Promotion-resistant JB6 mouse epidermal cells exhibit defects in phosphatidylethanolamine synthesis and phorbol ester-induced phosphatidylcholine hydrolysis

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The tumour-promotion-sensitive (P^+) and -resistant (P^-) variants of mouse JB6 epidermis-derived cells have often been used to study the requirements for the tumour-promoting effect of PMA. As part of an effort to identify the defect(s) in JB6 P^- cells that might prevent the promoting effect of PMA, stimulation of phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) by PMA as well as the rate of phospholipid synthesis were compared in three P^+ variants, two P^- variants and a transformed variant of the JB6 cell line. PMA (5–100 nM) had significantly less stimulatory effect on PtdCho hydrolysis in P^- cells than in P^+ or transformed JB6 cells. The effects of PMA on PtdEtn hydrolysis in the P^+ and P^- cell lines were similar, whereas in transformed cells PMA had slightly less effect. Each JB6 cell line was found to contain similar amounts of PtdCho. In contrast, P^- cells

contained significantly less PtdEtn and a correspondingly higher level of ethanolamine phosphate compared with P^+ and transformed cells. P^- cells also secreted ethanolamine phosphate into the medium; this process was greatly enhanced by PMA. In the two P^- variants the synthesis of PtdEtn from [14 C]ethanolamine was reduced to various extents, whereas the rate of PtdCho synthesis was comparable in each JB6 cell line. The synthesis of PtdCho, but not PtdEtn, was greatly stimulated by PMA in both the P^+ and P^- clones. The results indicate that decreased synthesis/level of PtdEtn and suboptimal functioning of a PtdCho-specific PLD are common characteristics of the P^- JB6 cells examined so far. The observed alterations in phospholipid metabolism may play a role in the resistance of P^- cells to the tumour-promoting action of PMA.

INTRODUCTION

The process of carcinogenesis, a multistage event, starts with the irreversible initiation stage followed by the promotion and progression stages [1]. Experimentally, initiation can be accomplished by exposure to a subthreshold concentration of carcinogen, and the growth of initiated cells can be promoted by repeated applications of tumour promoters such as the protein kinase C (PKC) activator PMA. Since the tumour-promotion phase is a rate-limiting step in carcinogenesis (reviewed in refs. [2,3]), understanding the molecular mechanisms involved has important implications with regard to human cancer.

Variants of the spontaneously immortalized mouse JB6 epidermis-derived cell lines have often been used to study the mechanism of PMA action on tumour promotion. The P^+ variants are spontaneously initiated and they can be efficiently promoted by PMA to achieve neoplastic transformation, whereas the P^- variants are resistant to the tumour-promoting effect of PMA [4,5]. It is not clear which of the several mechanisms required for tumour promotion is non-functional in the P^- clones. So far, only one major causal difference between the P^+ and P^- variants has been found: PMA caused a significant increase in the activity of the transcriptional factor-activator protein-1 complex only in the P^+ and not in the P^- cells [6–8].

The major cellular target of PMA is the PKC system [9], and activated PKC is an important regulator of phospholipase D (PLD) [10]. In turn, increased activity of phosphatidylcholine (PtdCho)-specific PLD may be implicated in the cell-transformation process [11–13]. Since activation of PKC itself requires membrane phospholipids [9], the inability of PMA to promote P^-

cells may be due to aberrant expression and/or hydrolysis of specific cell membrane phospholipids. Because of these considerations, the aim of this work was to determine possible defects in phospholipid metabolism in the P^- variants. We report that P^- cells contain less phosphatidylethanolamine (PtdEtn) and hydrolyse significantly less PtdCho in response to PMA than P^+ or transformed JB6 cells.

MATERIALS AND METHODS

Materials

PMA, ethanolamine, choline and Dowex 50W (H^+ form) were purchased from Sigma (St. Louis, MO, U.S.A.). [14 C]Ethanolamine (55 mCi/mmol) and [14 C]choline chloride (50 mCi/mmol) were bought from Amersham (Arlington Heights, IL, U.S.A.); and tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY, U.S.A.).

