after 2 weeks of flow reduction may involve responses other than adjustments of smooth muscle tone. For example, there may be a structural reorganization of the vessel wall. To assess this possibility, we ligated left external carotid arteries in an additional four rabbits. After 4 weeks the rabbits were reanesthetized, both carotids were exposed, and the smooth muscle relaxant papaverine (3.25 mg/ml) was applied topically to each vessel. Both dilated slightly, but the diameter reduction of the left carotid relative to the right was not reversed or even reduced by this dose of papaverine, even though it abolished the maximal constrictor response to norepinephrine (*13*). We infer that long-term, flow-induced reductions in arterial diameter are due to a structural modification of the vessel wall, although it remains possible that changes in smooth muscle tone contribute to the early phase of the response.

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Ras-Transformed Cells: Altered Levels of Phosphatidylinositol-4,5-bisphosphate and Catabolites

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Steady-state cellular levels of phosphatidylinositol-4,5-bisphosphate (PIP_2), 1,2-diacylglycerol (DAG), and inositol phosphates have been measured in two different fibroblast cell lines (NIH 3T3 and NRK cells) before and after transformation with three different *ras* genes. At high cell density the ratio of DAG to PIP_2 was 2.5- to 3-fold higher in the *ras*-transformed cells than in their untransformed counterparts. The sum of the water-soluble breakdown products of the polyphosphoinositides, inositol-1,4-bisphosphate and inositol-1,4,5-trisphosphate, was also elevated in *ras*-transformed NRK cells compared with nontransformed NRK cells. These findings suggest that the *ras* (p21) protein may act by affecting these levels, possibly as a regulatory element in the PIP_2 breakdown pathway.

INTERMEDIATES IN THE PHOSPHATIDYLINOSITOL turnover cycle apparently act as second messengers to mediate diverse responses in cells. Phospholipase C hydrolysis of phosphatidylinositol (PI) and its phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2), is stimulated by hormones, neurotransmitters, serum, and purified growth factors (*1*). Recent work from several laboratories has indicated that PIP_2 is the primary substrate for this breakdown (*2*). The breakdown products of PIP_2—diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3)—appear to act as intracellular regulators. Diacylglycerol activates a Ca^{2+}- and phospholipid-dependent protein kinase (kinase C) and can also act as a substrate for arachidonic acid release for production of prostaglandins (*3*); IP_3 stimulates Ca^{2+} release from intracellular vesicles into the cytosol (*4*). Both the DAG-activated kinase C and the IP_3-stimulated Ca^{2+} release cause activation of a plasma membrane Na^+-H^+ exchanger, which elevates cytoplasmic Na^+ and *p*H (*5*). Recent evidence has suggested that generation of these signals through stimulation of PI turnover is a critical event in growth factor stimulation of cell proliferation (*6*).

The transforming proteins encoded by the *ras* genes of the Kirsten and Harvey sarcoma viruses are homologous 21-kD proteins. Mutated versions of cellular homologs of *ras* have been implicated in the etiology of many human tumors of diverse origin (*7*). The ras protein is located in the plasma membrane and possesses GTP-binding capacity and low guanosine triphosphatase (GTPase) activity (*8*). Analogies between ras, transducin, and the proteins that regulate adenylate cyclase suggest a role for *ras* in transmembrane signal transduction (*9*), but its actual function in mammalian cells remains to be elucidated. In this study, levels of intermediates in the PI turnover cycle were measured quantitatively in NIH 3T3 and NRK cells and in these cells transformed by H, N, and K alleles of *ras* (see legend to Fig. 1). The results indicate that alterations in the steady-state levels of these membrane components is characteristic of *ras* transformation, suggesting that the ras protein may be involved in the regulation of PIP_2 breakdown.

