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ARTICLES

Activation of Phospholipase D by Ras Proteins Is Independent of Protein Kinase C

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Abstract Growth factors activate phospholipases, causing the generation of diverse lipid metabolites with second messenger function. Among them, the phosphatidylcholine-preferring phospholipase D (PLD) has attracted great interest, since in addition to the transient activation by growth factors stimulation, it is constitutively activated in some of the *src*- and *ras*-transformed cells investigated. To establish further the functional relationship of *ras* oncogenes with PLD, we have investigated its mechanism of regulation. Growth factors such as PDGF or FGF activate the PC-PLD enzyme by a common, PKC-dependent mechanism. By contrast, *ras* oncogenes activate the PC-PLD enzyme by a PKC-independent mechanism. These results suggest the existence of at least two mechanisms for PLD activation, and *ras* oncogenes contribute to one of them.

Key words: *ras* proteins, growth factors, phospholipase D, PKC, phorbol esters

Mitogenic stimulation of fibroblasts with growth factors is mediated by the activation of a variety of signal transduction molecules. Some of the most important enzymes involved in signal transduction may be converted into potent oncogenes [Cantley et al., 1991]. The implicated molecules are very similar, regardless of the stimulus or cellular system studied, with an important redundancy of signals and cross-talk among them [Cantley et al., 1991]. It is now well established that different mitogenic stimuli activate multiple pathways of which several combinations seem sufficient to induce entry into the S-phase. A wealth of information indicates that phospholipases and other lipid-related enzymes may play a relevant role in mitogenesis at least in some cellular lineages [Billah, 1990; Divecha and Irvine, 1995; Exton, 1994; Nishizuka, 1992; van Blitterswijk et al., 1994].

Most growth factors rapidly activate phospholipases, such as PI-specific PLC and PC-specific PLD, activation that rapidly decreases to near-basal, nonstimulated levels [Berridge, 1993; Exton, 1994; Nishizuka, 1992]. As a consequence, several lipid second messengers are generated, including DAG and inositol phosphates, which activate intracellular signaling cascades. Activation of PLD generates choline and phosphatidic acid (PA), which are further converted into *P*Cho and DAG [Exton, 1994]. This growth factor-mediated PLD activation follows a protein kinase C (PKC)-dependent mechanism, as it is lost in the absence of functional PKC [Cook and Wakelam, 1992; Freeman and Tallant, 1994; Kiss, 1992; Martinson et al., 1990; Plevin et al., 1991; Suchard et al., 1994; van der Bend et al., 1992]. In fact, direct activation of PKC by phorbol esters is the most potent mechanism for the activation of PLD in almost every cell type investigated [Exton, 1994; Huang and Cabot, 1990].

A parallel increase in the level of *P*Cho and DAG is observed at late times after growth factors stimulation of quiescent cells [Exton, 1994; Nishizuka, 1992]. While this late peak of DAG production has been proposed as one of the key steps in the mitogenic stimulation mediated by growth factors [Exton, 1994], it has been shown recently that production of *P*Cho is an essential requirement for their mitogenic activity [Jiménez et al., 1995]. The finding of elevated DAG and *P*Cho at late times of mitogenic stimulation initially supported the hypothesis of PC-PLC involvement. However, the appearance of *P*Cho seems to be dependent on a PC-PLD and ChoK pathway, since it is completely abolished by hemicholinium-3 (HC-3), an inhibitor of the ChoK [Jiménez et al., 1995]. Furthermore, DAG pro-

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duction is sensitive to propranolol, an indication that it is mostly generated by the PLD/PA phosphatase pathway [Carnero et al., 1994].

Transformation by oncogenes may also lead to activation of PLD, resulting in the constitutive elevation of both DAG and *P*Cho by the PLD/ChoK/PA phosphatase pathway [Carnero et al., 1994; Song et al., 1991]. Some of the mitogenic effects of growth factors and *ras* oncogenes in 3T3 cells could be mediated by PLD-derived metabolites [Carnero et al., 1994; Jiménez et al., 1995]. Furthermore, in *Xenopus* oocytes, the biological effect of *ras* may be mediated by PLD, since there is activation of the enzyme after *ras*-p21 microinjection, and the PLD-derived products mimic *ras* effects and inhibition of PLD by neomycin reduces the biological activity of *ras* proteins in this system [Carnero and Lacal, 1995]. These results points to PC-PLD as an important molecule involved in mitogenic signal transduction and the possibility that it may also be an effector for *ras* function, as demonstrated for the activation of c-Raf-1 kinase [Leevers et al., 1994; Stokoe et al., 1994] and PI3-kinase [Rodriguez-Viciana et al., 1994]. In keeping with these results, a constitutive activation of PLD in *src*-transformed cells has been demonstrated [Song and Foster, 1993]. This activation is mediated by a G-protein [Jiang et al., 1994] that has been identified as the *ras*-p21 protein [Jiang et al., 1995].

In this study we have further investigated the mechanism of PLD activation by *ras* and compared it with that mediated by growth factors. Our study indicates that activation of PLD by *ras* is cell dependent, as found for other well known *ras* effectors, such as Raf1 and PI3K, and may therefore require other factor(s) for proper regulation. We also show that there are at least two alternative mechanisms for PLD activation and that *ras* contributes to one of such systems. A model for PLD activation by growth factors and *ras* oncogenes is discussed.