Cell culture

The P^- clone-30 [14,15] and clone-25 [14] lines, the P^+ clone-41 [14,16], clone 41-5a [7] and clone-22 [14] lines, and the tumorigenic clone-RT101 line [17], each derived from JB6 cells, were kindly supplied by Nancy H. Colburn (National Institutes of Health, Frederick, MD, U.S.A.). Each clone was maintained in Eagle's minimum essential medium as indicated previously [18]. These clones were previously characterized with respect to their responses to PMA [6,7,14–21].

Abbreviations used: PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PKC, protein kinase C; P^+ and P^- cells, promotion-sensitive and promotion-insensitive cells respectively.

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Determination of PLD-mediated formation of [^{14}C]choline and [^{14}C]ethanolamine from prelabelled phospholipids

JB6 cells, grown in 12-well plates, were labelled with [^{14}C]choline (0.5 $\mu\text{Ci}/\text{ml}$) or [^{14}C]ethanolamine (0.35 $\mu\text{Ci}/\text{ml}$) for 60 h. At the end of the labelling period, the cell cultures were confluent. The cells were washed and then incubated for 3 h in fresh medium to complete incorporation of the remaining radioactive precursors into cellular phospholipids. Then cells were treated with PMA for 60 min in the presence of 20 mM choline or 2 mM ethanolamine, as appropriate. Incubations were terminated by scraping the cells into 2 ml of ice-cold methanol followed by rapid transfer of the methanol extract to 2 ml of chloroform. After phase separation, [^{14}C]choline, [^{14}C]ethanolamine and their metabolites were separated on Dowex 50W-packed columns (Bio-Rad Econo-columns; 1 ml bed volume) as described previously [22]. Briefly, the initial flow through (4.5 ml) along with a following 3.5 or 5 ml water wash contained glycerophosphoethanolamine or glycerophosphocholine respectively. Ethanolamine phosphate and choline phosphate were eluted by 15 and 20 ml of water respectively. Finally, ethanolamine and choline were eluted by 12 and 20 ml of 1 M HCl respectively. The metabolites of [^{14}C]ethanolamine and [^{14}C]choline were further identified by TLC, and phospholipids were separated as previously indicated [22].

Measurement of PtdCho and PtdEtn synthesis in JB6 Cells

Confluent JB6 cell cultures, grown in 12-well plates, were treated with PMA for up to 3 h in the presence of [*methyl*- ^{14}C]choline (1 $\mu\text{Ci}/\text{ml}$) or [2- ^{14}C]ethanolamine (0.3–1 $\mu\text{Ci}/\text{ml}$). Incubations were terminated as above, and PtdCho and PtdEtn were separated from other phospholipids on silica gel H plates by one-dimensional TLC using chloroform/methanol/28% ammonia (65:25:5, by vol.) as solvent. [^{14}C]Choline, [^{14}C]ethanolamine and their phosphorylated derivatives were separated on Dowex 50W (H^+ form) columns as above. ^{14}C -labelled CDP-choline and CDP-ethanolamine were separated from other products on silica gel G plates using the solvent system 0.5% NaCl/methanol/conc. NH_3 (50:50:5, by vol.). The phosphorus content of PtdCho and PtdEtn was determined as indicated previously [23].

RESULTS

The P^+ , P^- and transformed JB6 clones used in this work express the α -, δ -, ϵ - and ζ -PKC isoenzymes, but not the other PKC isoenzymes, at similar levels (N. H. Colburn, personal communication). These clones were chosen for this work because they were expected to simplify the interpretation of data by excluding differences in the PKC isoenzyme composition as a cause of the differential effects of PMA. Because of the possible role of PLD in signal transduction (reviewed in ref. [24]), we first examined the status of this enzyme in the JB6 cell lines. For the initial screening of possible differences in phospholipid hydrolysis, we used the RT101 transformed cell line as well as the P^+ clone-41 and P^- clone-30 cells.

