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Carbachol stimulates a different phospholipid metabolism than nerve growth factor and basic fibroblast growth factor in PC12 cells

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We have examined 1,2-diglycerides (DGs) generated in PC12 cells in response to the muscarinic agonist carbachol and compared them with those generated in response to the differentiation factors nerve growth factor and basic fibroblast growth factor. Whereas carbachol stimulates a greater release of inositol phosphates, all three agonists generate similar levels of DGs. In this report, we have analyzed the molecular species of PC12 DGs generated in response to these three agonists. Additionally, we have analyzed the molecular species of PC12 phospholipids. The data indicate that 1) after 1 min of either nerve growth factor or basic fibroblast growth factor stimulation, DGs arise primarily from phosphoinositide hydrolysis; 2) in contrast, after 1 min of carbachol stimulation, DG are generated equally by both phosphoinositide and phosphatidylcholine hydrolysis; and 3) after 15 min of stimulation by any of these agonists, DGs are generated largely by phosphatidylcholine hydrolysis, with a smaller component arising from the phosphoinositides. These results suggest that at least part of the mechanism by which PC12 cells distinguish between different agonists is via alterations in phospholipid sources and kinetics of DG generation.

Introduction

The stimulation of phospholipid hydrolysis resulting in the generation of 1,2-diglycerides (DGs)[1] is an important and widely distributed mechanism by which agonist-induced signals are transduced. (For reviews see Fisher and Agranoff, 1987; Berridge and Irvine, 1989; Exton, 1990.) In many systems, different agonists can stimulate phospholipid hydrolysis in the same cell to produce unique cellular responses. The basis for these alternate responses may involve, in part, a difference in the lipid metabolism induced by each distinct agonist. This may occur via a number of mechanisms: 1) variations in the type of phospholipid hydrolyzed; 2) variations in the metabolism of the resulting DGs; or 3) variations in the subcellular compartment in which the lipid metabolism occurs. In addition, a difference in the kinetics and/or magnitude of the stimulated lipid metabolism may play an important role.

The rat pheochromocytoma cell line, PC12, is a useful system to address how a cell distinguishes between two different agonists that both stimulate phospholipid turnover. PC12 cells respond to the muscarinic acetylcholine agonist carbachol by opening receptor-activated Ca^{2+} channels, which results in catecholamine release within a few minutes (Inoue and Kenimer, 1988). In contrast, these cells respond to the neurotrophic agents nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) by a number of both rapid and delayed responses. The rapid responses include cell flattening and tyrosine hydroxylase phosphorylation, whereas the delayed responses involve differentiation into sympathetic neuronlike cells (e.g., cessation of mitosis, neurite outgrowth, and elevation of acetylcholine-esterase activity) (Greene and Tischler, 1982; Rydel and Greene, 1987). Several studies have shown that carbachol, NGF, and bFGF all stimulate the hydrolysis of phosphoinositides (PIs) (Vicentini *et al.*, 1986; Contreras and Guroff, 1987; Altin and

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[1] Abbreviations: bFGF, basic fibroblast growth factor; DG, 1,2-diglyceride; HPLC, high-pressure liquid chromatography; NGF, nerve growth factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI phosphoinositide; PS, phosphatidylserine.

Bradshaw, 1990). Interestingly, Altin and Bradshaw (1990) found that carbachol stimulates a 6- to 10-fold greater rise in inositol trisphosphate levels than either NGF or bFGF. They also noted that all three agonists stimulate a 2- to 3-fold rise in DG levels. NGF and bFGF caused a biphasic increase, whereas the response to carbachol stimulation was monophasic. These studies suggested that the DGs generated after carbachol stimulation are derived primarily from hydrolysis of PIs, whereas the majority of DGs generated after NGF or bFGF stimulation arise from another source. Thus, this would provide a possible explanation for how PC12 cells discriminate between these two different agonist types, i.e., different phospholipids are employed. However, these results could also be obtained if each agonist type stimulated the metabolism of the DGs differentially, e.g., carbachol may stimulate a faster degradation of DGs.

