Dissociation of Inositol Trisphosphate from Diacylglycerol Production in Rous Sarcoma Virus-transformed Fibroblasts

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Abstract. The metabolism of phosphatidylinositol (PI) and related intermediates was studied in uninfected and Rous sarcoma virus-(RSV) infected chicken embryo fibroblasts (CEFs). Cells infected with wild-type RSV exhibited twofold increases in steady-state concentrations of inositol trisphosphate (IP_3) and inositol bisphosphate (IP_2) as compared to uninfected CEFs. In addition, increased concentrations of IP_3 and IP_2 were observed in CEFs infected with the RSV temperature-sensitive transformation mutant NY72-4 when maintained at the permissive temperature (35°C) for >24 h. Slight increases were observed in the amounts of inositol lipids in RSV-transformed cells. Phosphoinositol metabolic changes were related to transformation and not to viral infection since CEFs infected with NY72-4, maintained at the nonpermissive temperature (41.5°C), revealed amounts of phosphoinositols similar to that of uninfected cells. CEFs infected with a transformation-defective virus exhibited PI metabolic changes intermediate between those of transformed and nontransformed cells. NY72-4 CEF exhibited no increase in phosphoinositol concentrations before 8 h incubation at 35°C, indicating that the transformation-specific changes in inositol metabolism were a delayed event. Furthermore, inositol turnover was not activated during this time. In contrast to the case of inositol metabolism, significant increases in diacylglycerol (DAG) concentrations were observed within 15–30 min after shift of NY72-4 CEFs to 35°C.

These findings suggest that (*a*) the major changes in inositol metabolism are specific for RSV-transformed cells; (*b*) transformation-specific changes in phosphoinositol content in RSV-infected CEFs are not an early effect of the expression of $pp60^{v\text{-}src}$; and (*c*) increases in the DAG content of transformed cells occur before changes in inositol metabolism, indicating that DAG may be derived from other lipid sources.

THE transforming gene product of Rous sarcoma virus (RSV)[1], $pp60^{v\text{-}src}$, exhibits tyrosine-specific protein kinase activity, a property it shares with a number of oncogene products and growth factor receptors (for review see 25). However, the molecular events leading from tyrosine phosphotransferase activity to cellular mitogenesis remain obscure since phosphorylation of substrates identified appears to be incidental (29, 30).

Attempting to unravel the steps culminating in mitogenesis, investigators have concentrated on the early biochemical events that follow proliferative stimuli. Recently, several studies have implicated the importance of protein kinase activities specific to serine/threonine residues after mitogenic stimulation of cells. For example, a ribosomal protein S6-specific serine phosphotransferase has generated much interest since it is one of the earliest responding enzymatic activities after cellular stimulation by several oncogenes and growth factors (9, 22, 28). However, the link between the proliferative stimuli including tyrosine phosphotransferase activity and the identity and transduction of the mitogenic signal responsible for the stimulation of the S6 kinase activity have remained elusive.

The components of the phosphatidylinositol (PI) metabolic cycle appear to be signal transducers used by a number of hormones and growth factors including α-adrenergic agents (50), thyrotopin-releasing hormone (21, 24), vasopressin (11), muscarinic cholinergic agents (11), bombesin (12), and platelet-derived growth factor (6). Agents that stimulate PI turnover can potentially generate two intracellular second messengers; namely, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (3). DAG in the presence of calcium ion can influence the phosphorylation state of a number of intracellular proteins by activating protein kinase C (3). Protein kinase C is of particular significance since 12-O-tetradecanoylphorbol 13-acetate, a known mitogen for some cells, can activate the kinase by substituting for its endogenous effector, DAG (3, 38). Likewise, IP_3 is of interest

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1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; DAG, diacylglycerol; IP, IP_2, and IP_3, inositol mono-, bis-, and trisphosphate; PA, phosphatidic acid; PI, phosphatidylinositol; PIP and PIP_2, phosphatidylinositol mono- and bisphosphate; RSV, Rous sarcoma virus; SRA, Schmidt-Ruppin strain A of RSV.

since it is thought to be involved in the release of Ca^{2+} from internal cellular stores after its binding to specific receptors (6, 8, 26, 46, 47). Koch and Diringer (33) showed that transformed cells exhibit increased PI turnover when compared to their nontransformed parent cells. These studies were extended to include transformation by RSV as well (19). More recently, several investigations have demonstrated that treatment of quiescent cells with growth factors or serum results in rapid changes in PI turnover. Since RSV-transformed cells display a number of properties similar to those of growth factor-stimulated cells, initial investigations concentrated on the in vitro activity of key inositol-metabolizing enzymes. These studies indicated that transformation by RSV and related oncogenes resulted in elevated PI kinase activity (31, 36, 48, 49), phospholipase C activity, PI turnover (27), and elevation of DAG (51). Although the activities of different inositol-metabolizing enzymes were quantified in vitro, few data regarding the in vivo steady-state concentrations of phosphoinositides in RSV-transformed cells were available. Furthermore, no data was available with regard to the temporal relationship of phosphoinositide metabolism and expression of $pp60^{v\text{-}src}$, the transforming protein. Therefore, the quantitation of phosphatidylinositol metabolic intermediates was performed in uninfected and RSV-infected chicken embryo fibroblasts (CEFs).

The data reported below indicate that indeed, transformed cells exhibited altered amounts of phosphoinositols when compared to their nontransformed counterparts. However, experiments using temperature-sensitive, transformation-defective RSV-infected fibroblasts indicated that the change in inositol metabolism was a relatively late event. In fact, nontransforming mutant RSV-infected CEFs revealed some PI metabolite changes that were similar to their transformed counterparts. In contrast, rapid increases in DAG concentration were observed after expression of $pp60^{v\text{-}src}$ and were temporally dissociated from the transformation-specific changes in PI metabolism.

