling experiments apparently reflect stimulation of the 'de novo' pathway, rather than hydrolysis of pre-formed lipids. The latter conclusion seems inescapable if results of 2 h-prelabelling experiments are compared with those of 10-day-prelabelling experiments, i.e. IGF-I effects on [^3H]glycerol labelling of DAG were transient in the former, but persistent in the latter case.

Rapid increases in [^3H]choline labelling of phosphocholine were observed in the actions of both EGF and IGF-I. Presumably this reflects increased hydrolysis of PC by activation of a specific phospholipase C (see [16]), but decreases in total PC content as revealed by chronic choline labelling content were not evident, and it therefore seems likely that the hydrolysis of PC is equally balanced by its resynthesis from DAG or phosphatidylethanolamine. Evidence for PC resynthesis was in fact obtained in experiments in which [^3H]choline was added 1 h before agonist addition: increases in PC labelling were observed, presumably reflecting increased hydrolytic turnover of PC and its resynthesis. Although total PC did not decrease in response to EGF and IGF-I, we observed substantial decreases in a myristate-labelled pool of PC. This pool may be relatively small, and its resynthesis may involve fatty acids other than [^3H]myristate. As reported recently, [17], insulin also provokes closely coupled increases in PC synthesis and hydrolysis in BC3H-1 myocytes, but, with insulin, the increase in PC synthesis apparently exceeds that of PC hydrolysis, since increases in PC content are evident. Along these lines, the similarity between time-related changes in apparent PC hydrolysis (Fig. 5) and increases in [^3H]glycerol labelling of total glycerolipids (PL + monoacylglycerol + DAG + triacylglycerol) (Figs. 2 and 3) is striking, and suggests that activation of PC hydrolysis and synthesis of PA *de novo*

are closely coupled responses in the actions of both EGF and IGF-I, as well as insulin (see [17]). As a corollary, the lesser effects on PA synthesis *de novo* noted with EGF and IGF-I correlated with the lesser increases in PC synthesis, which are probably derived from synthesis of PA and DAG *de novo*.

Like insulin [2], EGF and IGF-I did not increase cytosolic Ca^{2+} or inositol phosphate generation appreciably in BC3H-1 myocytes. Thus it seems likely that EGF and IGF-I, as well as insulin, increase DAG content primarily by mechanisms other than hydrolysis of PI, PIP and PIP_2 . On the other hand, we have recently found that all three agonists provoke increases in the hydrolysis of the PI-glycan [18]. Obviously, hydrolysis of the PI-glycan may also contribute to the increases in DAG observed in the present work, in addition to PA synthesis *de novo* and PC hydrolysis.

EGF, like insulin [2,6], provoked increases in [^3H]inositol labelling of PI and of $\text{PIP} + \text{PIP}_2$ (PPI). Presumably this reflects an increase in the synthesis and mass of these substances (also see [1]), as the cells were pre-labelled for their entire 10-day growth period, before treatment with EGF or insulin. Since there is only limited synthesis of PI from newly synthesized PA in BC3H-1 myocytes (see [6,7]), it seems likely that, during the actions of EGF and insulin, substantial amounts of PI and other inositol phospholipids are synthesized from the DAG and PA which are derived both from the 'de novo' pathway and from hydrolytic cleavage (by phospholipase C or D) of pre-existing phospholipids such as PC and the PI-glycan. However, with IGF-I, increases in DAG did not necessarily lead to net increases in PI. Whether this difference between EGF and IGF-I reflects that DAG is increased in different compartments, or that other factors control its conversion into PI, is uncertain. The lesser conversion of DAG into PI during IGF-I action is further noteworthy, since it could explain how DAG content remains elevated, despite rapid abatement of both PA synthesis *de novo* and apparent PC hydrolysis responses.

The fact that insulin, EGF and IGF-I operate through receptors that have intrinsic tyrosine kinase activity is of interest. Apparently, activation of all three receptors provokes increases in DAG, probably largely derived from hydrolysis of phospholipids other than PIP_2 , and, to a variable extent, from PA synthesis *de novo*. Since these agonists also increased particulate protein kinase C activity, it is possible that activation of receptors having intrinsic tyrosine kinase activity frequently leads to increases in DAG-protein kinase C signalling by mechanisms other than PIP_2 hydrolysis. Further studies will be required to test this hypothesis.

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