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Kinetic and Molecular Species Analyses of Mitogen-Induced Increases in Diglycerides: Evidence for Stimulated Hydrolysis of Phosphoinositides and Phosphatidylcholine

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A wide variety of agonist-induced events appear to be mediated through an increase in cellular diglyceride levels. With regard to the ability of diglycerides to mediate these events, three important parameters must be considered: a) the kinetics of diglyceride generation, b) the absolute mass levels, and c) their molecular species. While this increase is often due to a stimulated hydrolysis of phosphoinositides, there is increasing evidence that the stimulated hydrolysis of phosphatidylcholine also contributes to agonist-induced increases in diglyceride levels. The kinetics of mass increases in diglyceride levels stimulated in cultured fibroblasts are agonist-dependent. High concentrations of α-thrombin stimulate a biphasic increase in diglyceride levels with the first phase peaking at 15 s and the second phase peaking at 5 min. In contrast, stimulation with epidermal growth factor, or platelet-derived growth factor, results in a monophasic increase in cellular diglyceride levels. Furthermore, the molecular species and phospholipid source of the stimulated diglycerides are also agonist-dependent. While the hydrolysis of phosphoinositides is major source of diglycerides initially generated in response to some agonists (15 s with α-thrombin at 500 ng/ml), phosphatidylcholine is hydrolyzed as well. Following longer incubations, or at all times following stimulation by epidermal growth factor or platelet-derived growth factor, phosphatidylcholine hydrolysis is the principal source of the stimulated diglycerides.

Key words: kinetics, molecular species, capillary gas chromatography, mitogen-stimulated diglycerides

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It is now well established that the stimulation of cellular diglycerides plays an important role in the transduction of a number of agonist-stimulated events (see [1] for review). Since diglycerides are present in unstimulated cells, activated events mediated by diglycerides must require: a) a certain threshold level of diglyceride for activation, or b) the appearance, disappearance, or change in the level of a certain molecular species upon stimulation, or c) both a and b. In addition, the subcellular compartment in which the diglyceride changes occur may also be important.

Initial studies designed to examine agonist-induced diglycerides suggested that these diglycerides were derived from the stimulated hydrolysis of phosphoinositides, in particular, phosphatidylinositol 4,5 biphosphate (PIP_2). However, there is increasing evidence that many agonist-induced diglycerides may be derived from the stimulated hydrolysis of phosphatidylcholine (PC) (see [2] for review). In fact, in some cases, hydrolysis of PC is the sole source of agonist-induced diglycerides [3,21].

In view of the above, we have examined the kinetics of diglyceride stimulation in cultured IIC9 fibroblasts in response to α-thrombin,[1] epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) [3,16]. In addition, we have analyzed the molecular species of these stimulated diglycerides by capillary gas chromatography, as well as the molecular species profiles of the endogenous phosphatidylinositol (PI), PC, phosphatidylethanolamine (PE), and phosphatidylserine (PS) [3,21]. The molecular species profiles of the stimulated diglycerides were compared to the profiles of the endogenous phospholipids [3,21]. We also analyzed the release of radiolabeled water-soluble head groups in response to the various agonists [3,21]. The data demonstrate that: a) the kinetics of diglyceride generation are agonist-dependent; b) phosphoinositides are not the sole source of stimulated diglycerides; and c) PC is the likely source of non-PI-derived diglycerides. This manuscript is a review of these data.

MITOGEN-STIMULATED DIGLYCERIDES

Mitogens stimulate a variety of biochemical changes in quiescent fibroblasts. While some of these biochemical changes occur in response to all mitogens in a particular cell type, others are unique. Furthermore, for a particular mitogen, there are not only quantitative differences, but also distinct temporal patterns. For example, DNA synthesis occurs many hours after the initial addition of the mitogen, while the induction of a number of specific genes occurs within minutes [4–6]. A number of studies have implicated an increase in cellular 1,2-diglycerides as an important event mediating the transmission of the mitogenic signals to the cell nucleus. These studies demonstrated that addition of mitogens to quiescent cells resulted in an increase in cellular diglyceride levels [7–11]. Furthermore, the addition of cell-permeable diglyceride analogs is capable of stimulating some of these responses [12,13].