Materials and Methods

Materials

Phytic acid was obtained from Sigma Chemical Co. (St. Louis, MO). AG-1-X8 anion exchange resin was purchased from Bio-Rad Laboratories, Digilab Div. (Cambridge, MA). *myo*-1,2[^{3}H]inositol, [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid and [^{3}H]acetic anhydride were supplied by New England Nuclear (Boston, MA). [$^{32}PO_4$] was purchased from ICN Radiochemicals, Div. ICN Biomedicals Inc. (Irvine, CA). Bovine calf serum was from HyClone Laboratories, Sterile Systems, Inc. (Logan, UT) and DME was from Gibco Laboratories (Grand Island, NY). 10-14-d-old chicken embryos were obtained from SPAFAS, Inc. (Norwich, CT). RSV mutants NY72-4 and NY315 were kind gifts from H. Hanafusa (The Rockefeller University, New York). All other chemicals were reagent grade or better.

Cell Culture

Primary CEFs and CEFs infected with a wild-type RSV (i.e., Schmidt-Ruppin strain A [SRA]) or RSV transformation-defective mutants, NY72-4 or NY315, were grown at 41.5°C on 100-mm culture dishes (Costar, Cambridge, MA) using DME supplemented with 5% bovine calf serum as described previously (10). To assess the progress of viral infection, cellular extracts were assayed for their ability to phosphorylate IgG from tumor-bearing rabbit serum (39). All experiments used cells that were between the fifth and tenth passages and at least two passages after infection with retrovirus.

Cellular Labeling with myo-1,2-[^{3}H]inositol

For these experiments, 60-mm culture dishes (Costar) were seeded at either 0.25 or 1.0 × 10^6 cells per dish in 3 ml inositol-free DME (prepared according to Gibco Laboratories, Grand Island, NY, but *myo*-inositol was eliminated from the vitamin mixture) supplemented with 5% calf serum. After 5 h, 1 ml of inositol-free DME containing 5% calf serum and 5 μCi/ml *myo*-1,2-[^{3}H]inositol was added to each dish. After 3 d of culture at 41.5°C, the cells were confluent, and the medium was then replaced with inositol-free DME containing 0.5% calf serum. The cells were maintained for 16–24 h in low serum before initiation of temperature shift or serum stimulation experiments. Cells could be maintained for at least three passages in inositol-free DME with no detrimental effects. To estimate the relative turnover of phosphoinositols in CEFs, some plates of cells were incubated for 30 min with 10 mM LiCl just before cell lysis. Lithium ion prevents inositol monophosphate hydrolysis by inhibiting phosphomonoesterase (4, 21). Hence, changes in inositol monophosphate concentration after lithium treatment of cells should reflect the relative activity state of the PI metabolic cycle.

Cellular Labeling with [^{32}P]PO_4

Conditions for labeling with [^{32}P]PO_4 were identical to those described above for inositol labeling except that unmodified DME and 10 μCi/ml [^{32}P]PO_4 was used in place of inositol-free DME and [^{3}H]inositol, respectively.

Synthesis of myo-[^{32}P]inositol-1,4,5-trisphosphate

Preparation of *myo*-[^{32}P]inositol-1,4,5-trisphosphate was essentially as described by Downes et al. (20). After the release of inositol phosphates from erythrocyte membranes with Ca^{2+}, the resulting supernatant was chromatographed on AG-1-X8-formate columns, and desalted by lyophilization (16). Thin layer electrophoresis on cellulose sheets in 100 mM sodium oxalate, pH 1.5, showed that the radiolabeled compound migrated with authentic IP_3 generated by partial acid hydrolysis of phytic acid. Contamination with [^{32}P]PO_4 never exceeded 5% of the total radioactivity.

Hydrolysis of Phytic Acid

Partial acid hydrolysis of phytate can generate inositol phosphates containing 1–5 mol PO_4/mol of inositol (44). (It should be noted that inositol phosphates generated in this way contain mixed forms of IP, IP_2, IP_3, IP_4, and IP_5 [e.g., inositol-1-monophosphate, inositol-2-monophosphate, etc.]. Hence, it is assumed that enhanced recovery occurs for all classes of phosphoinositols, exclusive of their form.) The method of Desjobert and Petek (18) was used to hydrolyze phytic acid, and this phytate hydrolysate was then used as carrier to aid in the recovery radiolabeled inositol phosphates. As previously shown (44) and confirmed by thin layer electrophoresis, when 40% of the phytic acid was completely hydrolyzed to PO_4, ~15–20% of the original phytate was converted to an inositol species containing three phosphates.

Quantitation of Inositol Metabolites

Measurement of Water-soluble Inositol Phosphates. After temperature shift or incubation with serum, experiments were terminated by the addition of 0.75 ml of ice cold 5% (wt/vol) TCA. Before scraping the cells from the plates, 200 μl of phytate hydrolysate (i.e., corresponding to ~0.4 μmol of IP_3-like inositol) containing 2,000–3,000 dpm of [^{32}P]IP_3 was added to each plate. The TCA/cell mixtures were transferred to 1.5 ml conical polypropylene tubes and centrifuged for 5 min at high speed in a microcentrifuge. The soluble extract was transferred to a test tube and the cells were washed once more with 0.5 ml 5% TCA. The aqueous layers were combined and the TCA was removed by five extractions with 4 vol of diethyl ether. The aqueous samples were applied to AG-1-X8-formate columns to separate the water-soluble phosphoinositols as described previously (5). Radioactivity in the column eluates was analyzed by dual channel liquid scintillation spectrometry and corrected for radioisotope channel spillover and for recovery of [^{32}P]IP_3. The cellular precipitates were stored at −20°C until analyzed for lipid inositol.