PMA-induced formation of [^{14}C]choline and [^{14}C]ethanolamine from the corresponding prelabelled cellular phospholipids under conditions when further metabolism of these products is blocked (i.e. in the presence of high concentrations of unlabelled choline or ethanolamine [25,26]) occurs by a PLD-mediated mechanism. Using this method, we found that, although each of the three JB6 cell lines responded to the stimulatory actions of PMA on PLD activity, there were significant differences. Thus although PMA stimulated the hydrolysis of [^{14}C]PtdCho in the [^{14}C]choline-labelled RT101 and clone-41 cells to roughly similar

Figure 1 Stimulatory effects of PMA on PtdCho hydrolysis in JB6 cell lines

Confluent cultures of RT101 (●), clone-41 (▲) and clone-30 (■) cells were labelled with [^{14}C]choline as described in the Materials and methods section, followed by treatment with PMA (0–100 nM) for 30 min. Each point represents the mean $\pm$ S.E.M. from nine incubations performed in three groups with the same passage of cells on the same day. Similar differences in the effects of PMA were obtained in four other experiments performed in triplicate or quadruplicate. * Significantly ($P < 0.01$) different from values obtained with P^+ cells (Student's t test).

extents, in clone-30 cells it had much smaller effects on this PLD activity (Figure 1). For example, in the transformed, clone-41 and clone-30 cells, 5 nM PMA enhanced PtdCho hydrolysis about 4.6-, 3.6- and 2.9-fold respectively, and 100 nM PMA had 8.2-, 8.4-, and 4.8-fold stimulatory effects in the same order (Figure 1). Since the role of PKC in the induction of 'basal' (unstimulated) release of [^{14}C]choline is not known, it is more instructive to compare the total amounts of PtdCho hydrolysed in response to a maximally effective (100 nM) concentration of PMA. By this comparison, in the transformed, clone-41 and clone-30 cells, PMA induced the hydrolysis of about 8.8, 7.1 and 3.6% of the total cellular [^{14}C]PtdCho respectively over a 30 min incubation period (Figure 1). Similar differences in PMA-induced PtdCho hydrolysis were observed when the incubation period was extended to 60 min. In none of these cell lines did PMA stimulate the formation of [^{14}C]choline phosphate.

When the effects of 100 nM PMA were examined on PLD-mediated formation of phosphatidylethanol in [^{14}C]palmitic acid-labelled cells in the presence of 150 mM ethanol as described previously [26], we observed that PMA-stimulated clone-30 cells produced about 35–40% less phosphatidylethanol than PMA-stimulated clone-41 cells (results not shown). Since [^{14}C]palmitic acid also labels PtdEtn, it was of interest to compare PMA-induced PtdEtn hydrolysis in the three cell lines. As shown in Figure 2, the effects of PMA on [^{14}C]PtdEtn hydrolysis over a 30 min incubation period were again somewhat smaller in the transformed cells than in the clone-41 cells, but the effects of PMA in the clone-30 and clone-41 lines were very similar. During the same incubation period, the formation of [^{14}C]ethanolamine phosphate from the prelabelled PtdEtn pool (involving a phospholipase C activity) was practically undetectable in these cells both in the absence and presence of PMA.

Studies on PLD-mediated PtdCho hydrolysis were also extended to the P^+ clones 41-5a and 22, and the P^- clone 25 cells; in these cells 100 nM PMA induced the hydrolysis of about 9.3, 8.2 and 4.9% of the total cellular [^{14}C]PtdCho, in that order, over a 30 min incubation period. Thus the stimulatory effect of PMA in the two P^- clones were similarly depressed compared

Figure 2 Stimulatory effects of PMA on PtdEtn hydrolysis in JB6 cell lines

Confluent cultures of RT101 (●), clone-41 (▲) and clone-30 (■) cells were labelled with [^{14}C]ethanolamine as described in the Materials and methods section, followed by treatment with PMA (0–100 nM) for 30 min. Each point represents the mean $\pm$ S.E.M. from six incubations performed in two groups with the same passage of cells on the same day. Similar results were obtained in four other experiments performed in triplicate or quadruplicate.