As stated above, the mass level as well as the molecular species of the stimulated diglycerides may be an important parameter in determining the ability of the diglycerides to mediate certain responses. In addition, the temporal relationship between the onset of the diglyceride changes and the induced responses is also important. Therefore, in order to assess the role of diglycerides in the responses induced by different

[1]The highly purified human α-thrombin used in the studies described in this review had a specific activity of 3,000–4,000 NIH Units/mg.

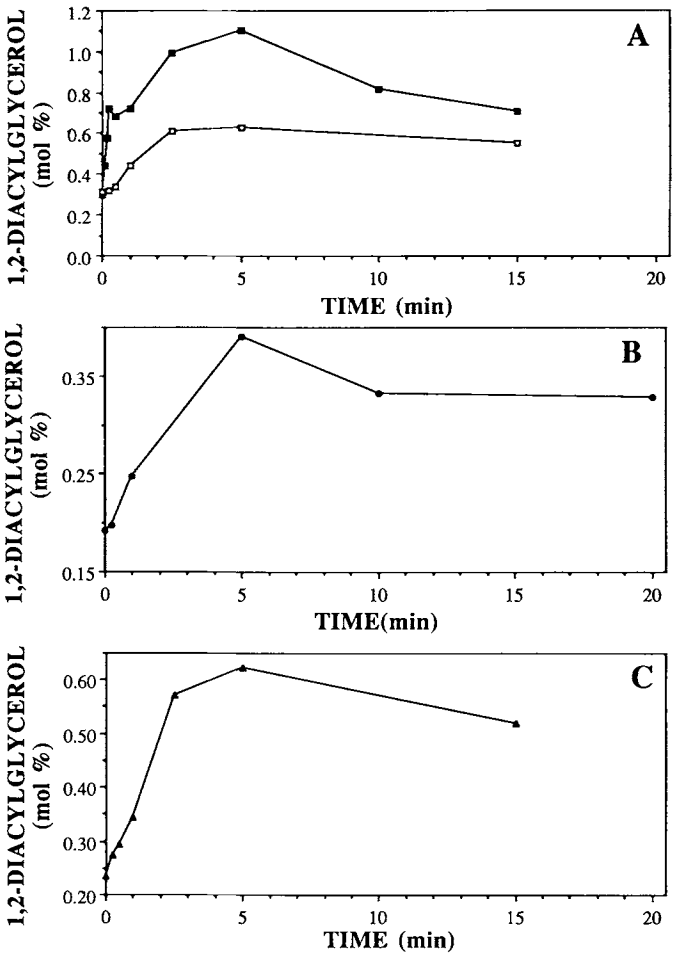

Fig. 1. Time course of mitogen-induced diglycerides in IIC9 cells. Quiescent cultures of IIC9 cells were stimulated with: (**A**) α-thrombin (500 ng/ml) alone (■) or following a 10 min pretreatment at 37°C with 200 ng/ml chymotrypsin (□); (**B**) 50 ng/ml EGF; or (**C**) 10 ng/ml PDGF. At the indicated times, the incubations were terminated and mass levels of diglyceride were quantitated as previously described [16]. (Reprinted with permission in part from Wright et al. (1988) 263:9374, and Pessin and Rabin (1989) 264:8729 Journal of Biological Chemistry, The American Society for Biochemistry and Molecular Biology, Bethesda, Maryland.)

mitogens, a detailed kinetic and biochemical analysis of the diglycerides stimulated by the various mitogens is required.

Treatment of quiescent IIC9 fibroblasts with α-thrombin, EGF, or PDGF results in the stimulation of DNA synthesis [14,15]. Additionally, α-thrombin stimulates an increase in inositol phosphates and the release of arachidonic acid. These later activities of α-thrombin are inactivated if the cultures are pretreated with 200 ng/ml chymotrypsin [7]. This treatment does not prevent α-thrombin-stimulated mitogenesis but does reduce the maximal stimulated level of diglycerides [16]. EGF and PDGF also stimulate an increase in cellular diglyceride levels. As shown in Figure 1, only high