Measurement of Lipid Inositols. The lipid inositols were extracted from cell precipitates with 2 ml of chloroform/methanol/concentrated HCl (200:100:1, by volume). The lipids were deacylated to their respective glycerophospho-derivatives with monomethylamine (15), and separated by anion exchange chromatography as described by Berridge (2).

[³H]Arachidonic Acid Labeling of Neutral Lipids

Uninfected and NY72-4-infected CEF were labeled for 4 h at 41.5°C with 0.5 μCi/ml [³H]arachidonic acid in DME containing 10% delipidated BSA. The cells were washed twice with PBS and incubated for another hour with DME at 41.5°C. The cells were then incubated at 35°C for the indicated times before lipid extraction. To augment identification by iodine vapor, authentic neutral lipid standards were added to each sample just before separation by TLC (45). Spots corresponding to mono-, di-, and triglycerides were scraped from the plates and analyzed with a liquid scintillation counter.

Measurement of DAG

DAG was measured by the method of Banschbach et al. (1) as altered by Farese et al. (23) with slight modifications. Briefly, the 1,2-DAG from each culture dish was purified and separated from 1,3-DAG by TLC (45) and quantitatively acetylated with [³H]acetic anhydride. The acetylated [³H]DAG was then rechromatographed on thin layer plates and quantitated by liquid scintillation spectrometry. With this procedure, DAG-associated radioactivity is directly proportional to the total mass of the lipid.

Measurement of Phosphatidic Acid (PA)

Lipids were extracted as described above for lipid inositols. Phospholipids were separated by TLC (45) and [³²P]PA was located by co-migration of autoradiographic spots with authentic unlabeled PA. The [³²P]PA spots were scraped into vials and analyzed for [³²P] radioactivity.

Miscellaneous Procedures

Phosphate was measured using the method of Lanzetta et al. (35). BSA was rendered free of fatty acids by the method of Chen (14). In experiments with radiolabeled cells, after organic extraction of lipid, the remaining protein was very difficult to solubilize and quantify. Therefore, cells were released from parallel culture dishes by trypsin and quantitated using a Coulter counter (Coulter Electronics Inc., Hialeah, FL). All data, unless specified otherwise, were expressed per 10^6 cells.

Results

Phosphoinositol Metabolism

Effects of Viral Infection/Transformation on PI Metabolism. To examine alterations in PI metabolism in response to transformation or viral infection, phosphoinositols and polyphosphoinositides were analyzed from [³H]inositol-labeled CEFs. Preliminary experiments indicated that [³H]inositol uptake by CEFs was relatively slow with steady-state labeling of PI being attained at ~48 h. Hence, cells were cultured in the presence of [³H]inositol for 48–72 h. Initially [³²P]IP_3 was included in each sample analyzed, but there was significant variability in recovery of ³²P. Partially hydrolyzed phytic acid was tested and was found to be a more suitable carrier for recovery of phosphoinositols. After the inclusion of phytate hydrolysate in all samples, smaller fluctuations in the recovery of [³²P]IP_3 were observed.

Fig. 1 and Table I show the amounts of phosphoinositols in uninfected cells or CEFs infected with either NY315, NY72-4, or wild-type SRA. To determine the relative turnover of the PI cycle intermediates, [³H]inositol-labeled cells were incubated for 30 min in the presence or absence of 10 mM LiCl before cell extraction. The data in Fig. 1 show that SRA-transformed CEFs and NY72-4–infected CEFs maintained at 35°C, a temperature permissive for transformation, had greater amounts of IP in the presence of Li^+ ion than in its absence. Correspondingly, the ratio of IP concentration in the presence and absence of Li^+ ion was much less for

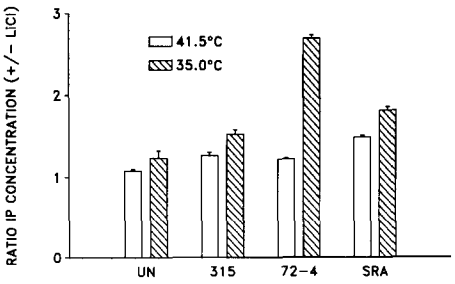

Figure 1. PI turnover in uninfected and SRA-infected CEFs. The conditions for cell manipulation were identical to those described in Table I. However, half of the plates were made 10 mM LiCl 30 min before analysis to evaluate PI turnover. The bars show the ratio of IP concentration in the presence and absence of Li^+ ion and error bars represent the SEM. *UN*, uninfected CEFs. *315*, *72-4*, and *SRA*, CEFs infected with NY315, NY72-4, and SRA viruses, respectively.

NY72-4–infected CEFs maintained at 41.5°C or uninfected CEFs. Table I demonstrates that RSV-transformed CEFs maintained higher steady-state concentrations of IP_3 and IP_2 than uninfected CEFs. Approximately 1.5–2-fold increases in IP_3 concentrations were observed in cells transformed by SRA or NY72-4. However, the data show also that NY315-infected CEFs exhibit phosphoinositol concentrations somewhat similar to those of transformed CEFs. Although NY315 virus is not transforming, PI turnover was slightly elevated and IP_2/IP_3 concentrations were similar to those observed in transformed cells.