PIP_2 and DAG levels in normal and *ras*-transformed cells were determined by growing cells in [^{3}H]glycerol. Cellular lipids were extracted in acidic chloroform-methanol and analyzed by thin-layer chromatography (TLC). Radioactivity remaining at the origin on the TLC plates developed in benzene–ethyl acetate (60:40) solvent (diacylglycerol plate) was used as a measure of total cellular [^{3}H]glycerol–containing phospholipid. PIP_2 and DAG were expressed as a percentage of this total, and a ratio of DAG to PIP_2 was obtained for each cell type. Figure 1 shows the results of experiments with cells at high density (0.8×10^7 to 1.0×10^7 cells per 100-mm plate). All three types of *ras*-transformed cells had signifi-

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Table 1. Ratios of DAG and PIP_2 to total cellular lipid. Ratios determined by isotopic labeling with [^{3}H]glycerol and [^{3}H]inositol and by direct mass measurements are compared. Values are expressed as percent ±SEM. The numbers in parentheses give the percentage of change in the transformed cells as compared to their normal counterparts; ND, not determined.

Cell line	DAG		PIP_2	
	Glycerol-lipid*	Phospholipid†	Glycerol-lipid‡	Glycerol-lipid§
NRK	4.5 ± 0.4	3.5 ± 0.04	0.46 ± 0.03	0.59 ± 0.1
kNRK	7.2 ± 0.2 (+60%)‖	4.3 ± 0.06 (+23%)‖	0.29 ± 0.02 (−37%)‖	0.23 ± 0.1 (−61%)‖
NIH 3T3	4.2 ± 0.3	3.5 ± 0.3	0.60 ± 0.04	ND
a_1-1	7.4 ± 0.5 (+76%)‖	4.5 ± 0.1 (+29%)	0.34 ± 0.04 (−43%)‖	ND
1B	5.9 ± 0.6 (+40%)¶	4.3 ± 0.4 (+23%)	0.33 ± 0.02 (−45%)‖	ND

*Values for ratio of DAG to glycerol-lipid are those given in Fig. 1. Cells were labeled with [^{3}H]glycerol. †Values for ratio of DAG to phospholipid were obtained by a DAG kinase assay using diacylglycerol kinase partially purified from bovine brain (*23*). The activity of diacylglycerol kinase was measured in an 0.1 ml reaction mixture containing 5 m*M* $MgCl_2$, 120 m*M* KCl, 30 m*M* NaCl, 50 m*M* Hepes (*p*H 7.1), 0.1 percent Triton, 0.3 m*M* [γ-^{32}P]ATP (10 to 15 μCi/0.1 ml), 1,2-diolein (Sigma) or cell lipid extract, and enzyme. Diolein was sonicated into phospholipid vesicles (PS-PI-PIP-PIP_2; 0.25 m*M*: 0.25 m*M*: 0.04 m*M*: 0.018 m*M*). After incubation of the mixture at 25°C for 60 minutes the reaction was terminated by adding 0.4 ml of $CHCl_3$-CH_3OH-HCl (200:400:5). The lipids were extracted for 30 minutes at 25°C, followed by a phase split induced by adding 156 μl of $CHCl_3$ and 156 μl of 0.1*N* HCl. The lower chloroform phase was removed and spotted onto TLC plates and developed as described in Fig. 1. Phosphatidic acid was identified by autoradiography, and the radioactivity was counted. A linear relationship between diolein concentration and phosphatidic acid formation was obtained. The level of DAG in the cell lipid extract was estimated from this standard curve and normalized to lipid phosphate; the number given represents nmol DAG per nanomole of phosphate. The number of determinations was six for NRK cells, four for kNRK cells, and two for the other three cell lines. Phosphate was determined by the method of Hess and Derr (*24*). ‡Values for PIP_2 are from Fig. 1. Cells were labeled with [^{3}H]glycerol. §To obtain this value the ratio of PIP_2 to PI determined in [^{3}H]inositol-labeled cells (Fig. 2A) was multiplied by the percent of PI of the total [^{3}H]glycerol lipid (18 ± 1.9; n = 3). To resolve PI in [^{3}H]glycerol extracts from other lipids, two-dimensional TLC was done in $CHCl_3$-CH_3OH-NH_4OH-H_2O (45:35:1.5:8.5) in the first dimension and $CHCl_3$-CH_3OH-CH_3OOH-H_2O (75:45:12:3) in the second dimension. PI was identified with [^{3}H]inositol-labeled standards in both dimensions and quantitated as described previously. ‖By Student's t-test, deviation of transformed cells from nontransformed controls was significant by $P < 0.001$. ¶By Student's t-test, deviation from nontransformed controls was significant by $P < 0.05$.

