Phorbol ester and bryostatin differentially regulate the hydrolysis of phosphatidylethanolamine in Ha-*ras*- and *raf*-oncogene-transformed NIH 3T3 cells

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Previously it was reported that transformation of NIH 3T3 fibroblast by the Ha-*ras*, *v-src*, *v-fms*, and A-*raf* oncogenes decreased the stimulatory effects of phorbol 12-myristate 13-acetate (PMA; 'TPA'), an activator of protein kinase C (PKC), on the phosphorylation of an endogenous 80 kDa substrate and on ⁸⁶Rb uptake [Wolfman, Wingrove, Blackshear & Macara (1987) J. Biol. Chem. **262**, 16546–16552], as well as on sphingomyelin synthesis [Kiss, Rapp & Anderson (1988) FEBS Lett. **240**, 221–226]. Here, we investigated how transformation affects the PMA-stimulated hydrolysis of phosphatidylethanolamine (PtdEtn), a recently characterized mechanism which may contribute to the generation of the second messengers phosphatidic acid and 1,2-diacylglycerol. The effects of PMA were compared with those of bryostatin, a non-tumour-promoter activator of PKC. Transformation of NIH 3T3 cells with Ha-*ras*, *v-raf*, or A-*raf* enhanced the stimulatory effect of PMA on the phospholipase D-mediated hydrolysis of PtdEtn. On the other hand, the effects of bryostatin on PtdEtn hydrolysis were only slightly increased, if at all, in cells transformed with these oncogenes. In crude membrane preparations isolated from these transformed cells, PMA, but not bryostatin, enhanced the combined stimulatory effects of ATP and the GTP analogue guanosine 5'-[γ-thio]triphosphate on phospholipase D-mediated PtdEtn hydrolysis. The PKC inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine inhibited the stimulatory effect of PMA only in intact cells. These results indicate that transformation of cells by certain oncogenes differentially affects phospholipase D-mediated hydrolysis of PtdEtn induced by PMA and bryostatin, suggesting that the action of PMA might involve two different mechanisms.

INTRODUCTION

Protein kinase C (PKC) is a phospholipid- and Ca²⁺-dependent serine/threonine-specific kinase [1,2] that is also markedly activated by the potent tumour promoter phorbol 12-myristate 13-acetate (PMA; 'TPA') [3]. This enzyme appears to be of fundamental importance in the regulation of normal cell growth [1] and also may play an important role in oncogene-induced malignant transformation of cells [4–7]. This view is supported by a number of observations. For example, the steady-state level of 1,2-diacylglycerol (1,2-DAG), a generally accepted natural activator of PKC [1,2], has been found to be elevated in a number of transformed cell populations [6,8–11]. Further, transfection of NIH 3T3 fibroblasts with PKC-1 cDNA enhances tumorigenicity of these cells [12,13], and cells that overproduce PKC have been shown to be more susceptible to transformation by Ha-*ras* oncogenes [14]. More recently, on the basis of studies involving the expression of a mutant form of α-PKC in Balb/c 3T3 fibroblasts, Megidish & Mazurek [15] suggested that altered PKC genes themselves may serve as oncogenes.

Interestingly, transformation of NIH 3T3 cells by the Ha-*ras*, *v-fms* and *v-src* oncogenes enhanced the cellular levels of 1,2-DAG and also caused a decrease in the total cellular content of PKC [9,11]. Perhaps owing to a partial down-regulation of PKC, transformation by these oncogenes caused an attenuation of cellular responsiveness to PMA, including PMA effects on the phosphorylation of the PKC substrate 80 kDa protein and on ⁸⁶Rb⁺ uptake [9,11]. Recently, Slivka *et al.* [16] found that in Ha-

ras-transformed Madin–Darby canine kidney cells the PMA-stimulated release of arachidonic acid and subsequent production of prostaglandin E₂ were also decreased. Confirming the emerging general scheme that transformed cells demonstrate an attenuated response to phorbol ester, we previously found that transformation of NIH 3T3 cells by A-*raf* diminished the stimulatory effect of PMA on sphingomyelin synthesis [17].