As shown in Table II, differences were observed in PI and the polyphosphoinositides as well. SRA-transformed cells differed in the amount phosphatidylinositol bisphosphate PIP_2 from uninfected or temperature conditional NY72-4–infected CEFs maintained at 41.5°C. Similarly, NY72-4–infected CEFs maintained at 35°C showed increased amounts of phosphatidylinositol monophosphate (PIP) and PIP_2 as

Table I. Polyphosphoinositol Levels in Uninfected and RSV-infected CEF

Cells	Temperature	Percent total inositol	
		IP_2	IP_3
Uninfected	41.5°C	0.085 (0.003)	0.183 (0.004)
	35°C	0.080 (0.003)	0.189 (0.012)
NY315	41.5°C	0.116 (0.005)	0.290 (0.034)
	35°C	0.132 (0.004)	0.350 (0.022)
NY72-4	41.5°C	0.069 (0.001)	0.176 (0.026)
	35°C	0.190 (0.003)	0.300 (0.010)
SRA	41.5°C	0.130 (0.003)	0.280 (0.022)
	35°C	0.150 (0.013)	0.380 (0.103)

Cells were maintained at either 41.5°C or 35°C and incubated with [³H]inositol as described in Materials and Methods. After a 24-h incubation in inositol-free DME containing 0.5% serum, the cells were washed, lysed, and analyzed for inositol content. Parallel cultures indicated that cell numbers ranged from 1.3 to 1.8 × 10^6 cells/60-mm dish. The data are expressed as the average percent of total inositol for triplicate plates and values in parentheses are SEM.

Table II. Phosphoinositide Levels in Uninfected and RSV-infected CEF

Cells	Temperature	Percent lipid inositol PI	PIP	PIP_2
Uninfected	41.5°C	91.65 (8.17)	5.08 (1.31)	3.27 (0.55)
	35°C	91.78 (4.97)	4.97 (0.70)	3.25 (0.43)
NY315	41.5°C	92.27 (6.17)	4.70 (0.61)	3.03 (0.23)
	35°C	91.39 (5.83)	5.43 (0.28)	3.18 (0.53)
NY72-4	41.5°C	92.54 (1.80)	4.33 0.33)	3.13 (0.33)
	35°C	90.42 (2.50)	5.98 (0.46)	3.60 (0.49)
SRA	41.5°C	91.24 (1.40)	5.09 (0.19)	3.66 (0.32)
	35°C	91.04 (2.92)	5.14 (0.44)	3.83 (0.76)

Conditions for cell labeling and inositol determinations were as described in Table I. Data are expressed as the average percent of total lipid inositol for triplicate determinations and values within parentheses represent SEM.

compared to uninfected CEFs. When contrasted to morphologically normal cells, CEFs transformed by SRA and NY72-4 viruses exhibited 10–25% increases in PIP and PIP_2. Little differences were observed in the amounts of PI present in these cells. Although NY315-infected CEFs exhibited elevated phosphoinositol concentrations, the pattern of polyphosphoinositides resembled that of uninfected cells.

Effect of pp60$^{v\text{-}src}$ Expression on PI Metabolite Concentration. To investigate the relationship between the expression of pp60$^{v\text{-}src}$ activity and the onset of changes in PI metabolism, temperature-sensitive transformation mutant NY72-4-infected CEF were used. After [^{3}H]inositol labeling, NY72-4-infected CEFs maintained at 41.5°C were made quiescent with serum deprivation. The data in Figs. 2 and 3 were obtained from cells shifted for the indicated times from 41.5°C to 35°C before quantitation of inositol metabolites as described in Materials and Methods. Duplicate plates of NY72-4-infected CEF cells were tested for pp60$^{v\text{-}src}$ activity as well. As shown, there were slight to no changes in the steady-state concentrations of many of the inositol metabolites before eight hours of incubation at the permissive temperature. The only exception was an increase in polyphosphoinositides that occurred between 5 and 8 h after temperature shift (Fig. 3). However, as shown in Figs. 2 and 3 and as indicated previously in Tables I and II, major changes in PI metabolism were observed only after a 24-h incubation at 35°C. Furthermore, these changes in PI metabolism occurred many hours after the expression of immunoglobulin phosphotransferase activity specific for pp60$^{v\text{-}src}$. This delayed response in PI metabolism could not be attributed to cell density since equivalent results were obtained from cells examined at fivefold lower density. No significant change in [^{3}H]inositol metabolites were observed in uninfected CEFs after temperature shift (Tables I and II and data not shown).

Effects of Serum on PI Metabolism. The response of PI metabolism to serum stimulation was examined in quiescent serum-starved uninfected and NY72-4-infected CEFs maintained at 41.5°C. Fig. 4 demonstrates that IP_2 and IP_3 con-