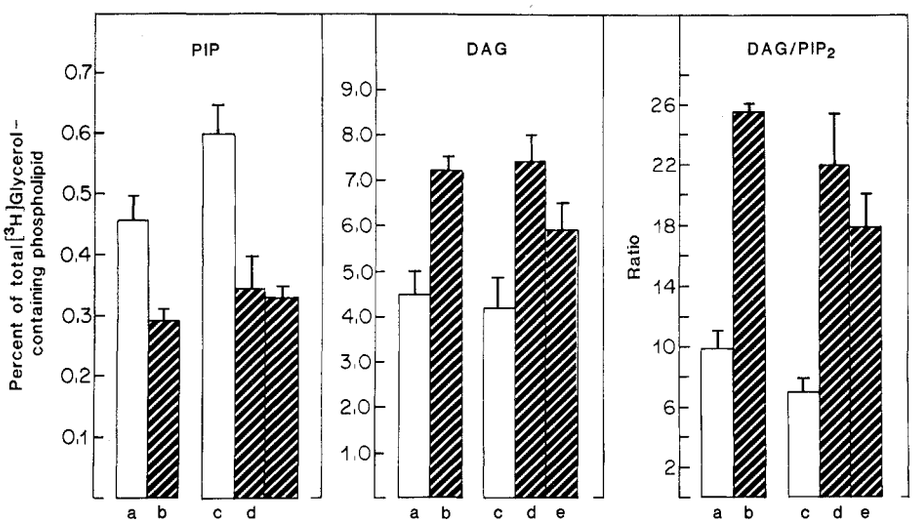

Fig. 1. Steady-state levels of 1,2-DAG and PIP^2 in ras-transformed and nontransformed cells grown at high cell density. Cells were grown to a density of 0.8×10^7 to 1.0×10^7 cells per plate in 100-mm dishes and labeled for 48 hours with [1,2,3,-3H]glycerol (20 μCi per 10 ml per 100-mm plate). Labeled medium was removed and cells were rinsed twice with cold phosphate buffered saline (PBS) (*p*H 7.4). One milliliter of 0.5*N* HCl was added to the plate and cells were scraped into 2 ml of cold chloroform. The plate was rinsed with 1 ml of 1*N* HCl, then 2 ml of methanol, and the washes were combined with the cell extract. Samples were then vortexed, allowed to stand for 30 minutes and briefly centrifuged to separate the two phases. The lower (organic) phase was removed and the aqueous phase was reextracted with 1 ml of chloroform. Pooled organic phases were then washed with 2 ml of 1*N* HCl-MeOH (1:1 by volume). The organic phase was dried under N_2, dissolved in $CHCl_3$-MeOH-H_2O (2:1:0.01 by volume), and applied to TLC plates. For analysis of polyphosphoinositides, samples were spotted on silica gel 60 thin-layer plates (Merck) impregnated with 1 percent potassium oxalate and separated in chloroform-methanol–4*N* NH_4OH (9:7:2 by volume). Spots were identified by comparison with [^{3}H]inositol-labeled standards and unlabeled standards visualized by iodine vapor. For analysis of diacylglycerol, lipids were spotted on untreated plates and separated in benzene–ethyl acetate (60:40 by volume). Lipids were visualized by autoradiography after treatment of plates with Enhance (New England Nuclear). The radioactive spots were cut out and assayed by liquid scintillation counting. At longer exposures small amounts of the 1,3 isomer of diacylglycerol could be detected migrating between 1,2-DAG and TAG; the relative positions of 1,2 and 1,3-DAG were confirmed by use of standards (Sigma) visualized by iodine vapor and were well resolved in this TLC system. Total [^{3}H]glycerol incorporated into phospholipid was approximately 8750 cpm per 10^6 cells. (a) Confluent NRK cells; (b) kNRK cells (Kirsten virus–transformed NRK cells); (c) confluent NIH 3T3 cells; (d) a, −1 cells (T24-H-ras–transfected NIH 3T3 cells; (e) 1B cells (SK-N-SH-N-*ras*–transfected NIH 3T3 cells). Averages and SEM's of three to nine determinations are shown. In all experiments described cells were grown in Dulbecco's modifed Eagle's medium supplemented by 10 percent fetal calf serum. Errors due to variability in cell number, extraction efficiency, or amount of sample applied to the thin layer system were eliminated by taking the ratio of counts in DAG or PIP_2 to the total counts in all glycerol-containing phospholipids.