To discriminate between the possible mechanisms underlying PC12 cells' responses to these various ligands, it is necessary to determine the exact source(s) of the generated DGs. Using techniques developed in studies on IIC9 fibroblasts after mitogen stimulation (Pessin and Raben, 1989), we have determined the molecular species of the DGs generated in PC12 cell cultures after stimulation with carbachol, NGF, and bFGF. As a basis for identifying the origins of the DGs, we have determined the molecular species of phospholipids present in PC12 cells. These studies suggest that 1) DGs generated after 1 min of NGF or bFGF stimulation arise primarily from hydrolysis of PIs; 2) DGs generated after 1 min of carbachol stimulation arise from an essentially equal contribution of PIs and phosphatidylcholine (PC) hydrolysis; and 3) after 15 min of NGF, bFGF, or carbachol stimulation, DGs are generated from a combination of PIs and PC hydrolysis, with PC contributing the majority of the DGs.

Results

Identification of the molecular species of agonist-induced DGs in PC12 cells

DGs generated in PC12 cell cultures preincubated in phosphate-buffered saline (PBS), and stimulated with 200 ng/ml NGF, 50 ng/ml bFGF, 0.5 mM carbachol, or PBS (control) for 1 or 15 min were isolated and chromatographed. As shown in Figure 1, there are 19 different molecular species found in both control and stimulated cultures. These profiles were generated by calculating the contribution of each molecular species to the total "stimulated" DG population (Pessin and Raben, 1989). Species 22 (18:0–20:4) is the dominant (45–50%) DG found in cultures stimulated with either NGF or bFGF for 1 min (Figure 1, B and C). This molecular species is also the largest component of the DGs from cultures stimulated with carbachol for 1 min, but other DGs are present in increased abundance (Figure 1D). After 15 min of stimulation by NGF, bFGF, or carbachol, the DG molecular species profiles are changed from those seen at 1 min (Figure 1, F–H). In all cases, the contribution of species 22 is much lower, and that of the other molecular species is increased.

Another important parameter defining DG molecular species is the nature of the linkage of the two fatty chains to the glycerol backbone. The fatty acyl chain at position 2 of a DG is joined by an ester linkage. The fatty acyl chain at position 1 of a DG can be joined to the glycerol backbone via either an ester, an alkenyl ether, or an alkyl ether linkage. When we examined the linkages of the DGs in either control (unstimulated) PC12 cultures or in PC12 cultures that had been stimulated with NGF, bFGF, or carbachol for 1 or 15 min, we found that >99% of the DGs contained an ester linkage at the 1 position and thus were diacylglycerols (data not shown).

Identification of the molecular species of phospholipids in PC12 cells and comparison with agonist-induced DGs

To determine the origins of the DGs produced by agonist-stimulated phospholipid hydrolysis, we first examined the molecular species profiles of total cellular phosphatidylinositol (representative of all the PIs), PC, phosphatidylethanolamine (PE), and phosphatidylserine (PS) of control PC12 cultures (Figure 2). Each phospholipid has a distinct profile with phosphatidylinositol containing almost 70% 18:0–20:4 (species 22), PC containing very little 18:0–20:4 (4%), and PE and PS containing significant amounts of a number of larger species (species 27–31). We also examined the phospholipid profiles after stimulation of the PC12 cultures with either NGF or carbachol for 1 and 15 min and found no significant changes in any of the phospholipid profiles (data not shown). To complete this analysis, we examined the linkages contained in PC12 cell phosphatidylinositol, PC, PE, and PS. Approximately 99% of all four phospholipids contained two ester linkages (data not shown).