Table 1 Cellular content of PtdCho and PtdEtn in various JB6 cell lines

(A) The phosphorus content of PtdCho and PtdEtn was determined in confluent cultures of JB6 cell lines grown in 60 mm dishes. Data are means $\pm$ S.E.M. from eight determinations performed in two groups with the same passage of cells in the same day. (B) Confluent cultures of JB6 cell lines were labelled with [^{14}C]choline (0.5 $\mu\text{Ci}/\text{ml}$) or [^{14}C]ethanolamine (0.5 $\mu\text{Ci}/\text{ml}$) for 60 h, and then the respective labelled phospholipids were determined. The data are means $\pm$ S.E.M. from six incubations in one representative experiment. Similar results were obtained in two other experiments each performed in quadruplicate. * Significantly ($P < 0.05$) different from the lowest value obtained with P^+ cells (Student's t test).

JB6 clone	(A) Phosphorus content (nmol/ 10^6 cells)		(B) $10^{-3} \times$ Radioactivity (d.p.m./well)	
	PtdCho	PtdEtn	[^{14}C]PtdCho	[^{14}C]PtdEtn
RT101	15.24 $\pm$ 1.38	9.61 $\pm$ 0.99	436 $\pm$ 28	544 $\pm$ 66
22	16.82 $\pm$ 1.80	9.92 $\pm$ 1.41	418 $\pm$ 33	487 $\pm$ 30
41	15.82 $\pm$ 1.19	9.11 $\pm$ 0.80	449 $\pm$ 40	452 $\pm$ 39
41-5a	15.22 $\pm$ 2.03	8.83 $\pm$ 1.06	397 $\pm$ 55	508 $\pm$ 22
30	16.62 $\pm$ 1.72	5.01 $\pm$ 0.72*	502 $\pm$ 47	263 $\pm$ 28*
25	16.10 $\pm$ 2.26	6.58 $\pm$ 0.91*	486 $\pm$ 41	371 $\pm$ 32*

with the three P^+ clones. On the other hand, PMA had similar effects on the hydrolysis of PtdEtn in the clone 41-5a, clone 22 and clone 25 cells (results not shown).

Since PtdEtn was shown to be an essential component of the PtdCho-hydrolysing PLD system *in vitro* [27], we considered the possibility that this PLD enzyme in P^- cells was activated by PMA less efficiently because these cells contain less PtdEtn. Indeed, whereas confluent cultures of all the six cell lines were found to contain similar amounts of PtdCho, both P^- clones contained significantly less PtdEtn than the other cell lines (Table 1). It should be added that reduction in PtdEtn content appeared to be greater in the clone-30 than in the clone-25 cells.

We also determined the relative levels of PtdCho and PtdEtn in cells labelled with [^{14}C]choline or [^{14}C]ethanolamine for 60 h to achieve near isotopic equilibrium. Although this method did not allow direct comparison of the cellular content of PtdCho with

Figure 3 Synthesis of PtdEtn in untreated and PMA-treated JB6 cell lines

Confluent cultures of RT101 (○, ●), clone-41 (△, ▲) and clone-30 (□, ■) cells were incubated in the presence of [^{14}C]ethanolamine (0.3 $\mu\text{Ci}/\text{ml}$) for up to 3 h in the absence (○, △, □) or presence (●, ▲, ■) of 100 nM PMA. The cellular contents of [^{14}C]PtdEtn (a), [^{14}C]ethanolamine phosphate (b), [^{14}C]ethanolamine (c) and [^{14}C]CDP-ethanolamine (d) were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. from six incubations in two experiments performed with 24 h difference with the same passage of cells. Similar results were obtained in a third experiment performed in triplicate.

that of PtdEtn (because of the different specific radioactivities of the precursor pools), it allowed simultaneous comparison of the relative amounts of the same phospholipid in the JB6 cell lines used here. As shown in Table 1, each JB6 cell line contained comparable amounts of [^{14}C]PtdCho, with P^- cells accumulating slightly more of this labelled phospholipid than the others. On the other hand, P^- cells clearly contained less [^{14}C]PtdEtn than the other cell lines. Again, reduction in the content of [^{14}C]PtdEtn was more pronounced in the clone-30 than in the clone-25 cells (Table 1).

