Early response gene signalling in bryostatin-stimulated primary B chronic lymphocytic leukaemia cells *in vitro*

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The protein kinase C activator bryostatin induces differentiation and antagonizes the effects of tumour-promoting phorbol esters in a number of different cell types. We show here that bryostatin preferentially inhibits phorbol 12-myristate 13-acetate (PMA)-induced proliferation compared with differentiation in a number of different B chronic lymphocytic leukaemia (BCLL) cell populations examined. By using a panel of 11 early-response gene probes in Northern hybridization analysis, we found that the profile of genes induced in response to bryostatin and PMA was qualitatively similar and displayed comparable sensitivities to inhibition with the serine–threonine kinase inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride (H7), consistent with common signalling through protein kinase C.

However, the nuclear oncogene, *c-myc*, which was induced strongly in response to PMA treatment, was only marginally up-regulated by bryostatin. In addition, bryostatin selectively inhibited the magnitude of PMA-responsive induction of *c-myc*, to a degree commensurate with its antagonistic effects seen at the biological level. Finally, an anti-sense oligonucleotide blockade of *c-myc* inhibited PMA-induced proliferation but not the differentiation of BCLL cells, implicating this nuclear oncogene as an important determinant distinguishing PMA from bryostatin-coupled biological responses and also as a candidate third-messenger effector target for the anti-tumour effects of bryostatin.

INTRODUCTION

Protein kinase C (PKC) is a key second-messenger enzyme involved in the control of growth and differentiation in many cell types [1]. This enzyme is activated in response to signalling through diverse cell-surface receptors, including the antigens on B and T lymphocytes. Signalling through these receptors initiates a biochemical cascade by the activation of phospholipase C, which hydrolyses membrane inositol phospholipids to generate inositol 1,4,5-trisphosphate and diacylglycerol (reviewed in [2]). Diacylglycerol can then function to directly activate distinct isoforms of PKC, which mediate further downstream signalling events in the cell [3,4]. A number of agents, including phorbol esters such as phorbol 12-myristate 13-acetate (PMA), can substitute for diacylglycerol in directly activating PKC [5]. Bryostatins, which are structurally unrelated to phorbol esters, represent another class of PKC activator; they were originally isolated from *Bugula neritina*, a member of the phylum *Ectoprocta* ([6]; reviewed in [7]). In a number of cellular systems the prototype, bryostatin-I, has been shown to have various agonistic effects on cell function and to mimic some effects of PMA on cells, for example in stimulating normal and leukaemic B-cell activation [8–10] and in mitogenic stimulation of Swiss 3T3 cells [11]. Intriguingly, however, bryostatin can also antagonize some PMA-induced effects such as the differentiation of promyelocytic HL60 [12] and primary B chronic lymphocytic leukaemia (BCLL) cells [9], the mitogenic responses of human T cells [13,14] and tumour promotion [6,15]. Indeed this agent has shown potential as an anti-cancer agent in treating lymphoid malignancies in recent pre-clinical evaluation studies [16]. However, precisely how bryostatin elicits such anti-tumour effects and what dis-

tinguishes the signalling pathways activated by this agent from those coupled to the tumour-promoting phorbol esters is not known (reviewed in [17]).

BCLL is characterized by the accumulation of small resting B lymphocytes arrested at discrete stages of B-cell differentiation [18]. Although BCLL cells are generally refractory to stimulation *in vitro* through their antigen receptors, certain agents such as PMA and bryostatin have been reported to induce activation/differentiation of these cells *in vitro* [9,19].

In previous studies we have used a panel of gene probes for detecting up-regulated expression of early-response genes (ERGs) after stimulation of B cells through a variety of ligand–receptor interactions [20–23]. These experiments have shown that the spectrum of ERG transcripts induced after B-cell stimulation is highly specific to the particular signalling pathway being activated. Consequently this gene probe approach has proved particularly useful for delineating relatedness between different signalling pathways at the third-messenger level. We have now extended this analysis to compare the ERG profiles coupled to the PKC activators PMA and bryostatin in BCLL cells, in particular to identify any ERGs whose encoded products might be uniquely coupled to the contrasting biological responses elicited by these two agents. Our results show that, as in some other cell types [13,14], bryostatin antagonizes PMA-induced BCLL cell proliferation. Significantly, expression of the *c-myc* proto-oncogene was strongly induced by PMA but only weakly up-regulated by bryostatin, and this drug also partly inhibited PMA-induced *c-myc* expression. Finally, we showed that an anti-sense oligonucleotide blockade of *c-myc* expression inhibits PMA-induced proliferation but not the differentiation of BCLL cells, implicating this nuclear oncogene as an important de-

Abbreviations used: BCLL, B chronic lymphocytic leukaemia; ERG, early-response gene; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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terminant distinguishing PMA from bryostatin-coupled biological responses and also as a candidate third-messenger effector target for the antitumour action of bryostatin.