To determine the probable sources of the DGs, we compared the DG molecular species

profiles with the profiles of the cellular phospholipids. To perform this comparison, we calculated the correlation coefficient between the molecular species profile of the DGs of interest and the total cellular phospholipid being considered as the putative source, assuming a nonselective hydrolysis of the cellular phospholipids (see Methods). If the DGs were generated by a nonspecific hydrolysis of a single phospholipid, then the correlation coefficient between the DG profile and the phospholipid profile would be 1.00, indicating identical profiles. When this analysis was performed (Table 1), only the DGs generated after 1 min of NGF or bFGF stimulation matched well a single phospholipid, PI (correlation coefficients of 0.990 and 0.976, respectively). Although the profile of the DGs generated after 1 min of carbachol stimulation somewhat matches the profile of total cellular PE (correlation coefficient of 0.913), the DG molecular species (Figure 1D) do not contain detectable amounts of the larger molecular species (species 27–31) found in cellular PE (Figure 2C). Therefore, although it is possible that PE may be hydrolized in response to the agonists used in this study, the data would require either that this hydrolysis must exclude the large molecular species found in this phospholipid or that selective metabolism of the large molecular species must occur. These possibilities might occur as a result of enzymatic specificity or subcellular compartmentation, but we have no evidence for the existence of any of these mechanisms at this time. In view of this, the simplest interpretation of our data is that it is unlikely that PE hydrolysis is the primary source of these DGs.

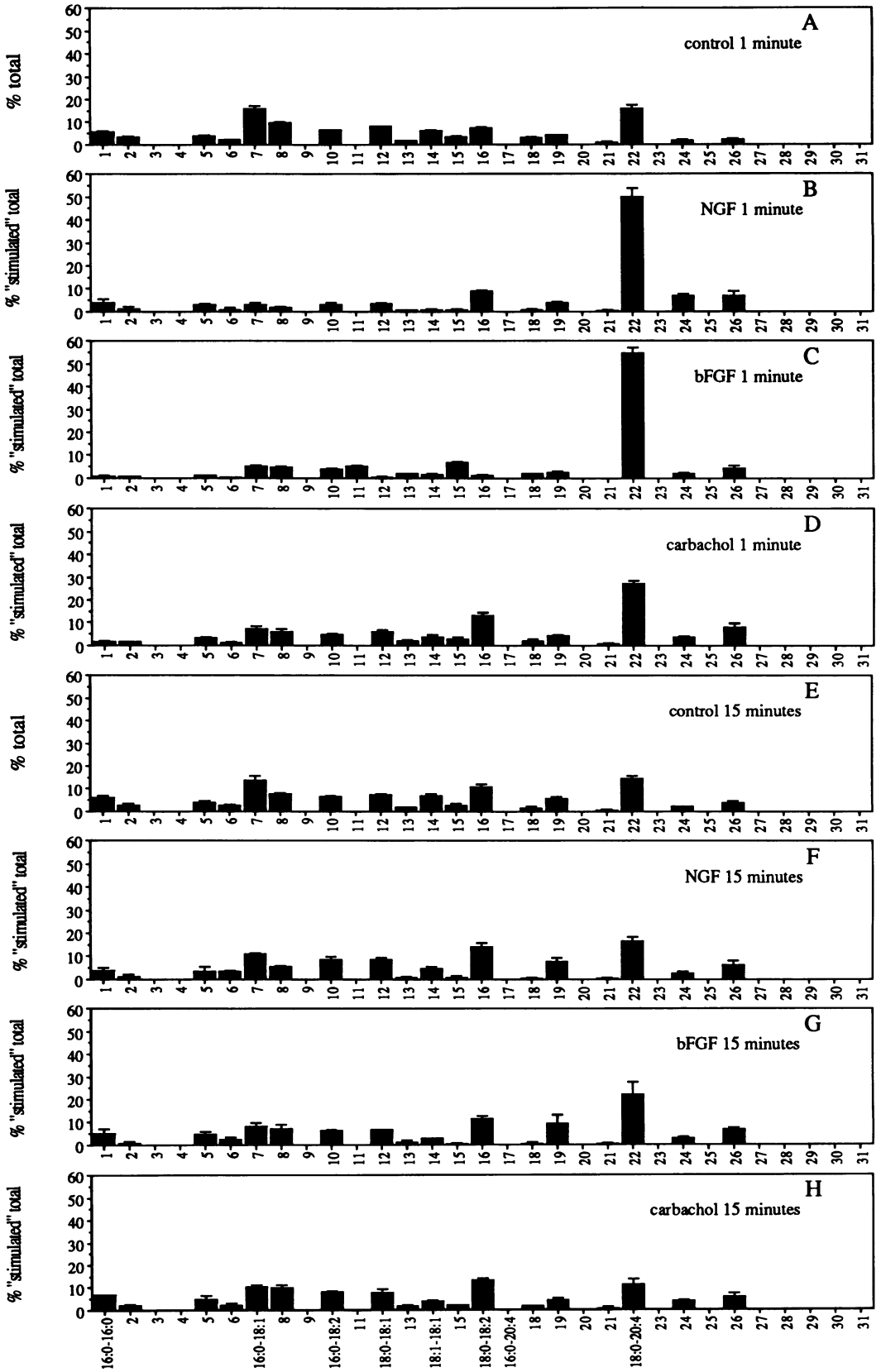
These data indicate that PIs and PC are the major sources of the agonist-induced DGs because 1) stimulated inositol phosphate release has been measured, 2) the smaller molecular species (species 1–6) that are present in the cellular DGs are present only in PC, and 3) PE and PS contain significant amounts of larger molecular species (species 27–31) not detectable in the stimulated DGs (see above). Small contributions from the hydrolysis of PE and/or PS cannot be ruled out because these larger molecular species would not be present in detectable amounts. In support of this conclusion, stimulated hydrolyses of PIs and PC have been implicated in a number of agonist-stimulated responses (1–3). The relative contributions of the PIs and PC molecular species profiles to each DG profile were calculated (see Methods). When this analysis was performed for all of the stimulated DG profiles (Table 1), combinations of PI and PC were found that gave good matches to the cellular DG profiles than any single phospholipid alone. In all but one case (carbachol after 15-min stimulation), these combinations had a correlation coefficient of >0.9. For each agonist tested, PI and PC contribute to the generation of DGs in proportions that differ significantly when comparing 1 versus 15 min of stimulation. In addition, the relative contributions of these phospholipids to the DGs generated in response to carabachol stimulation for 1 min were significantly different from the contributions of these phospholipids in response to NGF or bFGF at 1 min.