MATERIALS AND METHODS

Cell Culture and Reagents

Normal NIH 3T3 mouse fibroblasts and Rat-2 cells as well as *ras*-transformed derivatives were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with either 10% newborn (NIH 3T3 cells) or fetal (Rat-2 cells) calf serum (Gibco) under standard conditions of temperature (37°C) humidity (95%) and carbon dioxide (5%). The cell lines were obtained from independent transfections as follows: LP8-1 is a mass culture of NIH 3T3 cells transfected with EXV plasmid plus RSV5-hygro plasmid coding for hygromicin resistance, LP8-1A is a clone isolated from a cotransfection of EXV plasmid plus RSV5-hygro plasmid, LP8-3 is a mass culture of the same transfection using EXV-H-*ras*^{Val12} plus RSV5-hygro, and LP8-3A and LP8-3C are two independent clones obtained by cotransfection of EXV-H-*ras*^{Val12} plus RSV5-hygro. Cell lines were analyzed for *ras*-p21 expression by Western blot, using a specific monoclonal antibody for Ras proteins (Y13-259, Oncogene Science, Uniondale, NY), and those showing high expression levels selected for further characterization. Cell lines H-*ras* and K-*ras* (K2) are NIH 3T3 cells expressing transforming Harvey- or Kirsten-*ras* genes, respectively, and correspond to those described before [Carnero et al., 1994]. The cell lines T-794-97-1, T-808-70M, T-MAR-1-13, and T-MAR-1-14 were obtained by transfection of EXV-H-*ras*^{Val12} on NIH 3T3 cells and selected as transformed foci. Each cell line consisted of a pool of more than 100 transformed foci, except T-794-97-1, which correspond to a single isolated foci. The Rat-2 cell lines were generated as follows: RRAS is a mass culture of Rat-2 cells transfected with EXV-H-*ras*^{Val12} plus pZIP-neoplasmid coding for G418 resistance. LP9-3 and LP9-6 are mass cultures of Rat-2 cells transfected with EXV-H-*ras*^{Val12} plus RSV5-hygro; LP9-3A, LP9-3D, LP9-3C, and LP9-3H are independent clones obtained by

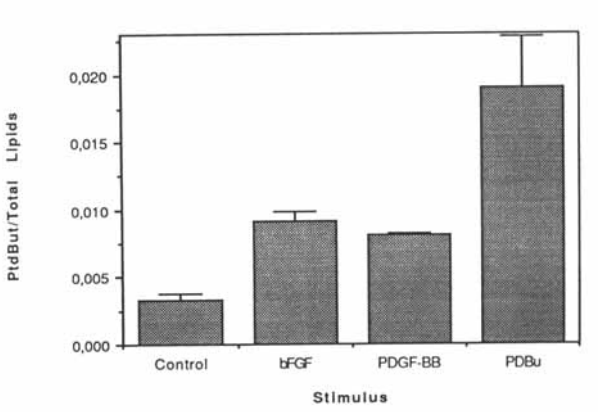

Fig. 1. Growth factors and PDBu stimulate PC-PLD activity. Cells were grown under standard conditions in DMEM supplemented with 10% serum. After 24 h of labeling with 10 μCi/ml [2-^{3}H]glycerol, cells were stimulated with the indicated mitogens (10 ng/ml of PDGF-BB, 10 ng/ml bFGF, or 350 nM PDBu) or left untreated (control) for 30 min, in the presence of 0.5% 1-butanol. Each point is the average of duplicate samples ± range, and the experiment was repeated three times with similar results.

transfection with the indicated plasmids. Rat-2-A and Rat-2-B are two isolated Rat-2 clones that do not express exogenous Ras proteins.

[2-^{3}H]Glycerol (1 Ci/mmol) and [methyl-^{3}H]choline chloride were from Amersham, Buckinghamshire, UK. 1-Butanol was from Merck, Darmstadt, Germany. Bisindolylmaleimide was purchased from Calbiochem, Lucerne, Switzerland. LK6D thin-layer chromatography plates were from Whatmann, Hillsboro, Oregon.

Assay of PLD Activity

Activation of PLD can be measured accurately by its transphosphatidylating activity on butanol to generate phosphatidylbutanol from PC [Song et al., 1991]. Cells were grown in 6-well plates in the presence of 10 μCi/ml [2-^{3}H]glycerol or 0.5 μCi [2-^{14}C]glycerol. Where indicated, cells were treated with 350 nM PDBU during the labeling period, to down-regulate the phorbol esters' sensitive endogenous PKC isoenzymes. To assay PLD activity, label media was discarded, and cells were washed with TD buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Tris, pH 7.4) and incubated 30 or 45 min in DMEM supplemented with or without 0.2% newborn calf serum containing 0.5% 1-butanol, in the presence or absence of 350 nM PDBu or 8 μM bisindolylmaleimide. The cells were then scrapped in 1 ml methanol and wells washed with 1 ml of methanol. The two methanol samples were collected and mixed with 2.5 ml chloroform and 1.25 ml water. The organic phase was dried in a 37°C heating block under a nitro-