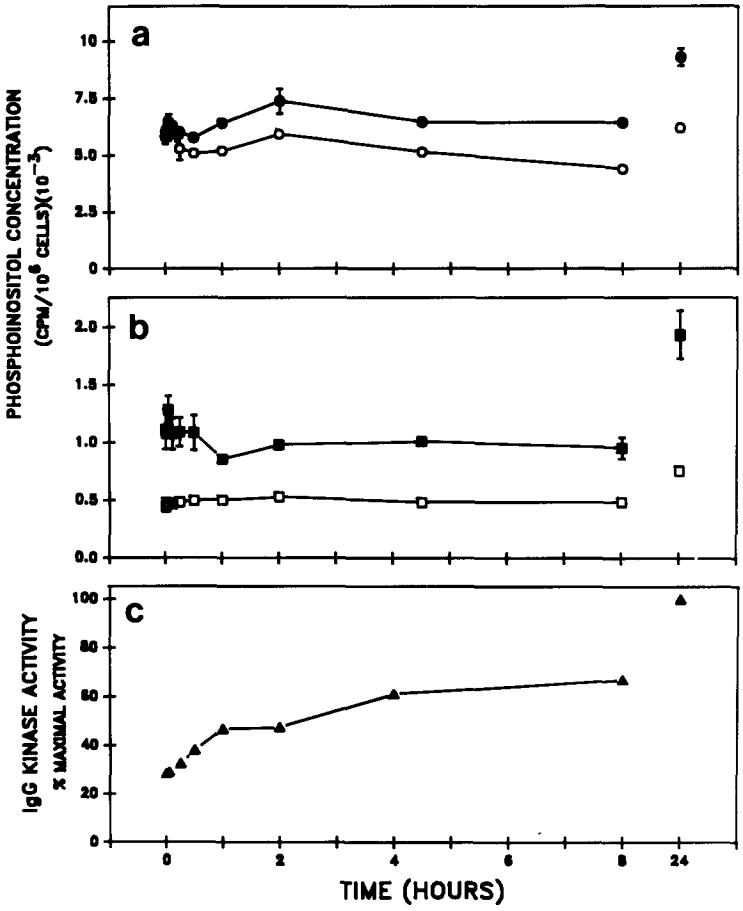

Figure 2. Effect of temperature on phosphoinositol concentration in NY72-4-infected CEFs. (*a* and *b*) Cells were seeded at 10^6 cells/60-mm plate in inositol-free DME containing 5% calf serum and 5 μCi/ml *myo*-1,2-[^{3}H]inositol as described in Materials and Methods. After 3 d of culture, the medium was changed to inositol-free DME containing 0.5% calf serum, and the cells were maintained for 24 h at 41.5°C. After a shift to 35°C for 0, 0.01, 0.05, 0.12, 0.25, 0.5, 1, 2, 4.5, 8, and 24 h the cells were analyzed for inositol metabolites. At the conclusion of the experiment, the cell number was determined to range from 2.5 to 3 × 10^6 cells per dish. The data are expressed as means ± SEM of triplicate determinations. (*a*) IP concentration in cells incubated in the absence (○) or presence (●) of 10 mM LiCl. (*b*) □ and ■ represent IP_2 and IP_3 concentrations, respectively. (*c*) IgG kinase activity from NY72-4-infected CEF extracts after incubating cells at 35°C for 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h. Data are expressed as the percent of maximum observed after 24 h at the permissive temperature.

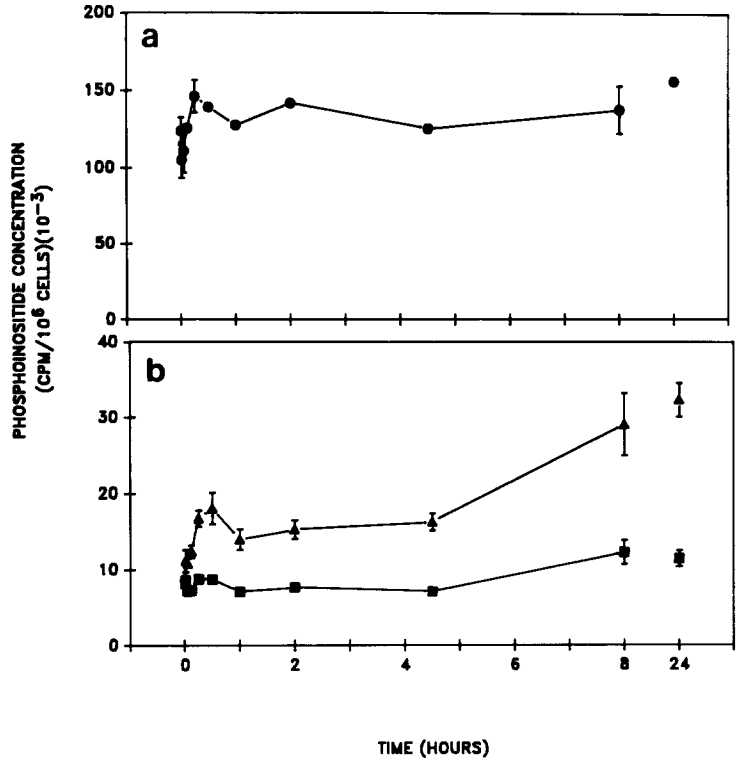

Figure 3. Effect of temperature on lipid inositol concentration in NY72-4-infected CEFs. The conditions for this experiment were identical to those described in Fig. 2. Lipid inositol was analyzed as described in Materials and Methods. (*a*) PI concentration (●). (*b*) PIP and PIP_2 concentrations are represented by ■ and ▲, respectively. All data are expressed as means ± SEM of triplicate determinations.