These correlation coefficients indicate that both PIs and PC hydrolysis combine to generate the stimulated DGs. To further evaluate these data, we examined the individual molecular species differences between the stimulated DG profiles and the combination PI and PC profiles. This was done by calculating, for each molecular species, the difference between the combination profile with the highest correlation coefficient and the stimulated DG profile of interest. Graphs of the absolute values of these differences are presented in Figure 3. For contrast, the DGs generated after 1 and 15 min of NGF stimulation are compared with the molecular species profiles of both total cellular PI and total cellular PC (Figure 3, A, B, D, and E). This analysis again shows that the stimulated DG profiles are best described by a combination of the total cellular PI and PC profiles.

Discussion

These studies demonstrate that hydrolysis of PIs is not the only source for the DGs generated in PC12 cells after stimulation by agonists mediating two different functions. The muscarinic agonist carbachol stimulates a monophasic rise in DG levels (Altin and Bradshaw, 1990) that is derived from hydrolyses of both PIs and PC. This is in agreement with a recent study by Horwitz (1990), who used metabolically labeled PC12 cells, as well as work done on astrocytoma cells (Martinson *et al.,* 1989). In contrast, the neurotrophic factors NGF and bFGF stimulate a biphasic rise in DG levels (Altin and Bradshaw, 1990) in which the first peak is derived primarily from hydrolysis of PIs (with a small PC component) and the second peak is from PIs and PC, with PC providing a greater proportion of the DGs.

It is important to note that, although combinations of PIs and PC hydrolyses are most likely the major sources of the stimulated DGs (Fig. 3),


A
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F
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H
carbachol 15 minutes
% total
% "stimulated" total
16:0-16:0
16:0-18:1
16:0-18:2
18:0-18:1
18:1-18:1
18:0-18:2
16:0-20:4
18:0-20:4

these combination profiles are not perfect representations of the DG profiles. Possible explanations for these small disparities are 1) PE and/or PS hydrolysis may be contributing to the DGs in a varying, but small level, as mentioned above; 2) subpopulations of PIs and PC that vary slightly in molecular species profile from the total cellular pools may be hydrolyzed in response to these agonists; and 3) selective metabolism of certain DG molecular species may be occurring, which changes the relative proportions of the DG molecular species that increase in the PC12 cultures.