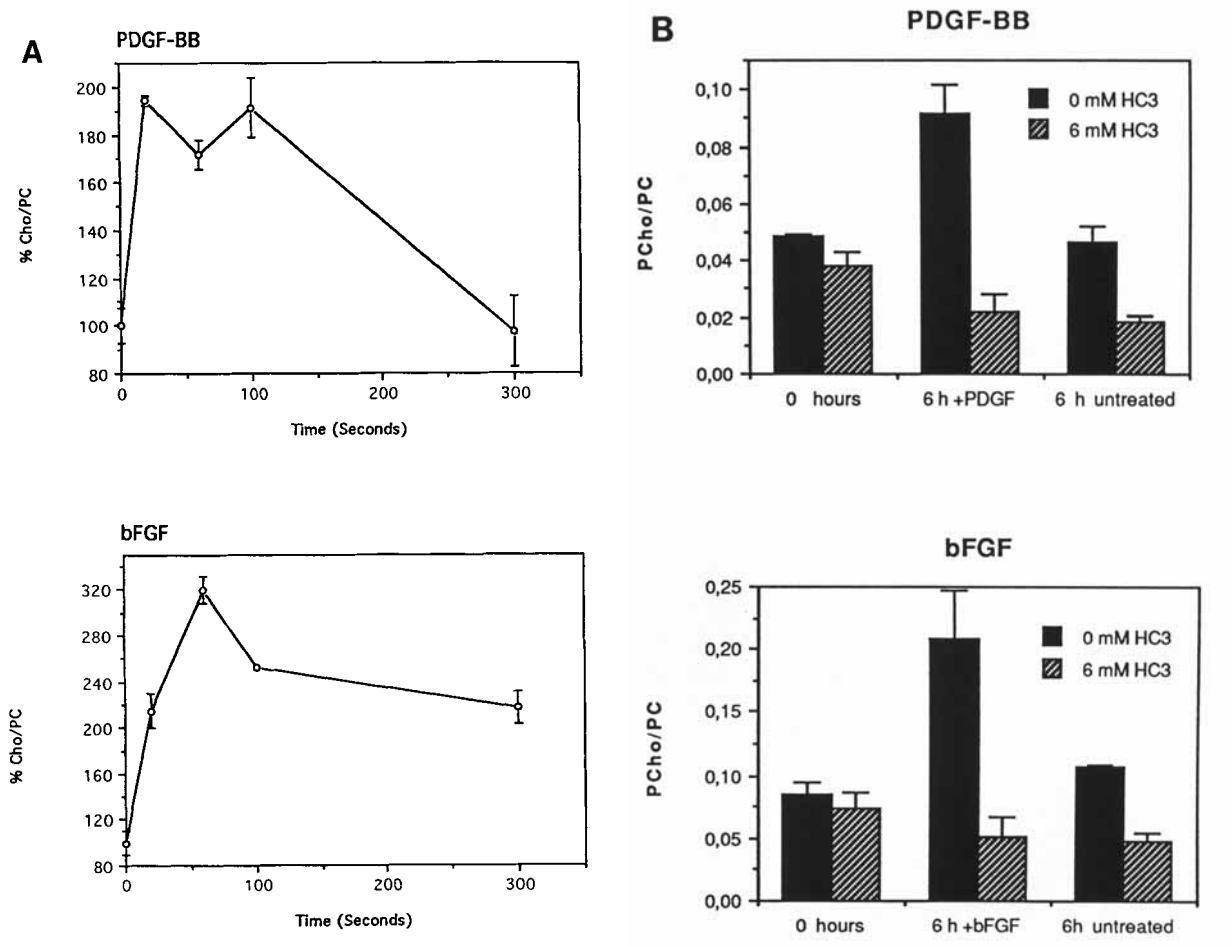

Fig. 2. Growth factors activate PC-PLD both at early and late times after stimulation. **A:** Cells were serum-starved and labeled with 10 μCi/ml of [methyl-^{3}H]choline in DMEM supplemented with 0.2% FCS, stimulated with either 10 ng/ml of PDGF-BB or bFGF during the indicated time, and then processed as described under Materials and Methods for choline release. Each point is the mean of triplicate samples ±SD. Similar results were obtained in two other independent experiments. **B:** Cells were treated as described in **A** with either PDGF-BB or bFGF in the presence (▨) or absence (■) of 6 mM HC-3. Analysis of the *P*Cho levels was performed as described under Material and Methods after 6 h of stimulation with the indicated growth factor or as basal levels at 0 and 6 h.

gen stream and the lipids separated by thin-layer chromatography (TLC), using Silica Gel 60A plates. The plates were developed with the upper phase of a mixture of ethyl acetate/iso-octane/glacial acetic acid/water (90:50:20:100) plus 1 ml of acetic acid. Plates were sprayed with enhancer (Dupont) and exposed to X-ray-sensitive films. Bands corresponding to phosphatidylbutanol were scraped off and scintillation counted. Phospatidylbutanol was identified using a standard generated in vitro using purified PLD with [^{14}C]phosphatidylcholine as substrate. PtdBut production was normalized by dividing the total cpm in PtdBut by total cpm incorporated into phospholipids. Where indicated, quantitation was carried out by analysis under an electronic autoradiography system (Instant-Imager, Packard, Meriden, CT).

Determination of Intracellular Choline

Cells were grown in 6-well plates and, when confluent, serum starved in DMEM supplemented with 0.2% newborn calf serum and labeled for 24 h in the presence of 10 μCi/ml [methyl-^{3}H]choline. The indicated stimuli were then added to the cells without replacing the labeling media and incubated for the indicated times at 37°C. After this time, media were aspirated, cells washed with ice-cold TD buffer and fixed with 16% TCA. The TCA-soluble material was washed four times with four volumes of water-saturated ethyl ether and then lyophi-