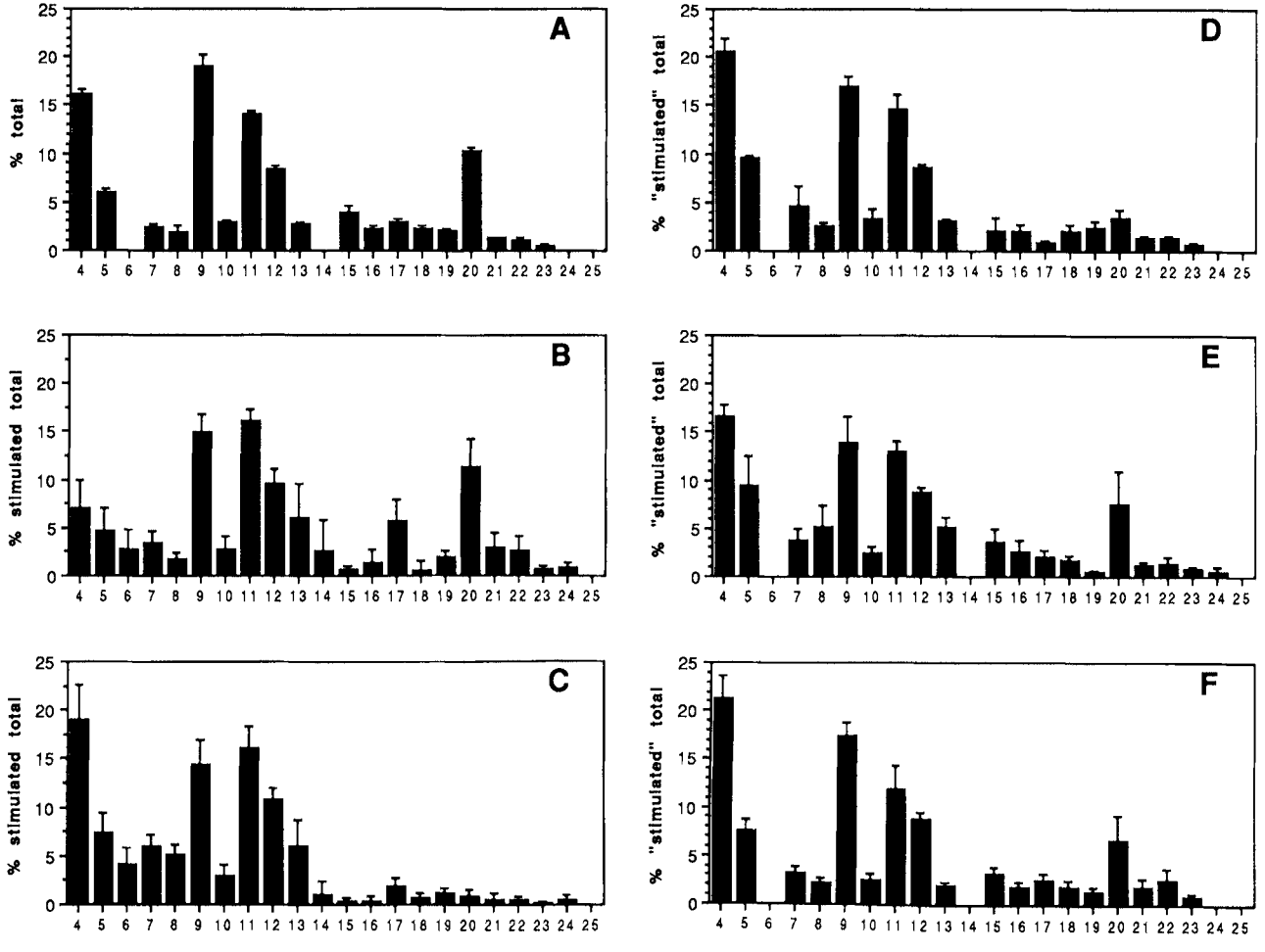
A
B
C
D
E
F
% total
% stimulated total
% "stimulated" total
0
5
10
15
20
25
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Figure 2.

concentrations of α-thrombin (500 ng/ml) stimulate a biphasic increase of these lipids with the first phase peaking at 15 s and the second phase peaking at 5 min after the addition of α-thrombin [16]. Monophasic increases in diglyceride are observed when IIC9 cells are stimulated by: a) low, mitogenic, concentrations of α-thrombin (100 pg/ml); b) high concentrations of α-thrombin added to cultures which had been pretreated with chymotrypsin; c) EGF; or d) PDGF (Fig. 1). These diglyceride elevations peak 5 min after the addition of the growth factor [3,16].