The findings of this study suggest one possible mechanism by which PC12 cells can distinguish between agonists that generate different responses. The kinetics of hydrolysis of both PIs and PC are different when comparing carbachol stimulation with that of NGF or bFGF. In the case of carbachol, hydrolysis of both PIs and PC is stimulated initially, with hydrolysis of PIs diminishing and PC hydrolysis increasing by 15 min. In contrast, in response to NGF or bFGF, only the hydrolysis of PIs is activated initially, but at later times, this hydrolysis decreases and PC hydrolysis increases and again is the dominant contributor to the stimulated DGs. Any proposed mechanisms must also incorporate the recent demonstration that inositol phosphate hydrolysis is much higher with carbachol stimulation, but DG elevations are the same (Altin and Bradshaw, 1990). One possible explanation for these results is that carbachol stimulates a greater hydrolysis of both PIs and PC as well as a faster metabolism of the generated DGs. The means by which the different molecular species generated by these agonists might lead to different physiological responses is unclear. However, it is intriguing to speculate whether the different profiles are involved in activating different isozymes of protein kinase C or as-yet-unidentified enzymes. Studies are currently ongoing in an attempt to address these possibilities.

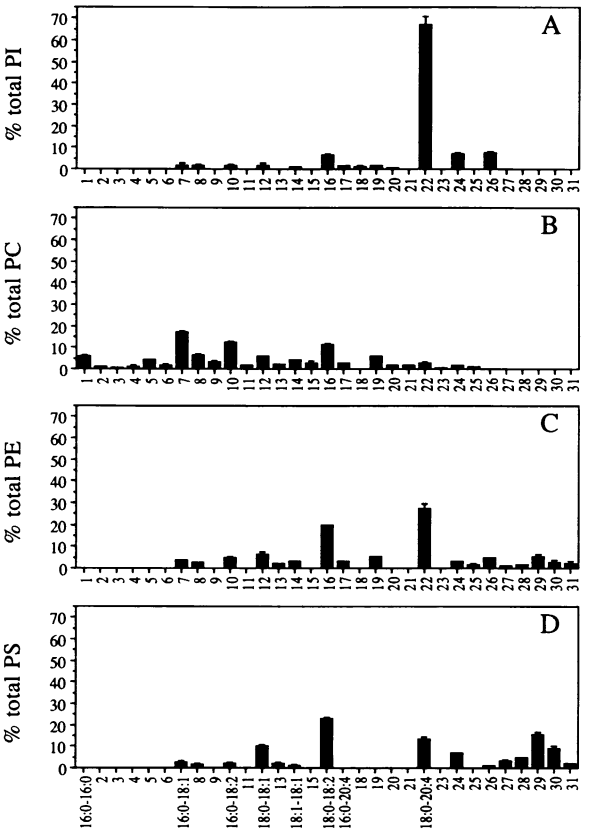

Figure 2. **Molecular species profiles of PC12 phospholipids.** PC12 cell cultures were grown and stimulated with phosphate-buffered saline (control); cellular (A) PI, (B) PC, (C) PE, and (D) PS were isolated by HPLC; headgroups were removed by phospholipase C hydrolysis; and the component DGs were converted into *tert*-butyldimethylsilane derivatives and analyzed by capillary gas chromatography as described in Methods. The molecular species profile of each cellular phospholipid was generated by calculating, for each molecular species, its percentage of the total phospholipid-derived DGs (% total), (mean ± SE, n = 2). Each number on the abscissa (A–D) represents a different molecular species.