cantly lower levels (see Table 1) of PIP_2 and higher levels of DAG than their normal counterparts. The actual amounts of DAG and PIP_2 found in the three types of transformed cells were strikingly similar. DAG and PIP_2 amounts in the two types of nontransformed confluent cells were also similar despite the fact that the cells examined originate from different species (mouse versus rat) and tissues (skin versus kidney). In other experiments, in which the ratios of DAG to PIP_2 were determined for cells under low density (exponential growth) conditions, the ratio of DAG to PIP_2 in uninfected NIH 3T3 cells was comparable to that of *ras*-transformed NIH 3T3 cells, whereas Kirsten-transformed NRK cells had a significantly higher ratio than normal NRK cells at both cell densities (*10*).

Experiments with [^{3}H]inositol-labeled cells revealed that the water-soluble breakdown products of PIP and PIP_2 were also elevated in *ras*-transformed NRK cells (Fig. 2a). The results are presented as ratios of IP_2, IP_3, PIP, or PIP_2 to phosphatidylinositol to minimize errors from variability in cell number and efficiency of extraction. In agreement with results obtained with [^{3}H]glycerol labeling (Fig. 1), the amounts of PIP_2 were significantly lower (see Table 1) in the kNRK cells compared with nontransformed NRK cells. When the [^{3}H]inositol radioactivity in PIP_2 was expressed as a percentage of that in PI the values obtained were 3.3 percent for the NRK cells and 1.3 percent for the kNRK cells (Fig. 2A). Although no significant change in IP_3 was detected in the *ras*-transformed cells, a large increase in IP_2 was apparent. In the NRK cells IP_2 expressed as a percentage of PI was 1.3 and in the kNRK cells it was 3.4. The sum of IP_2 plus IP_3 was 3.2 percent of PI in

the NRK cells and 5.0 percent of PI in the kNRK cells. The increase in IP_2 could have occurred because of increased phospholipase C hydrolysis of PIP, or because of a rapid inositol-1,4,5-trisphosphate phosphatase action on the IP_3 produced from PIP_2 breakdown, or by a combination of both effects. Very little difference in the amount of PIP was detected between normal and *ras*-transformed NRK cells (Fig. 2A). Thus, it seems likely that much of the IP_2 represents rapidly dephosphorylated IP_3, since the phosphatase that converts IP_3 to IP_2 has been shown to be an extremely active enzyme (*11*). Although some IP_2 may be produced from breakdown of PIP, the elevation of IP_2 in kNRK cells correlates better with the decreased steady-state level of PIP_2, suggesting derivation from PIP_2 breakdown (*12*).

Since transformed cells have altered rates of metabolism it is critical to eliminate differences due to rates of [^{3}H]glycerol and [^{3}H]inositol uptake and incorporation into the lipid pool. For this reason long labeling times were used in our studies. Control time-course experiments of [^{3}H]glycerol labeling of NRK, NIH 3T3, kNRK, and a_1-1 cells indicated that between 48 and 96 hours of labeling there was no significant change in the total incorporation of [^{3}H]glycerol into cell phospholipid or in the percentage of DAG or PIP_2 of this total. There was no significant difference per cell between the total [^{3}H]glycerol label incorporated into the lipid pool by a_1-1 and NIH 3T3 cells; kNRK showed about 30 percent higher total incorporation while that of NRK cells was about 20 percent lower. In cells labeled with [^{3}H]glycerol, approximately 18 percent of the total [^{3}H]glycerol-lipid was PI in both the normal and transformed cells, as determined by two-dimensional TLC. Thus, the [^{3}H]glycerol incorporation into the PI pool is similar in normal and transformed cells after 48 to 72 hours of labeling under conditions that permit at least three rounds of cell division.