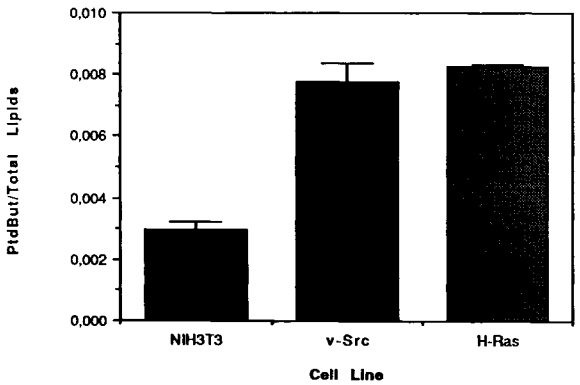

Fig. 3. Basal PLD activity in oncogene-transformed fibroblasts. After labeling for 24 h with 10 μCi/ml [2-^{3}H]glycerol, in DMEM supplemented with 0.2% FCS cells were washed with TD buffer and incubated for 45 min in DMEM supplemented with 0.2% newborn calf serum plus 0.5% 1-butanol. Phosphatidylbutanol production was determined as described under Materials and Methods. Data represent mean values of duplicate samples ± range. The experiment was repeated two more times with similar results.

TABLE I. Basal PLD Activity in Several *ras*-Transformed Cell Lines

Exp No.	Cell line	*ras* gene	PLD activity	% control
1	NIH 3T3	—	3.46 ± 0.24	100.0
	H-*ras*	Viral-H	9.13 ± 0.20	263.7
2	LP8-1	—	4.87 ± 0.54	100.0
	LP8-1A	—	4.35 ± 0.16	89.3
	LP8-3	H-Val^{12}	4.19 ± 0.07	86.0
	LP8-3A	H-Val^{12}	6.09 ± 0.22	125.0
	LP8-3C	H-Val^{12}	5.69 ± 0.27	116.8
	NIH 3T3	—	4.48 ± 0.35	91.9
	H-*ras*	Viral-H	13.74 ± 0.62	282.1
3	NIH 3T3	—	3.21 ± 0.23	100.0
	T-MAR-1-13	H-Val^{12}	3.05 ± 0.06	95.0
	T-MAR-1-14	H-Val^{12}	2.91 ± 0.11	90.6
	H-*ras*	Viral-H	5.58 ± 0.12	173.7
4	NIH 3T3	—	4.39 ± 0.00	100.0
	K2	Viral-K	7.06 ± 0.68	160.5
	H-*ras*	Viral-H	7.15 ± 0.88	162.6
	T-794-97-1	Viral-H	5.4 ± 0.13	123.8
	T-808-70M	Viral-H	3.93 ± 0.16	89.4
5	Rat-2	—	9.54 ± 2.50	100.0
	RRAS	H-Val^{12}	26.75 ± 4.60	280.3
6	Rat-2	—	5.30 ± 0.92	100.0
	Rat-2-A	—	4.41 ± 0.01	83.0
	Rat-2-B	—	5.61 ± 0.07	105.8
	LP9-3	H-Val^{12}	8.91 ± 0.68	167.9
	LP9-6	H-Val^{12}	8.27 ± 0.60	155.9
	LP9-3A	H-Val^{12}	6.40 ± 0.90	120.7
	LP9-3D	H-Val^{12}	6.74 ± 0.06	127.0
	LP9-3C	H-Val^{12}	5.86 ± 0.41	110.5
	LP9-3H	H-Val^{12}	6.34 ± 0.71	119.6
7	Rat-2	—	4.56 ± 0.76	100.0
	LP9-3	H-Val^{12}	8.22 ± 0.30	180.2

Cells were grown to confluence in 6-well plates, and then serum-starved and labeled with [^{3}H]glycerol in DMEM supplemented with 0.2% newborn calf serum during 24 hours. At this time, PLD activity was determined as described in Materials and Methods during 45 minutes of incubation with DMEM medium supplemented with 0.2% newborn calf serum and 0.5% 1-butanol. H-*ras*, T-794-97-1, LP8-3, LP8-3A, LP8-3C, TMAR-1-13 and T-MAR-1-14 are Harvey-*ras*^{val12} expressing NIH3T3 cells. K2 and T-808-70M are Kirsten-*ras* transformed NIH3T3 cells. LP8-1 and LP8-1A are control NIH3T3 cells transfected with an empty plasmid. RRAS, LP9-3, LP9-6, LP9-3A, LP9-3D, LP9-3C and LP9-3H are H-*ras*^{val12} expressing Rat-2 cells. Data represents mean values of duplicate samples ± standard deviations. The values for PLD activation in the *ras*-transformed cells are referred to their internal control, normal cells, processed under similar conditions for each experiment.

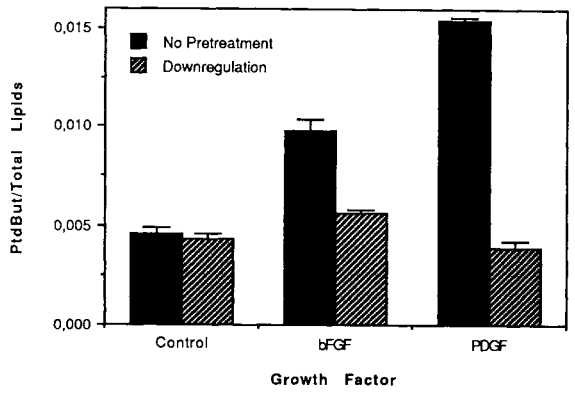

Fig. 4. PKC downregulation completely abrogates growth factor-induced PLD activity. Cells were serum starved and labeled as described in Materials and Methods in the presence () or absence (■) of 350 nM PDBu to induce PKC downregulation. Then, cells were stimulated with the indicated mitogens (10 ng/ml of bFGF or PDGF-BB) or left untreated (Control) for 30 min in the presence of 0.5% 1-butanol. Data represent mean values of duplicate samples ± range. The experiment was repeated two more times with similar results.

lized. Choline and derivatives were resolved by TLC, using Silica Gel 60A plates. The plates were developed with 0.9% NaCl/methanol/ammonium hydroxide (50:70:5). Dry plates were sprayed with enhancer (Dupont) and exposed to X-ray sensitive films. Bands corresponding to choline were scrapped and scintillation counted.