Methods

Materials

β-NGF was prepared according to the method of Mobley *et al.* (1976). A bFGF analogue in which all half-cystine residues

Figure 1. **Molecular species profiles of induced diglycerides.** PC12 cultures were grown and stimulated with phosphate-buffered saline (control) for 1 (A) or 15 min (E), 200 ng/ml NGF for 1 (B) or 15 min (F), 50 ng/ml bFGF for 1 (C) or 15 min (G), or 0.5 mM carbachol for 1 (D) or 15 min (H). Cellular DGs were isolated by thin-layer chromatography, converted into *tert*-butyldimethylsilane derivatives, and analyzed by capillary gas chromatography as described in Methods. The data are expressed for each molecular species as its percentage of the entire control (A and E, % total) or "stimulated" (B–D, F–H, % "stimulated" total) DG population. The level of each "stimulated" DG molecular species was obtained by subtracting the level contained in the control, quiescent cultures (A or D) from the total level of that molecular species. The data represent the mean ± SE of at least three separate experiments. Each number on the abscissa (A–H) represents a different molecular species. The total control DG masses were as follows (mean ± SE): control, 1 min 0.626 ± 0.104 mol%, 15 min 0.539 ± 0.146. Stimulated DG masses were the following percentages of control levels (mean ± SE): NGF, 1 min 143 ± 7%, 15 min 238 ± 8%; bFGF, 1 min 163 ± 32%, 15 min 161 ± 7%; carbachol, 1 min 215 ± 66%, 15 min 256 ± 45%.

Table 1. Comparison of stimulated DG molecular species profiles to phospholipid and phospholipid combination profiles by correlation coefficient

	Phospholipids				
Agonists	PI	PC	PE	PS	Best combination
1 min					
NGF	0.990	0.118	0.854	0.439	0.993 (87% PI + 13% PC)
bFGF	0.976	0.079	0.758	0.307	0.977 (79% PI + 21% PC)
carbachol	0.878	0.384	0.913	0.535	0.947 (50% PI + 50% PC)
15 min					
NGF	0.617	0.698	0.824	0.528	0.914 (26% PI + 74% PC)
bFGF	0.457	0.525	0.859	0.476	0.919 (41% PI + 59% PC)
carbachol	0.589	0.765	0.683	0.474	0.875 (19% PI + 81% PC)

Molecular species profiles of DG generated by stimulation of PC12 cells by either 200 ng/ml NGF for 1 or 15 minutes, 50 ng/ml bFGF for 1 or 15 minutes, or 0.5 mM carbachol for 1 or 15 minutes were compared with the molecular species profiles of total cellular PI, PC, PE, and PS or were compared with molecular species profiles of combinations of total cellular PI and total cellular PC. These combinations were obtained as described in Methods. These comparisons were performed by calculating the correlation coefficient between the DG profile of interest and the profile of the phospholipid (or combination) being considered as a putative source, as described in Methods.

were replaced by serine was used for all experiments with bFGF and was kindly provided by Dr. Gary M. Fox (Amgen, Thousand Oaks, CA). This analogue is more stable than recombinant native bovine bFGF, and its effects in producing inositol phosphates and DGs in PC12 cultures are indistinguishable from the recombinant bFGF (Altin and Bradshaw, 1990). Butylated hydroxytoluene and isopropyl ether of 99+% quality were obtained from Aldrich (Milwaukee, WI). All other organic solvents were GC_2 grade from Burdick and Jackson Laboratories. All organic solvents included 50 μg/ml butylated hydroxytoluene. Thin-layer chromatography plates were obtained from Analtech. *tert*-butyldimethylchlorosilane/imidazole was purchased from Alltech Associates. Zorbax PRO-10 Sil (4.6 mm ID × 25 cm) high-pressure liquid chromatography (HPLC) column was purchased from Du Pont-New England Nuclear (Boston, MA). SP-2380 capillary column (15 m × 0.32 mm) was purchased from Supelco (Bellefonte, PA). DG standards were obtained from Serdary Research Laboratories (London, Ontario, Canada). Other DG standards were obtained from phospholipase C (*Bacillus cereus*) hydrolysis of phospholipid standards (Pessin and Raben 1989). Phospholipid standards were purchased from Avanti Polar Lipids (Birmingham, AL). Phospholipase C (*Bacillus cereus*) was purchased from Boehringer Mannheim (Indianapolis, IN).