The above observations suggested that P^- cells may be defective in PtdEtn, but not PtdCho, synthesis. To examine this possibility, we next directly compared the rate of PtdEtn synthesis in the transformed, clone-41 and clone-30 cells over a 3 h period. In transformed cells the rate of PtdEtn synthesis from [^{14}C]ethanolamine was about twice as high as that in clone-41 cells and 11–18-fold higher than in clone-30 cells (Figure 3a). In none of these cell lines had PMA any stimulatory effect on PtdEtn synthesis (Figure 3a). The low level of [^{14}C]PtdEtn formation in clone-30 cells was accompanied by a large accumulation of [^{14}C]ethanolamine phosphate, the level of which was apparently unaffected by PMA (Figure 3b). In contrast, the cellular content (reflecting both uptake and metabolism) of [^{14}C]ethanolamine in the three cell lines was comparable (Figure 3c), whereas the rate of synthesis of CDP-ethanolamine, the immediate precursor of PtdEtn, was lower in the P^- cells than in the two other cell lines (Figure 3d).

One reason for the defect in PtdEtn synthesis could be increased secretion of ethanolamine phosphate into the medium. To examine this possibility, the same three clones were incubated

Figure 4 Distribution of [^{14}C]ethanolamine phosphate in JB6 cell lines

Confluent cultures of RT101 ($\circ$, $\bullet$), clone-41 ($\triangle$, $\blacktriangle$) and clone-30 ($\square$, $\blacksquare$) cells were incubated for up to 18 h in the presence of [^{14}C]ethanolamine ($0.6 \mu\text{Ci/ml}$) in the absence ($\circ$, $\triangle$, $\square$) or presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of 100 nM PMA. [^{14}C]ethanolamine phosphate present in the incubation medium (a) or associated with the cells (b) was determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. from six incubations performed in two groups with the same passage of cells on the same day. Similar results were obtained in another experiment performed in quadruplicate.

Figure 5 Differential labelling of PtdEtn in various JB6 cell lines with [^{14}C]ethanolamine in the absence and presence of PMA

In the same experiments as described in Figure 4, time-dependent incorporation of [^{14}C]ethanolamine into PtdEtn in RT101 ($\circ$, $\bullet$), clone-41 ($\triangle$, $\blacktriangle$) and clone-30 ($\square$, $\blacksquare$) cells in the absence ($\circ$, $\triangle$, $\square$) or presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of 100 nM PMA was also determined. Each point represents the mean $\pm$ S.E.M. from six incubations. Similar results were obtained in another experiment performed in quadruplicate.

with [^{14}C]ethanolamine for longer (6–18 h) incubation times. As shown in Figure 4(a), there was a relatively large efflux of [^{14}C]ethanolamine phosphate from the clone-30 cells, which was further enhanced by 100 nM PMA (2.6-, 5.25- and 5.6-fold after treatment for 6, 12 and 18 h respectively). In some experiments, the basal rate of [^{14}C]ethanolamine phosphate release from clone-30 cells could account for 30–40 % of the total [^{14}C]ethanolamine phosphate formed. Efflux of labelled ethanolamine phosphate from RT101 and clone-41 cells was less extensive (Figure 4a). Despite the high rate of secretion, the cellular content of

Figure 6 Synthesis of PtdCho in untreated and PMA-treated JB6 cell lines

RT101 ($\circ$, $\bullet$), clone-41 ($\triangle$, $\blacktriangle$) and clone-30 ($\square$, $\blacksquare$) cells were incubated in the presence of [^{14}C]choline ($1 \mu\text{Ci/ml}$) for up to 3 h in the absence ($\circ$, $\triangle$, $\square$) or presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of 100 nM PMA. The cellular contents of [^{14}C]PtdCho (a), [^{14}C]choline phosphate (b), [^{14}C]choline (c) and [^{14}C]CDP-choline (d) were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. from six incubations in two experiments performed with 24 h difference with the same passage of cells. Similar results were obtained in a third experiment performed in triplicate.