The observations that amounts of PIP_2 in the kNRK cells were similarly lowered in both [^{3}H]glycerol and [^{3}H]inositol labeling experiments suggests that an alteration in PIP_2 mass per cell is involved. In addition, when the ratio of PIP_2 to PI from [^{3}H]inositol labeling experiments is multiplied by the fraction of PI in total glycerol-containing lipid (0.18), the values obtained are similar to the ratio of PIP_2 to total glycerol-lipid measured in the [^{3}H]glycerol labeling experiments (Table 1, last column).

Since differences in glycerol or inositol (or both) metabolism in the transformed cells still may have affected these findings in unknown ways, we sought to measure DAG by an alternative method not involving metabolic labeling. The DAG in the cell lipid extracts was quantitated by phosphorylation with the use of [γ-^{32}P]ATP and a DAG kinase partially purified from bovine brain; samples of the same lipid extracts were analyzed for phosphate content. A comparison of the *ras*-transformed cells (kNRK, a_1-1, and 1B) to the untransformed cells (NRK and NIH 3T3) showed a 23 to 29 percent increase in ratio of DAG to lipid phosphate (Table 1). These findings confirm the increase in DAG amounts in the transformed cells and indicate that the differences in the metabolic labeling experiments reflect a significant difference in DAG mass (*13*).

The possibility that transformation by *ras* has an effect on the phospholipase C step in PI turnover is suggested by the data in Fig. 2B. The ratio of the primary breakdown products of polyphosphorylated phosphatidylinositol to the precursor PIP_2 are presented. Since the water-soluble inositol phosphates and diacylglycerol both increase while PIP_2 decreases, the ratios are increased three- to fourfold in *ras*-transformed cells.