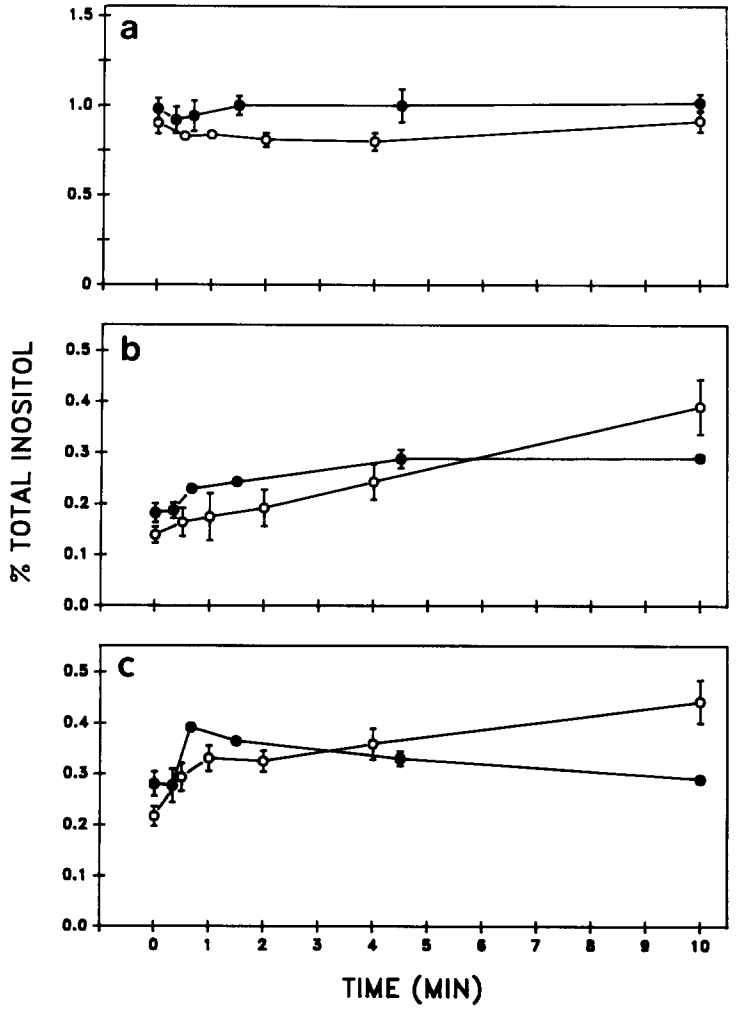

Figure 4. Effect of serum on phosphoinositol concentration in uninfected and NY72-4-infected CEF. Uninfected and NY72-4-infected CEFs were maintained at 41.5°C and labeled with [^{3}H]inositol as described in Materials and Methods. After 24-h incubation in inositol-free DME containing 0.5% calf serum, calf serum was added to a final concentration of 10% and the cells were incubated from 0 to 10 min as indicated. The data are expressed as a percent of the total inositol and error bars represent the range of duplicate determinations. (*a–c*) Concentrations of IP, IP_2, and IP_3, respectively. (○) Uninfected CEFs; (●) NY72-4-infected CEFs.

centrations in both groups of CEFs rapidly increased in response to mitogenic amounts of calf serum. However, NY72-4-infected CEF and uninfected CEFs did exhibit differences in the relative amounts of individual phosphoinositols. NY72-4-infected CEF showed a more rapid but less intense increase in IP_2 concentrations after the addition of serum. In contrast, uninfected CEFs exhibited an immediate increase in IP_3 followed by a more gradual increase in IP_2 in response to serum.

DAG and PA Metabolism

The effect of $pp60^{v\text{-}src}$ expression on incorporation of [^{3}H]-arachidonic acid into neutral lipids was examined in NY72-4-infected CEFs. The data in Fig. 5 indicates that a shift in incubation temperature from 41.5°C to 35°C did not alter the incorporation of [^{3}H]arachidonate into mono-, di-, and triglycerides of uninfected CEFs nor mono- and triglycerides of NY72-4-infected CEFs. However, NY72-4-infected CEFs exhibited approximately twofold increases in [^{3}H]arachidonate-labeled DAG after a shift of cells to the permissive temperature for 2 h. Detectable increases in [^{3}H]DAG were observed when cells were incubated at the permissive temperature for 1 h.

Since [^{3}H]arachidonate labeling of DAG reflects a combination of increased relative mass and precursor incorporation of the lipid, an alternative procedure was used to assess the temperature-induced changes in total mass of DAG alone. In Fig. 6 *a*, radioactivity is directly proportional to the total mass of DAG in cells since the lipid was quantitatively isolated and acetylated with [^{3}H]acetic anhydride. A shift of incubation temperature from 41.5°C to 35°C rapidly altered total DAG content of NY72-4-infected CEFs with no corresponding change in DAG content of uninfected CEFs. This increase in DAG occurred much earlier than the corresponding changes in phosphoinositol content shown above. Since DAG changes were not accompanied by changes in PI turnover, it was presumed that sources other than phosphoinositides exist for DAG. Recently, other studies have suggested that noninositide lipids may act as sources for DAG (23, 32, 34, 37). One purported source for DAG is PA. Consequently, NY72-4-infected or uninfected CEF cells were incubated with [$^{32}PO_4$] for 3 d to label PA to near equilibrium and the content of PA was analyzed. The data in Fig. 6 *b* indicate that after the expression of $pp60^{v\text{-}src}$ a 1.5–2.0-fold increase in total cellular PA was observed at the permissive temperature. The increase in total PA occurred after the change in DAG content of the transformed cells.

Discussion

Numerous studies have implicated the potential role of second messenger systems in mitogenesis and proliferation of cells in culture (for review see 41). Prior work with cells transformed by RSV indicated altered PI metabolism when compared to uninfected cells. To thoroughly define the changes in PI metabolism caused by the expression of $pp60^{v\text{-}src}$, phosphoinositols and related lipid metabolites were examined in mutant RSV-infected CEFs. Although the findings were generally consistent with previous studies, our use of temperature defective mutants of RSV indicated that the $pp60^{v\text{-}src}$ induced alterations in PI metabolism were temporally dissociated from DAG metabolism.

The use of transformation-defective mutants revealed some unexpected changes in the steady-state concentrations of the inositol metabolites. For example, NY315-infected CEFs are transformation negative by virtue of an amino-terminal deletion of 15 amino acids in $pp60^{v\text{-}src}$ that results in its corresponding lack of both myristylation and association with the plasma membrane (17). In vivo tyrosine-specific phosphotransferase activity and phosphotyrosine content varies little in cells expressing either wild-type or amino-terminal mutants of $pp60^{v\text{-}src}$ (17, 30). Although some aspects of PI metabolism in NY315-infected CEFs appear similar to those of uninfected CEFs, the amounts of phosphoinositols were comparable to concentrations found in transformed CEFs. These data suggest that the $pp60^{v\text{-}src}$-