The molecular species of a diglyceride, in addition to being an important determinant of its physiological role, is also an important aid in establishing the source and subcellular compartment of the diglycerides. Identification and mass analysis of agonist-stimulated diglycerides have often been accomplished by component fatty acid analysis [17,18] or argentation chromatography [19]. While both of these approaches yield important information, neither establishes the precise molecular species. Recently, we developed a procedure, modified from Myher and Kuksis [20], which allowed us to analyze the molecular species of diglycerides stimulated in tissue culture cells [21]. This procedure involves isolation of the cellular diglycerides followed by derivatization with *tert*-butyldimethylchlorosilane. The resulting TBDMS-diglycerides were chromatographed by capillary gas chromatography. Diglycerides in quiescent IIC9 cells and those generated in response to mitogenic concentrations of α-thrombin, EGF, or PDGF were isolated, derivatized, and analyzed. In addition, diglycerides generated in response to α-thrombin in cultures pretreated with chymotrypsin were analyzed. The results, shown in Figure 2, demonstrate that: a) at least 21 different molecular species are present in quiescent cultures (identified in Table I), and b) there is an increase in all molecular species in response to the above mitogenic conditions, without the addition of new species or complete loss of any existing species. In addition to these analyses, we have also demonstrated that the diglycerides are all ester-linked [3,21].

In order to establish the mechanism by which the stimulated diglycerides were generated, it was important to establish the potential source from which they were derived. Release of metabolically labeled headgroups or diglycerides [3,21,22] indicates that phosphatidylcholine is the major source of non-PI-derived diglycerides. However, these approaches are complicated by their low sensitivity and metabolism of the radiolabeled compounds. Therefore, we have examined the potential source(s) of the stimulated diglycerides by comparing the molecular species profile or "fingerprint" of the stimulated diglycerides to the molecular species profile of cellular phospholipids. These profiles were generated by calculating the percentage of each molecular species in the total population [3,21]. Analysis of IIC9 cellular phospholipids, shown in Figure 3, indicates that each phospholipid has a distinct molecular species profile. The profile of diglycerides stimulated by the conditions described above was compared to

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Fig. 2. Molecular species profiles of mitogen-stimulated diglycerides in IIC9 cells. Profiles of diacylglycerols present in: (**A**) control, quiescent IIC9 cells; (**B,C**) cells stimulated with α-thrombin (500 ng/ml); (**D**) α-thrombin (500 ng/ml) following a 10 min pretreatment at 37°C with 200 ng/ml chymotrypsin; (**E**) EGF (50 ng/ml); or (**F**) PDGF (10 ng/ml). Diacylglycerols were analyzed after 15 s (A) or 5 min (B–F) incubations as previously described [3,21]. Each molecular specie, identified in Table I, is presented as its contribution to the total diglyceride population (% total) [3,21]. (Reprinted with permission from Pessin et al. (1990) 265:7959–7966 and Pessin et al. (1989) 264:8729 Journal of Biological Chemistry, The American Society for Biochemistry and Molecular Biology, Bethesda, Maryland.)

TABLE I. Identification of 1,2 Diglycerides Separated by Capillary Gas Chromatography†

Peak No.	Relative retention time	Diglyceride molecular species
1	0.833	16:0–16:0*
2	0.862	16:0–16:1ω9
3	0.871	16:0–16:1ω7
4	1.000	16:0–18:1ω9*
5	1.011	16:0–10:1ω7
6	1.030	16:1ω9–18:1ω9
7	1.048	16:1ω7(9)–18:1ω9(7)
8	1.160	16:1ω7–18:1ω7
9	1.218	18:0–18:1ω9*
10	1.235	18:0–18:1ω7
11	1.273	18:1ω9–18:1ω9*
12	1,288	18:1ω7–18:1ω7
13	1.301	18:0–18:2ω6*
14	1.341	16:0–20:4ω6*
15	1.355	18:1ω9(7)–18:2ω6
16	1.517	18:0–20:3ω6
17	1.568	18:1ω9–20:3ω6
18	1.620	18:1ω7–20:3ω6
19	1.655	16:0–20.5ω6
20	1.703	18:0–20:4ω6*
21	1.791	18:1ω9–20:4ω6
22	1.818	18:1ω7–20:4ω6
23	1.863	18:0–20:5ω3
24	1.925	18:1ω9–20:5ω3
25	1.985	18:1ω7–20:5ω3