In the present study we investigated the effect of transformation of NIH 3T3 cells by Ha-*ras* and *raf* on PMA- and bryostatin-stimulated hydrolysis of phosphatidylethanolamine (PtdEtn). The ability of PMA to enhance phospholipid hydrolysis is a widespread phenomenon [18] and references cited therein and has been shown to occur in NIH 3T3 cells as well [19,20]. We found that transformation by Ha-*ras*, *v-raf* or A-*raf* elicited a GTP-dependent stimulatory effect of TPA on PtdEtn hydrolysis. These transformed cells failed to show a similar GTP-dependent enhancement of the stimulatory effect of bryostatin, a non-tumour-promoter activator of PKC [21–24].

MATERIALS AND METHODS

Materials

PMA, PtdEtn, phosphatidylcholine (PtdCho), Dowex-50-WH⁺, ATP, guanosine 5'-[γ-thio]triphosphate (GTP[S]), phospholipase C (type IX from *Clostridium perfringens*), phospholipase A₂ (from bee venom) and phospholipase B (from *Vibrio* species) were purchased from Sigma; [2-¹⁴C]ethanolamine (20 mCi/mmol)

Abbreviations used: PKC, protein kinase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; 1,2-DAG, *sn*-1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; GTP[S], guanosine 5'-[γ-thio]triphosphate.

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Fig. 1. Concentration-dependent effect of PMA on the degradation of [^{14}C]PtdEtn in untransformed and transformed NIH 3T3 cells

Untransformed and transformed cells prelabelled with [^{14}C]ethanolamine for 48 h were incubated in the presence of the indicated concentrations of PMA for 90 min, followed by the fractionation of water-soluble degradation products of [^{14}C]PtdEtn by ion-exchange chromatography. Each point represents the mean $\pm$ S.E.M. of three determinations. Similar results were obtained in two other experiments.

Fig. 2. Concentration-dependent effect of bryostatin on the degradation of [^{14}C]PtdEtn in untransformed and transformed NIH 3T3 cells

In an experiment similar to that described in the legend to Fig. 1, [^{14}C]ethanolamine prelabelled untransformed and transformed cells were treated with the indicated concentrations of bryostatin for 90 min. Each point represents the mean $\pm$ S.E.M. of three determinations. Similar results were obtained in two other experiments.

Cell culture

NIH 3T3 clone 7 fibroblasts were transformed with murine sarcoma virus carrying either the originally identified *v-raf* (NIH 3T3/F4 (*v-raf*); [26]) or human *A-raf* (NIH 3T3/6A-*raf* MSV; [27]). NIH 3T3 cells transfected with Harvey-murine-sarcoma virus [28] were obtained from Dr. Douglas R. Lowy (National Cancer Institute, Bethesda, MD, U.S.A.). Cells were cultured continuously in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (v/v) fetal-calf serum, penicillin/streptomycin (50 units/ml and 50 $\mu\text{g}/\text{ml}$ respectively) and glutamine (2 mM). Cells used in the experiments were seeded [$(1-2) \times 10^5/\text{dish}$] in 100-mm-diameter plastic dishes (Costar), and growing (50–70% confluent) cell populations were harvested after 2 days in culture.

Measurement of phospholipid hydrolysis in cells prelabelled with ^{14}C -labelled precursors

Cells were grown in the presence of [^{14}C]ethanolamine (0.2 $\mu\text{Ci}/\text{ml}$) for 48 h to about 70–80% confluency. After this incubation period, cells were washed twice in fresh medium, incubated in serum-containing medium for an additional 2.5 h to diminish the cell concentration of labelled precursors, and then washed again. Cells were harvested by scraping from the dish. The harvested cells, usually pooled from three to five dishes (150 cm^2), were again washed with fresh medium. Then, 0.3 ml aliquots [$(1.0-1.6) \times 10^6$ cells/ml] were incubated (final vol. 0.32 ml) at 37 $^\circ\text{C}$ in the presence of 2 mM unlabelled ethanolamine along with other agents as specified. Incubations were terminated by the addition of 4 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 3 ml of water. Released radioactivity was determined either before or after fractionation of the water-soluble products of phospholipid hydrolysis. Fractionation of ethanolamine metabolites was performed on columns packed with Dowex-resin-50-WH $^+$ (Bio-Rad Econo-columns; 1 ml bed volume) as described by Cook & Wakelam [29] for choline derivatives, with minor modifications. The initial flow-through (5 ml), along with a following 3 ml wash, contained glycerophosphoethanolamine. Ethanolamine phosphate and ethanolamine were eluted with 20 ml of water and 15 ml of 1 M-HCl respectively. For the preparation of appropriate ^{14}C -labelled standards, the prelabelled cells were treated with phospholipase A_2 (1 unit/ml) + phospholipase B (1 unit/ml) and phospholipase C (1 unit/ml) respectively for 60 min, and extracts were prepared as described above. For the determination of the elution profiles of standards (determined in each experiment), 2 ml fractions were collected. The metabolites of [^{14}C]ethanolamine present in the peak fractions were further identified by t.l.c. [19].