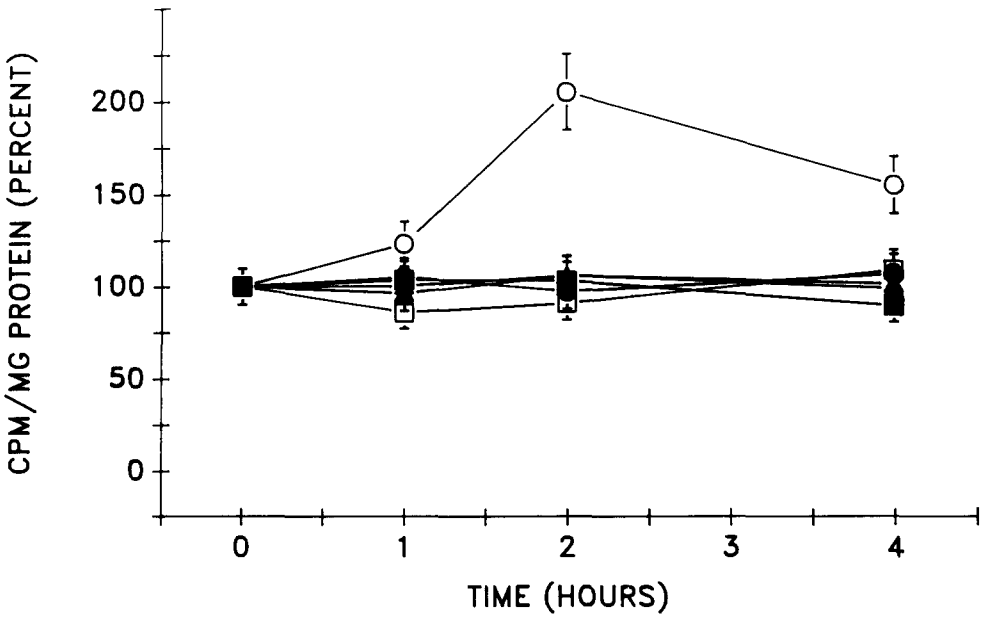

Figure 5. Effect of temperature on [^{3}H]arachidonic acid labeling of neutral lipids in uninfected and NY72-4-infected CEF. Cells were seeded at ~10^6 cells/60-mm dish and cultured at 41.5°C for 3 d. Cells were labeled for 4 h with [^{3}H]arachidonic acid before incubation for 0, 1, 2, and 4 h at 35°C. Data were expressed as a percent of total [^{3}H]cpm/mg protein obtained at 41.5°C. (▲-△) Monoglyceride; (●-○) diglyceride; (■-□) triglyceride; open symbols, NY72-4-infected CEFs; closed symbols, uninfected CEFs.

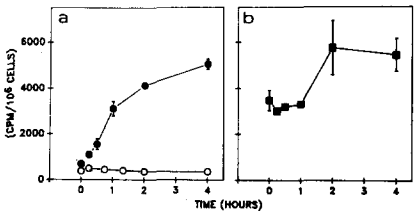

Figure 6. Effect of temperature on DAG and PA metabolism. (*a*) DAG; NY72-4-infected CEFs or uninfected CEFs were seeded at 10^6 cells/60-mm dish and cultured at 41.5°C for 3 d. The media was exchanged with DME containing 0.5% calf serum and incubation continued for 24 h. After the shift of temperature from 41.5°C to 35°C for the indicated times, neutral lipids were extracted from cells, and DAG was analyzed as described in Materials and Methods. The data are expressed as the mean of triplicate dishes and error bars represent SEM. Cell density, as determined from parallel cultures, reached 3×10^6 cells per dish. Open and closed circles represent uninfected and NY72-4-infected CEFs, respectively. (*b*) PA, NY72-4-infected CEF were seeded at 0.7×10^6 cells/60-mm dish and were cultured at 41.5°C in DME containing 5% calf serum and 10 μCi/ml [$^{32}PO_4$]. 3 d later, the medium was exchanged with DME containing 0.5% serum and 10 μCi/ml [$^{32}PO_4$]. After 24-h incubation, the cells were incubated at 35°C for the times indicated and analyzed for [^{32}P]PA as described in Materials and Methods. The data are expressed as the means ± SEM for triplicate plates of cells. Cell density at the time of analysis reached ~4.5×10^6 cells per plate.

associated increase in phosphorylated inositol metabolites resembles an analogous increase in tyrosine phosphorylation of other adventitious substrates (30). Therefore, it is unlikely an increase in phosphoinositols by themselves is sufficient to transform CEFs. However, the data presented here are not inconsistent with a potential role for increased phosphoinositols in the greater saturation density and growth rates observed for cells infected by RSV-expressing pp60$^{v\text{-}src}$s mutated at the amino terminus (13, 30).

The use of temperature-sensitive transformation-defective mutants of RSV allows for the rapid expression of pp60$^{v\text{-}src}$ activity in infected cells after a shift to a permissive, lower temperature. These viral mutants proved useful in delineating some of the early biochemical events after expression of pp60$^{v\text{-}src}$ activity that eventually culminate in cellular transformation of cells maintained at the permissive temperature (for review see 29, 52). In this study, temperature-sensitive transformation-defective NY72-4-infected CEFs were used to examine the dependence of PI metabolism on expression of pp60$^{v\text{-}src}$ activity. As predicted from previous studies, PI metabolism resembled that of uninfected CEFs when NY72-4-infected CEFs were cultured at the nonpermissive temperature. NY72-4-infected CEFs, cultured at a temperature permissive for transformation, resembled SRA transformed CEFs in both PI turnover and steady-state concentrations of inositol intermediates. However, temperature shift experiments indicate that these PI metabolic changes occur with a timecourse different from that of many other enzymatic processes known to be influenced by the expression of pp60$^{v\text{-}src}$. Activation of 2-deoxyglucose uptake, sodium/proton exchange, and ribosomal protein S6 phosphorylation are examples of processes that change before that of PI metabolism in these cells. The sluggish responsiveness of PI metabolism to temperature shift was not a general property of CEFs nor RSV-infected cells since they respond rapidly to mitogenic serum concentrations. Thus we conclude it is unlikely that RSV-stimulated increase in inositol phosphate metabolism provides an early trigger leading to CEF proliferation and transformation in culture.