Western Blot Analysis of PKC

Cells were grown to confluence in 10-cm dishes. After appropriate treatment, cells were washed in TD buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Tris, pH 7.4) and lysed by the addition of 300 μl of ice-cold lysis buffer (50 mM Tris, pH 7.4, 0.2% NP-40, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 15 mM β-glycerophosphate, 10 mM Na PPi, 50 mM Na F, 20 μM aprotinin, 1 mM PMSF). Nuclei and detergent-insoluble material was removed by centrifugation at 10,000*g* for 10 min. The resulting supernatants were assayed for estimation of total cell protein (BioRad) and equal amounts of cell lysate (typically 70 μg) were boiled at 95°C for 5 min in SDS–PAGE sample buffer. For Western blot analysis, proteins were resolved onto 12% SDS–PAGE gels poured in 20 × 20-cm glasses. Separated proteins were transferred to nitrocellulose, and blots were blocked for 1 h in 3% nonfat dried milk in T-TBS (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.005% Tween 20). Blots were washed once in T-TBS and incubated 2 h with an anti PKC-specific antibody (MC5, Amersham, Buckinghamshire, UK), and developed by ECL (Amersham).

RESULTS

PC-PLD Activation by Growth Factors and Oncogenes

Challenge of quiescent fibroblasts with growth factors such as PDGF-BB and bFGF or phorbol esters like PDBu leads to the activation of PLD activity when analyzed by its transphosphatidylating activity to butanol (Fig. 1). This rapid and transient activation reaches maximum levels at 1 min after stimulation and can be also monitored as the release of choline, an indication that phosphatidylcholine (PC) is the substrate for this PLD activation (Fig. 2A). The late increase in DAG and *P*Cho seems to be generated by the consecutive activation of PLD and ChoK as well, since *P*Cho production is drastically reduced in the presence of HC-3 (Fig. 2B) under conditions in which this drug does not affect phospholipase activity [Carnero et al., 1994].

Malignant transformation of NIH 3T3 cells by oncogenes such as *ras* and *src* is associated with a constitutive increase in PLD activity (Fig. 3). Besides elevated levels of DAG and *P*Cho, the constitutive activation of PLD by *ras* does not seem universal, since no increase in PLD activity has been reported in some of the *ras*-transformed cells studied (Fu et al., 1992). We have investigated further the origin of this discrepancy. To that end, we selected several individual clones and mass cultures from different transfection experiments using NIH 3T3 and Rat-2 fibroblasts as recipient cells. After characterization of *ras* expression levels and selection of high level expressors of the exogenous Ras protein (data not shown), we carried out analysis of basal PLD activity of each newly generated cell line. As shown in Table I, only some of the NIH 3T3 cell lines analyzed showed a significant constitutive increase in PLD activity of 20–180% over control cells. A better correlation could be established for the Rat-2 cell lines analyzed, with constitutive increases in almost all the *ras*-transformed cell lines ranging from 20% to 180% over control cells. In contrast with these results, no increase of basal PLD activity was observed in any of the cell lines analyzed overexpressing the wild-type H-*ras* gene in NIH 3T3 cells or Rat-2 cells (data not shown). These results indicate that a significant constitutive activation in PLD may not be observed in all