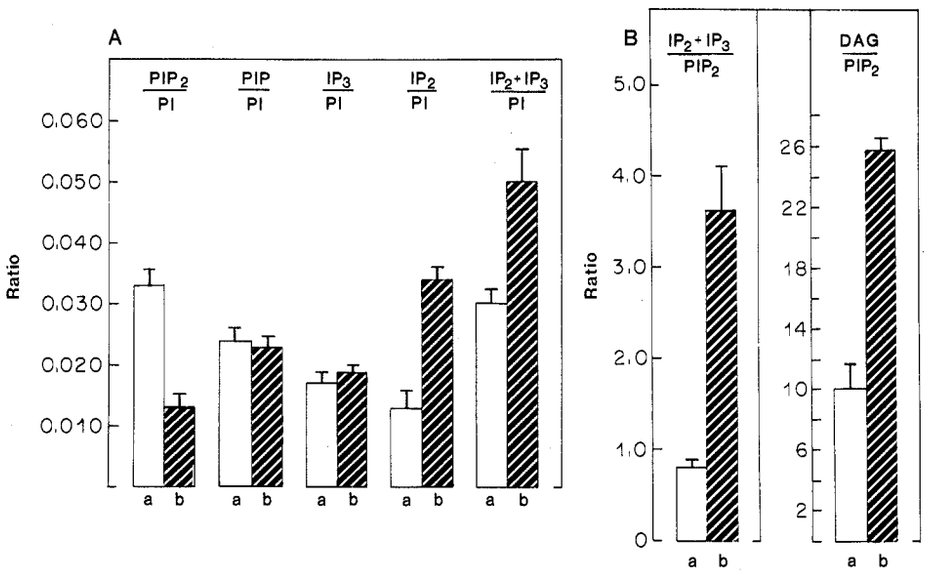

Fig. 2. Steady-state levels of inositol phosphates and polyphosphoinositides in NRK and kNRK cells. NRK and kNRK cells were seeded at 5×10^4 cells in 2 ml of medium into 33-mm dishes and used after 6 to 7 days and 4 to 5 days, respectively, at which time the cell densities were 0.25×10^7 to 0.3×10^7 cells per plate (a confluent density for the normal NRK cells). The cells were labeled 48 to 72 hours before harvesting with *myo*-[2-^{3}H]inositol (20 μCi per milliliter per 33-mm plate). The labeled medium was removed and the cells were rinsed three times with cold PBS to remove as much as possible of the free [^{3}H]inositol. One milliliter of cold 15 percent trichloroacetic acid was added and the dishes were kept on ice for 30 minutes to extract the water-soluble inositol phosphates. The inositol phosphates were separated by anion-exchange chromatography as described previously (*22*). The inositol lipids that remained on the dishes after removal of the inositol phosphates were extracted with 2 ml of chloroform-methanol–concentrated HCl (200:100:1 by volume). The dishes were rinsed with an additional 1 ml of the same solvent and the two extractions were combined. Phase split was induced by adding 1 ml of chloroform and 1 ml of 0.1*N* HCl. The sample was then mixed and briefly centrifuged to resolve the two phases. The lower (organic) phase was removed, dried under N_2, and analyzed by TLC (see Fig. 1). The average total incorporation was approximately 51,000 cpm per 10^6 cells for NRK cells and 59,000 cpm per 10^6 cells for kNRK cells. (a) NRK cells (confluent); (b) kNRK cells (high density). The numbers are averages of eight to nine determinations in two experiments. (B) Ratios of steady-state levels of polyphosphoinositide breakdown products to PIP_2 in NRK and kNRK cells. (a) Confluent NRK cells are compared to (b) kNRK cells at similar density. Labeling and lipid analysis were as described [Fig. 1 and (A)].

More information on the functions of DAG and PIP_2 in cell physiology is needed before the significance of the steady-state alterations observed here can be assessed. The ability of DAG to activate kinase C has been widely discussed, although the cellular concentration necessary for this stimulation is difficult to estimate (*14*). The magnitude of difference between the amounts of DAG in normal and *ras*-transformed cells (a 40 to 76 percent increase) (Fig. 1) appears to be small. However, mitogen stimulation of cells arrested at the G_0-G_1 boundary of the cell cycle typically gives only a transient 30 to 150 percent increase in DAG and appears to activate kinase C (*6*), indicating that the changes seen here may be significant for kinase C regulation. Activation of kinase C by elevation of cellular DAG may be a critical step in cell transformation by *ras*. It has been shown that mutated *ras* genes are only capable of transforming embryonic rat fibroblasts after the cells are immortalized by a class of oncogenes that includes the *myc* gene (*15*). Tumor-promoting phorbol esters

have also been shown to induce transformed foci in cells immortalized by *myc* (*16*). Since the phorbol esters are specific activators of kinase C, the results suggest that activation of kinase C is the critical step in this transformation. Elevated levels of DAG, IP_2, and IP_3 have recently been found associated with transformation of NIH 3T3 cells by polyoma virus (*17*). In this case PIP_2 amounts were increased as well, consistent with the finding that immunoprecipitated complexes containing polyoma middle T protein and $pp60^{c\text{-}src}$ contain PI kinase activity. Comparison with our results suggests that *ras* and polyoma may affect different steps in the PI turnover pathway.

Proteins homologous to mammalian ras have been studied in yeast and shown to be involved in the regulation of cyclic AMP; introduction of an activated yeast or mammalian *ras* gene stimulated an increase in cyclic AMP in yeast cells (*18*). However, recent attempts in several laboratories to demonstrate stimulation of adenylate cyclase by purified ras in mammalian cells have yielded negative results (*19*), consistent with earlier findings that cyclic AMP levels in *ras*-transformed cells are generally depressed or unchanged (*20*). The influence of *ras* on transmembrane signaling may indirectly affect adenyl cyclase and other enzymes involved in interconnected pathways. In the simplest model consistent with our results, the *ras* protein could directly activate a phospholipase C that hydrolyses PIP_2. These events would result in a decrease in PIP_2, an increase in inositol phosphates (IP_2 and IP_3), and DAG levels in *ras*-transformed cells as seen here. Other modes of action at other points in the PI cycle are also possible, such as inhibition of diglyceride lipase or diacylglycerol kinase, raising the DAG level (*21*). However, these inhibitions do not offer a simple explanation for the decrease in PIP_2 and the increase in IP_2.