Our initial impressions of the data obtained with RSV mutants seemed to contradict previous findings (27, 31, 36, 48, 49). However, the previous studies using cells transformed by oncogenes that encode or influence tyrosine kinases failed to examine the temporal relationship of phosphoinositol concentration with expression of the transforming factors. In this study, we report the actual changes in PI metabolite concentrations as opposed to the potential for change as measured in studies that solely examine in vitro enzymatic activity. Nevertheless, our data do support prior studies that analyzed PI metabolism in cells constitutively expressing oncogenes. Furthermore, data presented in this manuscript expand upon previous findings by dissociating early events of pp60$^{v\text{-}src}$ expression from PI metabolism. Thus it would appear that changes in steady-state PI metabolite concentrations, though tightly associated with the transformed state, probably reflect a cellular response to transformation and growth as opposed to a direct causal relationship. In fact, dissociation of the mitogenic effect of growth-promoting agents from phosphatidylinositol metabolism has been shown previously (7).

The diesteratic breakdown of phosphoinositides by phospholipase C generates DAG. Since NY72-4-infected CEFs exhibited slow changes in inositol metabolism on shift to the permissive temperature, it was presumed that DAG concentrations would increase in like manner. Although transformed cells were recently shown to exhibit higher levels of DAG than their nontransformed parental cells (34, 51), the temporal relationships between oncogene expression and changes in DAG were not determined. Our studies show that temporally, pp60$^{v\text{-}src}$-induced changes in PI metabolism can be dissociated from DAG metabolism. When the temperature of NY72-4-infected CEF was shifted from 41.5°C to 35°C, total DAG increased twofold within 30 min and began to plateau ~2 h later. This change in DAG was not a temperature-induced, nonspecific effect since other neutral lipids failed to show any change, and the increase in DAG was specific for cells expressing pp60$^{v\text{-}src}$. Furthermore, the change in DAG precedes any major alteration in PI turnover and would imply that phosphoinositides are not the source of DAG for this increase.

Saltiel et al. (43) reported that insulin-stimulated DAG production resulted in part from the breakdown of a novel PI glycan. In our studies, the water-soluble product of phospholipase C-catalyzed cleavage of any presumed PI glycan would comigrate with other labeled inositols in the glycerophosphoinositol fraction. We were unable to reproducibly observe changes in this inositol-containing fraction (data not shown), and therefore PI glycan was ruled out as a viable source for DAG. Furthermore, DAG accumulations determined from [^{3}H]arachidonate-labeled cells confirm this finding since myristate is the major fatty acid constituent of this novel PI glycan (43). Farese et al. (23) reported that insulin treatment of myocytes and rat adipocytes generates PA and DAG with little to no change in PI metabolism. These studies prompted our investigation into the likelihood that dephosphorylation of newly accumulated PA could act as a

potential source of DAG in RSV-infected CEFs. However, PA was an unlikely source for DAG in our system since increases in this phospholipid occurred subsequent to any increase in DAG concentration. Moreover, increases in PA probably represent enhanced DAG phosphorylation that is consistent with the increase availability of substrate and the reported increase in DAG kinase activity (48). Recently, Harvey^{ras}-transformed cells were shown to have higher amounts of DAG than their nontransformed parental cells (34). This accumulation of DAG was accompanied by an increase in the turnover of phosphatidylcholine and phosphatidylethanolamine but not in phosphatidylinositol, thereby suggesting that these phospholipids were the putative source. More recently, interleukin-1 treatment of T lymphocytes was shown to stimulate rapid DAG and phosphocholine production with no change in PI metabolism nor intracellular calcium concentrations (40). Moreover, tumor-promoting phorbol esters and bombesin were shown to increase phospholipase C–catalyzed hydrolysis of phosphatidylcholine in [^{3}H]choline-labeled Swiss 3T3 cells (37). However, preliminary experiments (Martins, T. J., and R. L. Erikson, unpublished observations) indicate that phosphocholine release in NY72-4–infected CEFs remained similar to that in uninfected CEFs regardless of the incubation temperature. Since more thorough analyses of DAG molecular species indicate that the majority of DAG synthesized during thrombin stimulation of rat mast cells was not derived from PI metabolism (32), future experiments must investigate the possibility that RSV-associated changes in DAG metabolism occur at the expense of other phospholipids. It is also important to note that despite the early increase in DAG observed in these studies, there is little data regarding early activation and redistribution of protein kinase C in RSV-transformed fibroblast. Although Wolfman et al. (51) indicated that stably transformed RSV-infected 3T3 cells exhibited down regulation of both protein kinase C and its major 80-kD substrate, another study demonstrated apparent activation of protein kinase C and increased 80-kD phosphorylation in CEFs after transient expression of pp60$^{v\text{-}src}$ (42). Thus, these important proximal events require additional attention.

This work was supported by grant CA42580 from the National Institutes of Health and from the American Business Research to R. L. Erikson. T. J. Martins was supported by Public Health Service fellowship grant No. 5 F32 CA07729 awarded by the National Cancer Institute, Department of Health and Human Services. R. L. Erikson is an American Cancer Society Professor of Cellular and Developmental Biology.

Received for publication 24 June 1988 and in revised form 22 September 1988.

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