†Cellular 1,2-diglycerides and component diglycerides from cellular PI were isolated, derivatized, and identified by capillary gas chromatography as previously described (see reference 21 for details). Each molecular species was numbered based upon its retention time. Relative retention times were calculated with respect to 16:0–18:1ω9 (peak 4). The actual diglyceride molecular species represented by each peak was determined by comparison either to the relative retention times of standard diglycerides (designated by asterisks) or to the predicted retention times calculated as described by Myher and Kuksis [20]. The relative retention times of the diglyceride standards were used to derive the retention factors used in these calculations. Relative retention times of diglyceride standards were highly reproducible with a standard deviation of ± 0.001 (n = 5). (Reprinted with permission from Pessin and Rabin (1989) The Journal of Biological Chemistry 264:8729, The American Society for Biochemistry & Molecular Biology, Bethesda, Maryland.)

the cellular phospholipid profiles in order to examine potential phospholipid sources. This comparison was made by calculating the correlation coefficients between the "stimulated" diglyceride profile of interest and the profile of a cellular phospholipid. This correlation coefficient is a measure of the closeness of the relationship between these two profiles [23]. If a population of "stimulated" diglycerides was derived from a non-selective hydrolysis of a cellular phospholipid, then the correlation coefficient between the diglyceride profile and the phospholipid would be 1.00 indicating identical profiles [21]. As shown in Table II, all the "stimulated" diglycerides appear to be derived primarily from phosphatidylcholine except for those stimulated 15 s after the addition of a high concentration of α-thrombin (500 ng/ml). These diglycerides are derived primarily (at least 80%) from the phosphoinositides with a small percentage