Cell culture and molecular species analyses

PC12 cells were grown, stimulated with agonists, and extracted as described (Altin and Bradshaw, 1990). Cellular DGs were isolated by thin-layer chromatography, derivatized into *tert*-butyldimethylsilane analogues, and separated by capillary gas chromatography on an SP-2380 capillary column as described (Pessin and Raben, 1989). DG molecular species were identified by comparison with known standards. Phospholipids were isolated by thin-layer chromatography, separated by HPLC on a Zorbax PRO-10 Sil Column, and headgroups were removed by phospholipase C hydrolysis as previously described (Pessin and Raben, 1989). The resulting DGs were derivatized and analyzed as for cellular DGs. DG linkage analysis was performed as described (Pessin and Raben, 1989).

Correlation coefficients

The correlation coefficient is defined by:

$$\sum (A \times B)/(\sqrt{[\sum A^2] \times [\sum B^2]})$$

(Snedecor and Chochran, 1980)

where, in our analyses, for molecular species i to n, A_i is the percentage that a particular molecular species (i) comprises of a specific phospholipid or phospholipid combination profile, and B_i is the percentage that this molecular species (i) comprises of a total stimulated DG profile (Pessin *et al.*, 1990).

Calculation of PI and PC contributions to stimulated DG profiles

The amounts that PIs and PC hydrolysis contribute to generate the stimulated DG profiles are calculated by solving for X and Y in the following equations:

$$\text{(stimulated DG 18:0–20:4 percentage)} = X \cdot \text{(PI 18:0–20:4 percentage)} + Y \cdot \text{(PC 18:0–20:4 percentage)} \quad (1)$$

$$\text{(stimulated DG 16:0–18:1 percentage)} = X \cdot \text{(PI 16:0–18:1 percentage)} + Y \cdot \text{(PC 16:0–18:1 percentage)} \quad (2)$$

where

$$\text{(PIs contribution)} = X/(X + Y) \quad \text{and} \quad \text{(PC contribution)} = Y/(X + Y).$$

Stimulated DG 18:0–20:4 percentage is the percentage that molecular species 18:0–20:4 contributes to the total stimulated DG pool for a given agonist and period of stimulation and is taken from the data in Figure 1. Stimulated DG 16:0–18:1 percentage is the percentage that molecular species 16:0–18:1 contributes to the total stimulated DG pool for a given agonist and period of stimulation and is