[^{14}C]ethanolamine phosphate in the clone-30 cell population was also much higher than in the two other clones (Figure 4b). PMA slightly lowered the [^{14}C]ethanolamine phosphate content in the P $^+$ cells after incubations for 12 and 18 h, whereas it increased the cellular content of this compound after 6 h treatment in the other two cell lines (Figure 4b). Again, the rate of labelling of PtdEtn by [^{14}C]ethanolamine in clone-30 cells was only about 7–22 % of that observed in the RT101 and clone-41 clones at different time points (Figure 5). Interestingly, the sum of the ^{14}C radioactivity missing from the cell-associated ethanolamine phosphate pool (Figure 4b) and the PtdEtn pool (Figure 5) in PMA-treated cells is much less than the amount of [^{14}C]ethanolamine phosphate secreted from cells into the incubation medium in response to PMA (Figure 4a). This suggests that PMA not only stimulated the secretion of ethanolamine phosphate, but also stimulated the formation of this compound possibly through the stimulation of activity/expression of ethanolamine kinase. This aspect of the PMA effect is not pursued further here.

The RT101, clone-41 and clone-30 clones synthesized comparable amounts of [^{14}C]PtdCho from [^{14}C]choline (Figure 6a). Also, in each cell line, PMA, a potent stimulator of PtdCho synthesis in other cells [10], had similar stimulatory effects on PtdCho synthesis (Figure 6a). Again, the transformed cells synthesized the most [^{14}C]choline phosphate (Figure 6b). In each clone, PMA decreased the level of [^{14}C]choline phosphate reflecting increased utilization of this intermediate for PtdCho

Figure 7 Comparison of PtdEtn synthesis in P⁺ and P⁻ JB6 cell lines

Confluent cultures of clone-22 (●), clone-41-5a (▲), clone-25 (■) and clone-30 (◆) cells were incubated in the presence of [¹⁴C]ethanolamine (1 μ Ci/ml) for up to 6 h. Each point represents the mean $\pm$ S.E.M. from six incubations in two experiments performed with 24 h difference with the same passage of cells. Similar results were obtained in a third experiment performed in quadruplicate.

synthesis (Figure 6b). Initially, PMA also stimulated cellular uptake of [¹⁴C]choline in each JB6 cell line; this effect of PMA was actually somewhat higher in the clone-30 cells than in the two other cell lines (Figure 6c). Finally, in each cell line PMA also slightly stimulated the formation of CDP-choline; the effects of PMA and the basal rate of CDP-choline formation were only slightly different in the three JB6 cell lines (Figure 6d). In other experiments, PMA-stimulated PtdCho synthesis was also examined in the clone-41-5a, clone-22 and clone-25 cells; after treatment for 3 h, 100 nM PMA stimulated PtdCho synthesis in these cell lines 3.1–3.4-fold.

Since in clone-30 cells the rate of PtdEtn synthesis was clearly reduced, we next extended these studies to the P⁻ clone-25 and

the P⁺ clone 41-5a and clone-22 cells to see if reduced synthesis of PtdEtn was a common characteristic of P⁻ cells. For a comparison, clone-30 cells were also included in these series of experiments. As shown in Figure 7, clone-25 cells clearly synthesized less [¹⁴C]PtdEtn than clone-41-5a or clone-22 cells over a 6 h incubation period. On the other hand, the rate of [¹⁴C]PtdEtn synthesis in clone-25 cells was higher than in clone-30 cells (Figure 7). These observations are in line with data (presented in Table 1) showing that the content of PtdEtn tends to be higher in clone-25 than in clone-30 cells.