Measurement of phospholipid hydrolysis in membranes isolated from prelabelled cells

Cells were labelled with [^{14}C]ethanolamine as described above. Washed cells were collected by scraping and disrupted by Dounce homogenization (A pestle; 100 strokes) in 5 ml of ice-cold 20 mM-Tris/HCl, pH 7.5. Remaining intact cells were removed by centrifuging the homogenates at 1000 g for 5 min. The resulting supernatants were further centrifuged at 20000 g for 10 min at 4 $^\circ\text{C}$; the membrane pellets were resuspended in Tris buffer and used immediately. The assay mixture (0.1 ml) for the determination of phospholipid hydrolysis contained 40 mM-Tris/HCl, pH 7.5, 5 mM-MgCl $_2$, 1 mM-ethanolamine, 1 mM-ethanolamine phosphate, 0.2 mM-CDP-ethanolamine, 80–120 μg of membrane protein, and other additions as indicated. Incubations were carried out at 37 $^\circ\text{C}$ for 20 min in a shaking water bath, and were terminated by the addition of 2 ml of chloroform/methanol (1:1,

was from Amersham Corp.; tissue-culture reagents were from GIBCO. Bryostatin 1 was purified from the marine animal *Bugula neritina* [25].

Table 1. Effects of ATP, GTP[S], PMA and bryostatin on the formation of ethanolamine in membranes prepared from [¹⁴C]ethanolamine-prelabelled untransformed and Ha-*ras*-transformed NIH 3T3 cells

Cells were prelabelled with [¹⁴C]ethanolamine for 48 h, and crude membrane fractions were isolated and then incubated for 20 min. [¹⁴C]Ethanolamine was separated by ion-exchange chromatography. The concentrations of ATP, GTP[S], PMA and bryostatin were 0.5 mM, 2 μ M, 100 nM and 100 nM respectively. The ¹⁴C content of PtdEtn in 3T3 and 3T3/Ha-*ras* cells was 189 000 and 161 000 d.p.m./100 μ g of protein respectively. Results are means $\pm$ S.E.M. for three incubations performed with the same membrane preparation. Similar results were obtained in two other experiments. *Significantly ($P < 0.05$) different from the value obtained in the presence of ATP + GTP[S].

Addition	Formation of [¹⁴ C]ethanolamine (d.p.m./20 min per 100 μ g of protein)					
	3T3 (clone-7)			3T3/Ha- <i>ras</i>		
	No activator	PMA	Bryostatin	No activator	PMA	Bryostatin
None	2110 $\pm$ 210	2210 $\pm$ 130	2190 $\pm$ 110	1730 $\pm$ 170	1810 $\pm$ 100	1670 $\pm$ 130
ATP	5650 $\pm$ 280	5680 $\pm$ 480	5340 $\pm$ 200	5000 $\pm$ 230	5970 $\pm$ 180	5010 $\pm$ 150
GTP[S]	2990 $\pm$ 170	3390 $\pm$ 180	3030 $\pm$ 160	2890 $\pm$ 130	3480 $\pm$ 250	3050 $\pm$ 120
ATP + GTP[S]	8150 $\pm$ 80	8740 $\pm$ 160	7870 $\pm$ 180	7690 $\pm$ 380	9380 $\pm$ 90*	7810 $\pm$ 120

Table 2. Effects of ATP, GTP[S], PMA, and bryostatin on the formation of ethanolamine in membranes prepared from [¹⁴C]ethanolamine-prelabelled cells transformed by *v-raf* or *A-raf*

For experimental details, see the legend to Table 1. The concentration of ATP, GTP[S], PMA and bryostatin was 0.5 mM, 2 μ M, 100 nM and 100 nM respectively. The ¹⁴C content of PtdEtn in 3T3/*v-raf* and 3T3/*A-raf* cells was 231 000 and 138 000 d.p.m./100 μ g of protein respectively. Results are the mean $\pm$ S.E.M. of three incubations performed with the same membrane preparation. Similar results were obtained in two other experiments.