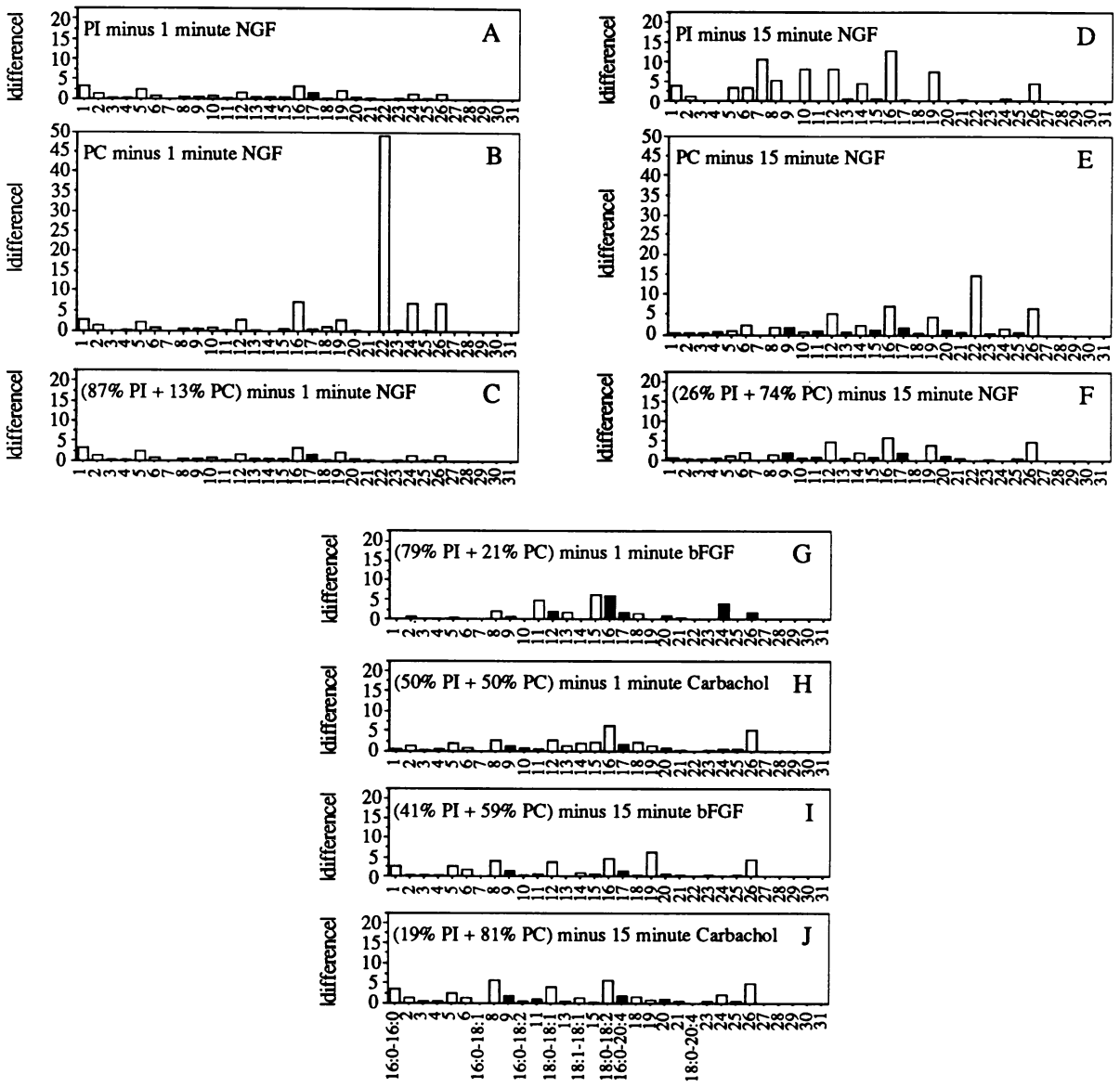

Figure 3. **Comparison of induced diglycerides and phospholipids.** The molecular species profiles of the "stimulated" cellular DGs were compared with the profile of their best combination (see Methods) of total cellular PI and total cellular PC (C, F–J). To better demonstrate the necessity for a combination profile, the molecular species profiles of the DGs generated after stimulation of PC12 cells by NGF for 1 (A and B) and 15 min (D and E) were compared with the molecular species profiles of total cellular PI (A and D) and total cellular PC (B and E). These comparisons were performed by calculating, for each molecular species, the absolute value of the difference between the stimulated cellular DG profile and the phospholipid (or combination) molecular species profile (|difference|). The black bars indicate positive differences and the white bars indicate negative differences for the calculation: phospholipid (or combination) profile minus "stimulated" DG profile as indicated in the figure. The level of each "stimulated" DG molecular species was calculated, for each molecular species, by subtracting the control level from the total level. Each number on the abscissa (A–J) represents a different molecular species.

taken from the data in Figure 1. PI 18:0–20:4 percentage is the percentage that molecular species 18:0–20:4 contributes to the total PI pool and is taken from the data in Figure 2. PC 18:0–20:4 percentage is the percentage that molecular species 18:0–20:4 contributes to the total PC pool and is taken from the data in Figure 2. PI 16:0–18:1 percentage is the percentage that molecular species 16:0–18:1 contributes to the total PI pool and is taken from the data in Figure 2. PC 16:0–18:1 percentage is the percentage that molecular species 16:0–18:1 contributes to the total PC pool and is taken from the data in Figure 2.

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