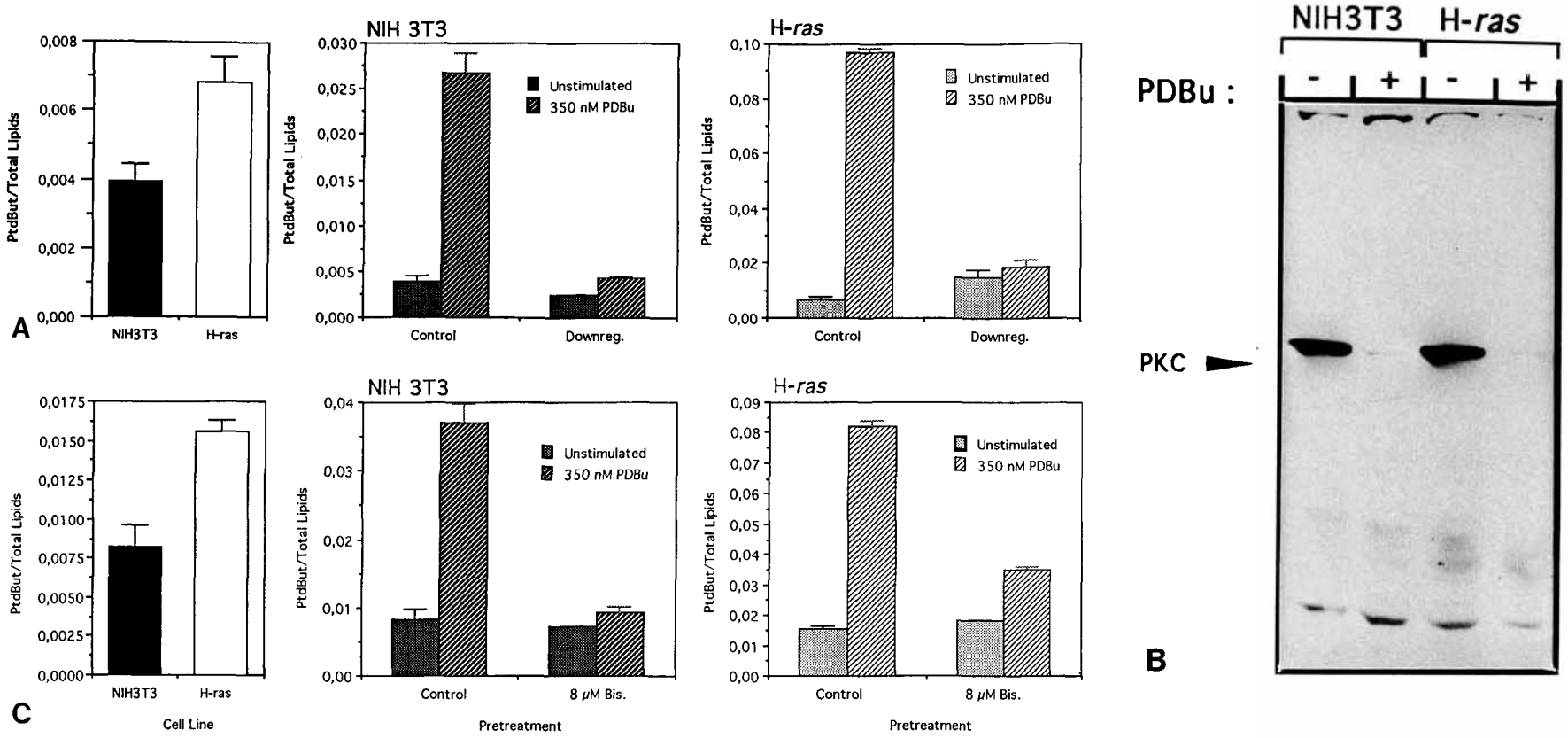

Fig. 5. *ras*-induced PLD activity is PKC independent. **A:** Cells were serum-starved and labeled with 10 μCi/ml of [2-^{3}H]glycerol and processed for PLD activity as described in Materials and Methods for 24 h. *Left:* basal levels of PLD in control (■) and *ras*-transformed cells (□). *Center; right:* Cells were labeled as above in the presence (downreg.) or absence (Control) of 350 nM PDBu to induce PKC downregulation. Cells were then stimulated with 350 nM PDBu (350 nM PDBu) or left untreated (unstimulated) for 45 min in the presence of 0.5% butanol and PLD activity determined. Data represent mean values of duplicates samples ± range. The experiment was repeated at least two more times with similar results. **B:** Control NIH 3T3 and *ras*-transformed cells were grown to confluence in 100-mm dishes and then incubated for 24 h with (+) or without (−) 350 nM PDBu. At this time, cells were processed for Western blot analysis using an anti-PKC antibody as described in Materials and Methods. *Arrow,* band corresponding to PDBu-responsive PKC isoenzymes. The experiment was repeated two more times with similar results. **C:** Cells were serum-starved and labeled with 10 μCi/ml of [2-^{3}H]glycerol and processed for PLD activity as described in Materials and Methods for 24 h. *Left:* basal levels of PLD in control (■) and *ras*-transformed cells (□). *Center; right:* Cells were labeled as above. The labeling media was then discarded, cells washed with TD buffer and incubated for 25 min in DMEM supplemented with 0.2% newborn calf serum and containing either 8 μM bisindolylmaleimide in DMSO (8 μM Bis.) or same amounts of DMSO alone (control). Medium was then replaced for DMEM supplemented with 0.2% newborn calf serum plus 0.5% butanol, supplemented with 8 μg/ml bisindolylmaleimide or DMSO, stimulated with 350 nM PDBu (350 nM PDBu) or left untreated (Unstimulated) for an additional 45 min, and processed for phosphatidylbutanol determination. Data represent mean values of duplicate samples ± range. The experiment was repeated once more with similar results.

stable *ras*-transformed cells, besides efficient transformation.

PLD Activation by Oncogenic *ras* Is PKC Independent

Phorbol esters are one of the most potent activators of PLD activity in several cell types [Exton, 1994; Huang and Cabot, 1990], indicating that PKC may be involved in the regulation of PLD activity. We first established whether the rapid and transient activation of PLD induced by growth factors and the constitutive activation induced by *ras* transformation required functional PKC. To that end, cells were treated for 24 h with high concentrations (350 nM) of the phorbol ester PDBu, to induce down-regulation of PDBu-sensitive PKC species. After down-regulation, cells were challenged with either PDGF-BB or bFGF, and the activity of PLD was assayed after 30-min incubation in the presence of 1-butanol. As shown in Figure 4, down-regulation of PKC by prolonged exposure to PDBu completely blocked the activation of PLD by growth factors, while untreated cells had a normal activation of the enzyme. These results demonstrate that PLD activation induced by growth factors was completely dependent on the availability of some of the phorbol ester-sensitive PKC isoenzymes in agreement with previous reports showing that PKCα may be responsible for this effect [Eldar et al., 1993]. It also readily demonstrates that the experimental conditions used in our assay efficiently removed all phorbol ester-sensitive PKC enzymes.