*Significantly ($P < 0.05$) different from the value obtained in the presence of ATP + GTP[S].

Addition	Formation of [¹⁴ C]ethanolamine (d.p.m./20 min per 100 μ g of protein)					
	3T3/ <i>v-raf</i>			3T3/ <i>A-raf</i>		
	No activator	PMA	Bryostatin	No activator	PMA	Bryostatin
None	2680 $\pm$ 280	2830 $\pm$ 120	2540 $\pm$ 300	1590 $\pm$ 100	1490 $\pm$ 220	1660 $\pm$ 270
ATP	5830 $\pm$ 310	6560 $\pm$ 80	5970 $\pm$ 220	5150 $\pm$ 180	5840 $\pm$ 180	5340 $\pm$ 440
GTP[S]	5140 $\pm$ 140	5086 $\pm$ 380	5290 $\pm$ 160	2980 $\pm$ 40	3350 $\pm$ 250	3190 $\pm$ 160
ATP + GTP[S]	11 300 $\pm$ 130	13 370 $\pm$ 310*	11 730 $\pm$ 170	8520 $\pm$ 180	10 480 $\pm$ 140*	8680 $\pm$ 210

v/v). Radioactivity was determined either in the water phase, as described above, or in the chloroform phase, as described previously [30,31].

RESULTS AND DISCUSSION

The aim of the present study was to determine the effects of PMA and bryostatin, two similarly potent activators of PKC [22,24], on the hydrolysis of PtdEtn in untransformed and transformed NIH 3T3 cells. Previous data indicated that, in NIH 3T3 cells, PMA-stimulated hydrolysis of PtdEtn, similar to that of PtdCho, was mediated by a phospholipase D activity [19]. At lower (2–5 nM) concentrations, PMA had either no effect or a slightly reduced effect on [¹⁴C]PtdEtn hydrolysis in the transformed cells (Fig. 1). In contrast, transformation with these oncogenes potentiated the stimulatory effects of higher concentrations of PMA on PtdEtn hydrolysis (Fig. 1). In the Ha-*ras*- and *A-raf*-transformed cells, the effect of bryostatin was not significantly different from that observed in control cells, and transformation with *v-raf* caused less potentiation of the bryostatin effect when compared with PMA (Fig. 2). Thus transformation by Ha-*ras*, *A-raf* and *v-raf* preferentially enhanced the stimulatory effect of PMA on PtdEtn hydrolysis.

We have reported that ATP and GTP synergistically stimulated

PtdEtn hydrolysis in crude membranes isolated from NIH 3T3 cells [20]. Thus we decided to investigate the possible inter-relationship between the effects of PMA and these nucleotides on PtdEtn hydrolysis in membranes isolated from untransformed and transformed cells prelabelled with [¹⁴C]ethanolamine. Special care was taken not to use chelators during membrane isolation, which would likely result in the removal of plasma-membrane-bound PKC [32–35]. For the same reason, we avoided extensive washing of membranes. As shown in Table 1, ATP and GTP[S] had similar synergistic effects on PtdEtn hydrolysis in membranes isolated from either untransformed or Ha-*ras*-transformed cells. In membranes isolated from the *ras*-transformed cells, PMA potentiated the combined stimulatory effects of nucleotides on PtdEtn hydrolysis, whereas PMA had no statistically significant effect in membranes from untransformed cells (Table 1). On the other hand, PMA also potentiated the effects of nucleotides on PtdEtn hydrolysis in membranes isolated from *v-raf*- or *A-raf*-transformed cells (Table 2). In contrast with PMA, bryostatin failed to stimulate PtdEtn hydrolysis in any of these membrane preparations (Tables 1 and 2).