The P⁻ clone-25 and clone-30 cell cultures contained much higher levels of [¹⁴C]ethanolamine phosphate than the P⁺ clone 41-5a and clone-22 cells (Figure 8). In these experiments about 40 % of the total [¹⁴C]ethanolamine phosphate produced by clone-30 cells was present in the incubation medium, whereas about 15 % of the total [¹⁴C]ethanolamine phosphate was secreted from the clone-25 cells (Figure 8). In another experiment we found that PMA had similar stimulatory effects on the efflux of ethanolamine phosphate in the clone-25 and clone-30 cell populations (results not shown). Whereas different passages of P⁻ cells showed some variation in the amount of ethanolamine phosphate secreted, clone-25 and clone-30 cells invariably secreted at least two to four times more [¹⁴C]ethanolamine phosphate than the P⁺ variants.

DISCUSSION

We have shown that P⁻ cells contain less PtdEtn than P⁺ and transformed JB6 cells, reflecting decreased rates of phospholipid synthesis. The lower rate of PtdEtn synthesis appears to be due to decreased synthesis of CDP-ethanolamine by the respective cytidyltransferase, because there was a reduction only in the content of CDP-ethanolamine and not in the uptake and/or phosphorylation of [¹⁴C]ethanolamine. It remains to be determined whether decreased *de novo* synthesis of PtdEtn was accompanied, at least in clone-30 cells, by a compensatory increase in its formation by decarboxylation of phosphatidylserine. Some increase in the synthesis of PtdEtn by this latter mechanism may be expected in the clone-30 variant, because in these cells the reduction in total PtdEtn (represented by the phosphate content) was less extensive than the decrease in the labelling of PtdEtn by [¹⁴C]ethanolamine. In contrast, in clone-25 cells the less drastic reduction in the rate of PtdEtn synthesis may fully account for the observed decrease in the cellular content of this phospholipid.

Decreased synthesis of PtdEtn in P⁻ cells was associated both by large increases in the cellular level of ethanolamine phosphate and, particularly in clone-30 cells, by an extensive release of ethanolamine phosphate into the medium. At longer incubation times PMA appeared to stimulate both the synthesis of ethanolamine phosphate by ethanolamine kinase and the release of ethanolamine phosphate into the medium. Since PMA had only a relatively small inhibitory effect on the formation of PtdEtn from [¹⁴C]ethanolamine, the large amount of [¹⁴C]ethanolamine phosphate released from the cells in the presence of PMA may derive from a cellular pool that does not have a major role in PtdEtn synthesis. On the other hand, untreated P⁻ cells may release a disproportionately high amount of ethanolamine phosphate from a cellular pool which is destined to supply precursors for PtdEtn synthesis, resulting thereby in a lower rate of phospholipid synthesis. The finding that in the two P⁻ clones the rate of ethanolamine phosphate release is inversely related to the rate of PtdEtn synthesis supports this possibility. Further experiments are required to determine the possible causal

Figure 8 Comparison of formation and release of [¹⁴C]ethanolamine phosphate in P⁺ and P⁻ JB6 cell cultures

In the same experiments as described in Figure 7, the cellular content of [¹⁴C]ethanolamine phosphate (a) and the amount released (b) was determined in clone-22 (●), clone-41-5a (▲), clone-25 (■) and clone-30 (◆) cell cultures over a 6 h incubation period. Each point represents the mean $\pm$ S.E.M. from six incubations. Similar results were obtained in a third experiment performed in quadruplicate.

relationship between increased ethanolamine phosphate release and decreased PtdEtn synthesis.