We next investigated the requirement for PKC in the constitutive activation of PLD by *ras*-transformation. For this, we used two different cell lines showing constitutive activation of the enzyme including both NIH 3T3 and Rat-2 cells. Functional PKC was eliminated by two alternative methods: (1) chronic treatment for 24-h exposure to phorbol esters, which will induce down-regulation of phorbol ester-sensitive PKC species; and (2) treatment with 8 μM bisindolylmaleimide, a highly selective PKC inhibitor capable of efficiently inhibiting all known PKC isoenzymes at this concentration. As shown in Figure 5A, down-regulation of PKC had no inhibitory effect on the increased PLD activity in the NIH 3T3 cell lines transformed by the Harvey-*ras* oncogene. By contrast, down-regulation of control NIH 3T3 cells totally abrogated PDBU-induced PLD activity, indicating the lack of functional PKC activity.

We previously showed that expression of the oncogenic *ras* gene in PC12 cells protects the PKC enzymes from down-regulation induced by PDBu treatment [Lacal et al., 1990]. To test

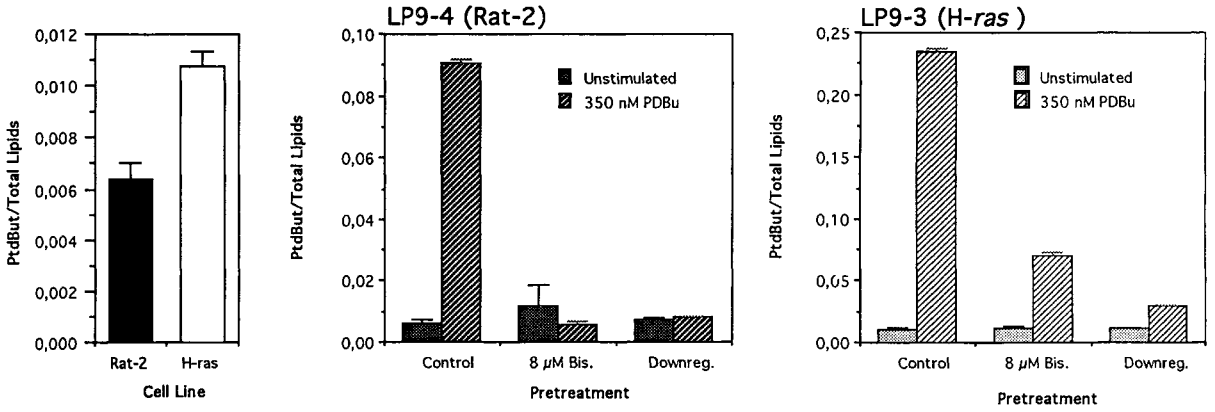

Fig. 6. Constitutive PLD activity in Rat-2 *ras*-transformed cells is PKC independent. Parental Rat-2 cells were transfected with plasmid RSV5-hygro alone and selected for hygromycin resistance as a pool (LP9-4), or cotransfected with the Harvey-*ras* oncogene and selected also as a pool of hygromycin-resistant colonies (LP9-3). Cells were serum-starved and labeled with 10 μCi/ml of [U-^{14}C]glycerol for 24 h and processed for PLD activity as described in Materials and Methods. *Left:* basal levels of PLD in control (■) and *ras*-transformed cells (□). *Center; right:* Cells were grown to confluence in DMEM supplemented with 10% FCS and 1 μCi [2-^{14}C]glycerol (72-h labeling). Where indicated, PKC was downregulated by treatment with 350 mM PDBu during the labeling period (Downreg). Then, media was discarded and cells incubated for 30 min in DMEM without serum, supplemented with either 350 nM PDBu to maintain PKC downregulation (Downreg), 8 μM bisindolylmaleimide (8 μM Bis.) to directly inhibit PKC, or DMSO alone as control (Control). Cells were then further incubated for an additional 30 min at 37°C in same media supplemented with 0.5% 1-butanol and stimulated with PDBu (350 nM PDBu) or DMSO alone (Unstimulated). Cells were processed as described in Material and Methods. Data represent mean values of duplicate samples ± range. The experiment was repeated once more with similar results.

whether the lack of an inhibitory effect of down-regulation of PKC in the increased PLD activity mediated by *ras* was a consequence of a similar mechanism, we analyzed the levels of the PKC enzymes after down-regulation in *ras*-expressing NIH 3T3 cells by Western blot. As shown in Figure 5B, all the immunoreactive PKC was efficiently eliminated at the same extent in Harvey *ras*-transformed cells and in control NIH 3T3 cells. Thus, the lack of an inhibitory effect of down regulation of PKC on the PLD activity in *ras*-transformed cells strongly indicates PKC-independent activation of PLD by *ras*. Finally, direct inhibition of the PKC activity by 8 μM bisindolylmaleimide confirmed the above results. As shown in Figure 5C, activation of PLD induced by PDBu in the parental NIH 3T3 cells was drastically reduced in the presence of the inhibitor, while the constitutively elevated PLD activity induced by *ras* was not affected, indicating a PKC-independent mechanism. The above results were confirmed using an independent cell system. Rat-2 cells transformed by the Harvey-*ras* oncogene were also investigated. Figure 6 shows that neither down-regulation by chronic treatment with 350 nM PDBu nor direct inhibition with 8 μM bisindolylmaleimide had any effect on the constitutive increased PLD activity by *ras* transformation.