Recently, several laboratories [36–38] reported that the potent PKC inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) [39] failed to inhibit the effect of PMA on phospholipase D-mediated hydrolysis of PtdCho. In the present studies, even high

Table 3. Inhibition by H7 of the stimulatory effects of PMA and bryostatin on PtdEtn hydrolysis in untransformed and Ha-*ras*-transformed cells

Untransformed and transformed cells prelabelled with [14 C]ethanolamine for 48 h were incubated for 90 min in the absence or presence of 100 nM-PMA, 100 nM-bryostatin and/or 200 μ M-H7. [14 C]Ethanolamine was separated by ion-exchange chromatography. The 14 C content of PtdEtn in 3T3 (clone-7) and 3T3/Ha-*ras* cells was 364000 and 416000 d.p.m./ 10^6 cells respectively. Similar results were obtained in another experiment.

Addition	Cells ... H7 ...	Formation of [14 C]ethanolamine (d.p.m./90 min per 10^6 cells)			
		3T3 (clone-7)		3T3/Ha- <i>ras</i>	
		—	+	—	+
None		4210 $\pm$ 230	4180 $\pm$ 330	3690 $\pm$ 210	4150 $\pm$ 280
PMA		22970 $\pm$ 170	12430 $\pm$ 190	27810 $\pm$ 610	14380 $\pm$ 250
Bryostatin		18790 $\pm$ 200	11200 $\pm$ 150	16910 $\pm$ 440	5690 $\pm$ 170

(200 μ M) concentrations of H7 failed to inhibit significantly PMA-stimulated (nucleotide-dependent) hydrolysis of PtdEtn in membranes (results not shown). In contrast, H7 inhibited, somewhat differentially, the effects of PMA and bryostatin on PtdEtn hydrolysis in the untransformed and *ras*-transformed cells. In the untransformed cells, PMA and bryostatin had similar stimulatory effects on PtdEtn hydrolysis, and H7 similarly inhibited their effects (Table 3). In the *ras*-transformed cells, PMA was more potent than bryostatin in stimulating PtdEtn hydrolysis, and the effect of PMA was also less efficiently inhibited by H7 (Table 3). Similar to the *v-raf*-transformed cells, the effect of PMA on PtdEtn hydrolysis was also less effectively inhibited by H7 (results not shown).

In conclusion, these studies suggest that, in transformed NIH 3T3 cells, PMA, but not bryostatin, may regulate PtdEtn hydrolysis through two different mechanisms, one of them being more sensitive to the inhibitory effect of H7 than the other. On the basis of the observed differences between the actions of PMA and bryostatin, tumour-promoting and non-tumour-promoting activators of PKC respectively, we cannot exclude the possibility that the action of PMA on PtdEtn hydrolysis involved, at least in transformed NIH 3T3 cells, both PKC-dependent and -independent mechanisms. This possibility is supported by observations indicating that transformation of NIH 3T3 cells usually leads to attenuated response to PMA [9,11,16,17]. Alternatively, the action of PMA may involve two different isoforms of PKC, one of them acting in a nucleotide-dependent manner and being insensitive to bryostatin and H7. Clearly, bryostatin, which appears to mimic only one action of PMA on PtdEtn hydrolysis, will be a valuable tool in identifying the mechanisms involved in the regulation of phospholipase D activity in transformed cells.

While this manuscript was in preparation, Meulen & Haslam [38] reported that PMA treatment of intact rabbit platelets further enhanced the stimulatory effect of GTP[S] on phospholipase D-mediated hydrolysis of PtdCho in isolated platelet membranes. Furthermore, inhibitors of PKC, including H7 and staurosporine, were relatively ineffective inhibitors of PMA action [38]. These data led Meulen & Haslam [38] to suggest that PMA may activate the phospholipase D enzyme through two different mechanisms. In this respect, their conclusion is similar to ours, except that, so far, we have not been able to detect significant GTP-dependent PMA effects on phospholipid hydrolysis in membranes from untransformed fibroblasts. Nevertheless, these data suggest that phospholipase D-mediated hydrolysis of PtdEtn and PtdCho may be similarly regulated. In fact, in preliminary experiments we found that in membranes isolated from [14 C]choline-prelabelled NIH 3T3 cells,

ATP and GTP[S] had synergistic stimulatory effects on PtdCho hydrolysis, and that PMA potentiated the effects of nucleotides by 20–25% if membranes were derived from Ha-*ras*-transformed cells.

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