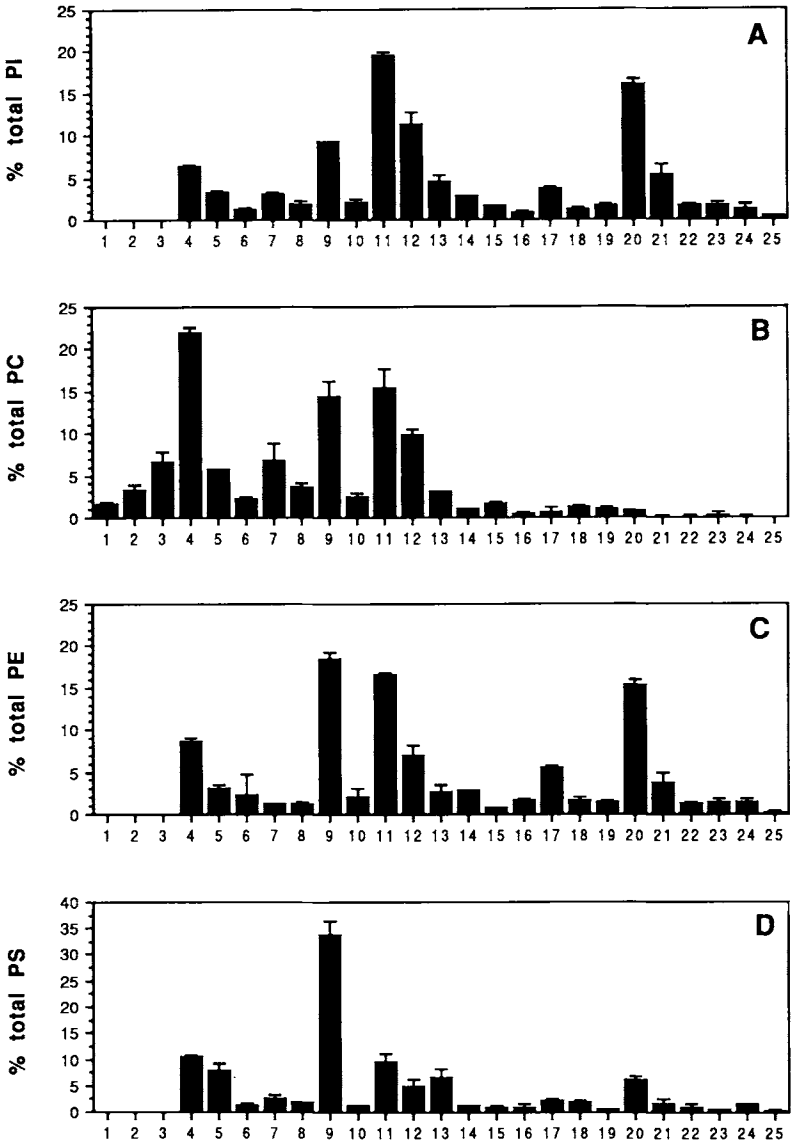

Fig. 3. Molecular species profiles of IIC9 phospholipids. Phospholipids from IIC9 cells were extracted, isolated by HPLC, and hydrolyzed with phospholipase C. The resulting diglycerides were isolated, derivatized, and analyzed by capillary gas chromatography as previously described [3,21]. Each molecular specie, identified in Table I, is presented as its contribution to the total diglyceride population (% total) [3,21]. (Reprinted with permission from Pessin et al. (1990) 265:7959–7966 and Pessin et al. (1989) 264:8729 Journal of Biological Chemistry, The American Society for Biochemistry and Molecular Biology, Bethesda, Maryland.)

TABLE II. Comparison of Molecular Species Profiles of Diglycerides Generated by Either α-Thrombin Alone, α-Thrombin Following Chymotrypsin Pretreatment, EGF, or PDGF to Profiles of Total Cellular PI, PC, PE, and PS*

	Phospholipids			
Mitogen	PI	PC	PE	PS
α-TH: peak 1	0.911	0.678	0.950	0.743
α-Th: peak 2	0.577	0.981	0.640	0.673
EGF: peak	0.670	0.913	0.753	0.738
chymo/α-th: peak	0.569	0.965	0.697	0.752
PDGF: peak	0.591	0.929	0.749	0.767
α-Th: sustained	0.589	0.930	0.647	0.787
EGF: sustained	0.635	0.905	0.733	0.792
chymo/α-th: sustained	0.512	0.876	0.710	0.797
PDGF: sustained	0.668	0.957	0.759	0.741

*Molecular species profiles of diglycerides generated by stimulation of IIC9 fibroblasts by either 500 ng/ml α-thrombin for 15 s (peak 1), 5 min (peak 2) or 60 min (sustained), 500 ng/ml α-thrombin for 5 min (peak) or 60 min (sustained) following 200 ng/ml chymotrypsin for 10 min, 50 ng/ml EGF for 5 min (peak) or 60 min (sustained), or 10 ng/ml PDGF for 5 min (peak) or 30 min (sustained) were compared to the molecular species profiles of total cellular PI, PC, PE, and PS [3, 21]. This comparison was performed by calculating the correlation coefficient between the diglyceride profile of interest and the profile of the phospholipid being considered as a putative source (see reference 3 for details). Correlation coefficients were calculated by using the StatView 512+ program on a Macintosh SE computer. α-Th = α-thrombin. Chymo/α-th = α-thrombin following chymotrypsin pretreatment. (Reprinted with permission from Pessin et al. (1990) 265:7959–7966 and Pessin et al. (1989) 264:8729 Journal of Biological Chemistry, The American Society for Biochemistry and Molecular Biology, Bethesda, Maryland.)

(20%) derived from the hydrolysis of phosphatidylcholine [21]. This profile (α-thrombin; peak 1) also shows a strong correlation to the profile of cellular PE (Table II). While we cannot absolutely rule out a contribution by a stimulated hydrolysis of PE, we feel it is unlikely for two reasons: a) in cultures which had been metabolically labeled with [^{3}H]ethanolamine, radiolabeled ethanolamine metabolites were not elevated in response to α-thrombin [21]; and b) the profile most likely resembles PE because the profile of cellular PE is strikingly similar to a profile consisting of 80% PI-derived diglycerides and 20% PC-derived diglycerides, sources which have been confirmed by examination of released head groups from metabolically labeled cultures [16,21].

FUTURE DIRECTIONS

The data generated to date indicate that hydrolysis of PC is an important event in the transduction of a variety of agonist-induced signals. Recent studies employing many of the above techniques have demonstrated that the kinetics and molecular species of diglycerides generated in stimulated PC12 cells are also agonist-dependent and also involve the stimulated hydrolysis of phosphatidylcholine (Pessin et al., submitted for publication). An understanding of the mechanism regulating this hydrolysis requires knowledge of the cellular enzymes involved. Recent studies in our laboratory indicate that IIC9 cells contain agonist-activated PC-phospholipases C and D. It will be important to determine the role of the PC-derived diglycerides in the activation of protein kinase C and gene expression. Furthermore, establishing the subcellular compartment in which the stimulated lipid metabolism is occurring will aid

in determining the physiological role of these diglycerides as well as the mechanism(s) by which they are regulated. These studies are currently in progress.

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