DISCUSSION

A wealth of data suggest a significant role for PLD in signal transduction: PLD is activated transiently in response to numerous mitogenic stimuli, some of the PLD products have a recognized role as second messengers, and exogenous added purified PLD to quiescent fibroblasts leads to DNA synthesis [reviewed in Exton, 1994; van Blitterswijk et al., 1994, Carnero et al, 1994]. Thus, if PLD were part of the normal signaling pathway leading to mitogenesis, it would be possible that oncogenic transformation would affect it, as has been shown for other components of the signaling cascade that behave as oncogenes when properly altered. Furthermore, the normal counterparts of these oncogenes would be implicated in the regulation of PLD.

Activation of PLD activity have been described by PKC, G-proteins, and Ca^{2+} [Exton, 1994; van Blitterswijk et al., 1994]. Several studies using toxins indicate that activation of some unidentified heterotrimeric G-proteins may be implicated in the regulation of PLD [Coorssen and Haslam, 1993; Geny and Cockcroft, 1992; Irving and Exton, 1987; Olson et al., 1991; Quian and Drewes, 1989; van der Meulen and Haslam, 1990]. By a more direct approach, small GTP-binding proteins have also been implicated in PLD activation. Recombinant ARF mediates GTP activation of PLD in HL60 both in in vitro systems and in whole permeabilized cells [Brown et al., 1993]. Rho proteins, another member of the superfamily of low-molecular-weight GTPases, have also been implicated in PLD regulation in neutrophils [Bowman et al., 1993]. Finally, two recent reports demonstrate that activation of PLD induced by the *src* oncogen is mediated by a G-protein [Siang et al., 1994] that can be inhibited by incubation with the *ras*-specific neutralizing antibody Y13-259 [Jiang et al., 1995]. All these results suggest that, depending on cell type and/or the PLD isozyme implicated, different GTPases may be involved in the observed activation of PLD by alternative mechanisms.

Ras proteins are small GTPases that have been implicated in the regulation of pathways critical for proliferation and differentiation processes [Lacal and Carnero, 1994]. Among these pathways, two enzymatic cascades have recently been implicated: the intracellular kinase cascade initiated by the activation of c-Raf-1, and the activation of the PI3-kinase, which triggers signaling through inositol–phospholipid metabolism. Previous studies in our group demonstrated that *ras*-transformation leads to an increase in the basal PLD activity in NIH 3T3 cells [Carnero et al., 1994], as well as an acute activation upon microinjection in *Xenopus* oocytes [Carnero and Lacal, 1995], suggesting that *ras* may be involved in the regulation of PLD activity.

Besides the evidence that in some cell types the basal activity of PLD is increased after transformation by the *ras* oncogene, this increase is not observed in all the cell lines analysed (Table I). These results could be explained if the activation of PLD induced by *ras* was a consequence of an indirect mechanism, involving other(s) component(s). A similar effect has been found for other well-characterized *ras* effectors. While c-*raf* was found to be constitutively activated in some cell lines investigated [Leevers and Marshall, 1992], reports from other groups fail to find a constitutive activation of either c-Raf [Reed et al., 1991] or MAPK [Gupta et al., 1992] in *ras*-transformed cells. Also, while a recent report clearly demonstrates the physical interaction

between Ras-GTP and PI3-kinase [Rodriguez-Viciana et al., 1994] and transient activation of PI3-kinase upon *ras* transfection into COS-7 cells, an indication that PI3-K might function as a *ras* effector, *ras*-transformed cells do not have a constitutively active PI3-K [P. Esteve and J.C. Lacal, unpublished data; J. Downward, personal communication]. Thus, from these results it seems clear that enzymatic activities of well-established *ras* effectors are not always constitutively activated in *ras*-transformed cells; therefore, the absence of an increase of basal activity in transformed cells may not be considered a sufficient criteria to discard any molecule as a putative *ras* effector.

When an increase in basal PLD activity was observed in *ras*-transformed cells, this activity was PKC independent, in contrast to the PKC-dependent activation mediated by growth factors. Similar results have been reported for the *src* oncogene [Song and Foster, 1993], in agreement with the proposed common pathway for *src* and *ras* in cell transformation. Two alternative hypotheses can explain the above results: (1) either *ras* (and *src*) uses a different pathway to activate PLD from that used by growth factors and PKC activation; or (2) they activate distinct PLD isoforms. While the results shown here clearly demonstrate that PLD activation mediated by *ras* is PKC independent, further investigation will clarify among these alternative hypotheses. In this regard, preliminary results from our group indicate that *ras*-transformed cells show a drastic attenuation of the PLD activation mediated by growth factors [del Peso et al., Submitted], suggesting that these alternative pathways may have a common intracellular intermediary. Furthermore, the lack of correlation between transforming activity by *ras* oncogenes and PLD activation suggests that PLD is a redundant or dispensible signal for oncogene-mediated cell transformation, similar to that shown for the PI3K or the PI-PLC enzymes in mitogenic stimulation by growth factors. However, more detailed study on growth properties will be needed to reach this conclusion.

Despite the observation of an elevated basal PLD activity in some of the cell lines analyzed, our previous studies and the results presented here strongly support the hypothesis that Ras oncoproteins affect PLD activity regulation in an indirect manner. As it stands for other *ras* effectors, a complete understanding of the relationship between *ras* and PLD is still to come. This problem is further complicated for PLD, since mammalian PLD genes have not yet been cloned. These genes will be instrumental in assessing which molecules are critical for its regulation and the precise role of PLD in the known mitogenic signaling pathways. Our study indicates that *ras* proteins regulate PLD by an alternative mechanism from that demonstrated for growth factors.

NOTE ADDED IN PROOF

After acceptance of this manuscript, Hammond et al. have reported the cloning of a human PC-PLD gene [J. Biol. Chem. 270:29640–29643, 1995]

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