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10. Nontransformed exponentially growing cells were compared to their transformed counterparts at low density (0.4×10^7 to 0.6×10^7 cells per 100-mm plate). Labeling and lipid analysis were as described in Fig. 1. Ratios of DAG to PIP_2 were 10.9 for NRK, 19.7 for kNRK, 18.3 for NIH 3T3, and 21.8 for a_1-1 cells (compare with results for confluent cells presented in Fig. 1). The DAG as a percent of total [^{3}H]glycerol-lipid was 5.8 (NRK), 6.1 (kNRK), 5.3 (NIH 3T3), and 7.4 (a_1-1); PIP_2 as a percent of total [^{3}H]glycerol-lipid was 0.53 (NRK), 0.31 (kNRK), 0.29 (NIH 3T3), and 0.34 (a_1-1). The numbers are averages of five to six determinations in two separate experiments. The SEM was ±0.006 for DAG measurements and ±0.0005 for PIP_2. In other experiments NIH 3T3 cells were investigated at low (0.4×10^7 to 0.6×10^7 cells per plate, intermediate (0.6×10^7 to 0.8×10^7 cells per plate, and maximum (confluent) (0.8×10^7 to 1.0×10^7 cells per plate) density. A decline in DAG/PIP_2 from 18.3 to 14.9 to 7.0 was observed as the density increased. PIP_2 as a percent of total [^{3}H]glycerol-lipid increased from 0.29 to 0.48 to 0.60 with increasing density; DAG as a percent of total [^{3}H]glycerol-lipid was 5.3 (low), 6.2 (intermediate), 4.2 (maximum density). The SEM was ±0.005 for DAG and ±0.0010 for PIP_2 for six determinations in two separate experiments. In other experiments in which cells were kept at confluent density for another 24 to 48 hours before harvesting ("post-confluent") a further decline in DAG to 2.5 to 3.5 percent of total lipid occurred in the nontransformed cells while DAG levels in the transformed cells did not decrease. These findings suggest that DAG and PIP_2 levels in *ras*-transformed cells are less affected by conditions of cell density than those of their nontransformed counterparts.
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12. Two isomers of IP_3 exist in some cells, inositol-1,4,5-trisphosphate and inositol-1,3,4-trisphosphate, the latter of which is more stable [R. F. Irvine *et al.*, *Biochem. J.* **223**, 237 (1984)]. The separation techniques that we used were not capable of resolving these isomers. The major isomer of PIP_2 is presumably phosphatidylinositol-4,5-bisphosphate although the separation technique employed would probably not resolve other possible isomers of this lipid, such as phosphatidylinositol-3,4-bisphosphate.
13. It is not clear why the increase in DAG measured by [^{3}H]glycerol labeling (40 to 76 percent) is greater than that estimated by DAG kinase (23 to 29 percent). It could reflect a small difference in utilization of extracellular glycerol in DAG synthesis between the normal and transformed cells. In an experiment in which metabolically labeled DAG was eluted from a TLC plate, deacylated, and analyzed for label in fatty acid, less than 5 percent of the label was present in this fraction in both the normal and transformed cells. This suggests that essentially all the label in DAG is in the glycerol backbone and that differences in labeling are not a consequence of changes in label incorporated into fatty acid.
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21. As we have emphasized, PIP_2 is likely to be a critical source of DAG; however, PI, PIP, and phosphatidic acid are also potential sources of DAG; the relative contributions of each to the cellular DAG level is unresolved in fibroblasts and is a difficult question to address. Also, DAG is rapidly converted to other metabolites in the cell and one of these reactions results in the release of arachidonic acid for prostaglandin synthesis. The by-product of this reaction, monoacylglycerol, was also elevated in the *ras*-transformed cells.
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