Previously, we observed that in NIH 3T3 fibroblasts ethanolamine was a co-mitogen when insulin was also present [28]. Since a large portion of ethanolamine is phosphorylated in these cells, ethanolamine phosphate could mediate the mitogenic effect of ethanolamine. However, JB6 P⁻ and JB6 P⁺ cells grow at about the same rate (doubling time is 22 h and 18 h respectively), suggesting that in these cell cultures the intracellular or extracellular level of ethanolamine phosphate does not regulate cell growth. Consistent with this possibility, addition of ethanolamine phosphate to P⁺ or P⁻ cells failed to exert any, stimulatory or inhibitory, mitogenic effects (results not shown). Also, in P⁺ cells, addition of 1 or 2 mM ethanolamine phosphate failed to prevent the tumour-promoting effect of PMA (results not shown). Thus the evidence collected so far does not indicate an inhibition of PMA-induced promotion by excreted ethanolamine phosphate.

Recently, PtdEtn was shown to be required for the optimal activity of a PtdCho-specific PLD activity [27] *in vitro*. In another study, cultivation of ethanolamine-requiring rat mammary cells (64-24 line) in the absence of ethanolamine resulted in both decreased cellular levels of PtdEtn and a reduction of the phorbol ester effect on PtdCho hydrolysis [29]. On the basis of these previous data, it is tempting to assume that in P⁻ cells there is a causal relationship between the reduced cellular content of PtdEtn and the reduced effect of PMA on PtdCho hydrolysis. This possibility remains to be proven. It should be noted that, in the PtdEtn-deficient mammary cell line, the effect of phorbol ester on PLD-mediated PtdEtn hydrolysis was also impaired [29]. In contrast, we found that the effects of PMA on PtdEtn hydrolysis in PtdEtn-deficient P⁻ cells were not inhibited. The exact reason for these differences is at present not known.

There are many other cellular mechanisms that may be affected by the reduced cellular level of PtdEtn observed in the P⁻ cell population. Several laboratories have shown that the presence of PtdEtn in membranes is required for optimal membrane binding/activation of PKC [30–35]. Thus a decrease in the membrane level of PtdEtn may affect critically important functions of PKC required for tumour promotion. PtdEtn is also required for several membrane functions, including promotion of membrane fusion (reviewed in refs [36–38]) and membrane binding and activation of numerous proteins [32,39–42]. It remains to be seen whether any of the cellular events that are potentially affected by PtdEtn play a role in the resistance of P⁻ cells to the tumour-promoting effect of PMA. It should be added here that, in rat mammary cells, a decreased level of PtdEtn (resulting from the absence of ethanolamine in the incubation medium) was associated with decreased growth (cited in ref. [29]), and deficiency in PtdEtn did not appear to significantly affect the growth of P⁻ cells. Interestingly, untransformed human mammary epithelial cells, but not transformed cells isolated from breast tumours, also require ethanolamine for growth [43]. From this limited amount of data it appears that cells of different origin respond differently to the absence of ethanolamine in the incubation medium and/or to decreased cellular levels of PtdEtn. The reason for this apparently different requirement of various cell types for PtdEtn is not known.

Activation of a PtdCho-specific PLD by Ha-ras- or v-Fps-induced cell transformation [11,12] or by v-Src [13] implicates this hydrolytic enzyme in cell transformation. Incidentally, phorbol ester-activated PKC is a major regulator of PLD [10,24], and, in the promoter-resistant P⁻ cells, PMA has considerably smaller effects on PtdCho hydrolysis than in the P⁺ cells. All these facts suggest that full activation of a PtdCho-specific PLD, but not a PtdEtn-specific PLD, may be a required event for the

tumour-promoting effect of PMA. This possibility is at present under investigation in our laboratory.

In summary, here we have shown that P⁻ cells contain less PtdEtn than their P⁺ counterparts, most probably because of decreased synthesis of this phospholipid. In the P⁻ cells, PMA is a less effective activator of the PtdCho-specific PLD. Further experiments are required to determine whether these deficiencies alone or in combination play any role in the resistance of P⁻ cells to the promoting effect of PMA. As a more immediate application, P⁻ cells may serve as experimental cellular model systems to study the effects of PtdEtn deficiency in cell membrane function.

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