EXPERIMENTAL

Cells and reagents

Mononuclear cells were purified from peripheral blood of BCLL patients with lymphocyte counts greater than 6×10^{10} /litre by single-step Ficoll-Hypaque gradient centrifugation (Lymphoprep, Nyegaard, The Netherlands). Cells were either used directly or stored in liquid nitrogen until use. All cell cultures were grown at 37 °C in a humidified atmosphere of 5% CO₂ in air in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol. Cell culture reagents were obtained from Gibco (Paisley, Scotland, U.K.).

Cells were incubated with PMA (Sigma, Poole, Dorset, U.K.) or bryostatin-1 (a gift from A. McGown, Paterson Institute, Manchester, U.K.) at various concentrations as indicated in the text. *c-myc* sense and anti-sense phosphorothioate oligonucleotides (sense, 5'-ATGCCCTCACCGTT-3'; anti-sense, 5'-AACGGTGAGGGGCAT-3') were synthesized on an Applied Biosystems oligonucleotide synthesizer and were purified by adsorption on Nensorb (NEN Research Products, Boston, MA, U.S.A.) purification cartridges.

Analysis of surface antigen expression on BCLL cells

Cells (10^6) were incubated with the indicated monoclonal antibody in PBS containing 0.02% NaN₃ and 0.1% BSA for 30 min at 4 °C. After they had been washed twice, fluorescein isothiocyanate (FITC)-labelled goat anti-mouse Ig (Becton Dickinson, Oxford, U.K.), diluted 1:50, was added to the cells for 30 min at 4 °C. Cells were washed twice and analysed immediately on a FACScan flow cytometer (Becton Dickinson). The antibodies used in this study were specific for CD3 (T3; Coulter Electronics Ltd., Luton, Beds., U.K.), CD20 (B1; Coulter Electronics Ltd.), IgM (anti-IgM; Becton Dickinson), PCA-1 (anti-PCA-1; Coulter Electronics Ltd.), CD23 (anti-CD23; Cymbus Bioscience Ltd., Southampton, Hants., U.K.), CD37 (anti-CD37; Cymbus Bioscience Ltd.) and FMC7 (anti-FMC7; Serotec Ltd, Oxford, U.K.). As controls, cell samples were incubated with second-step reagent alone and with irrelevant mouse monoclonal antibodies. The immunophenotypes of BCLL cells from different patients used in the study are shown in Table 1. BCLL cells stain positive for mature B-cell markers (CD20,

CD37), have varying amounts of surface IgM and are also generally CD5 positive. Seven of the BCLL populations (from patients 1, 2, 3, 4, 5, 6 and 8) in the study had a typical BCLL phenotype, and one BCLL (from patient 7) had a phenotype of a more mature/activated B-cell, staining positive for CD23 and FMC7 and the plasma cell marker PCA1 (Table 1).

Measurement of DNA and RNA synthesis

BCLL cells (2×10^5 per well) were cultured in triplicate in round-bottomed 96-well microtitre plates (MicroWell, Nunclon, Denmark) for 3 days in the presence or absence of different agents as indicated. For the measurement of DNA and RNA synthesis, 1 µCi of [³H]thymidine (1.78 TBq/mmol; Amersham, Bucks., U.K.) or 1 µCi of [³H]uridine (1.55 TBq/mmol; Amersham) respectively was added to each well of cells and after 4 h ([³H]thymidine) or 2 h ([³H]uridine) cells were harvested (Multimash 2000; Dynatech, Billingham, West Sussex, U.K.). Radioactivity in cells was counted with a liquid-scintillation counter (Rackbeta; Wallac, Turku, Finland).

Measurement of IgM secretion by ELISA

Microtitre plates (micro ELISA; Dynatech) were precoated overnight with 20 µg/ml rabbit anti-human IgM (Dako, High Wycombe, Bucks., U.K.) in coating buffer (0.05 M carbonate buffer, pH 7.6). Supernatants from triplicate cell cultures that had been incubated for 3 days in the presence or absence of different agents were taken and added at 1:2 dilution to the plates and incubated in a humidified chamber for 2 h at room temperature. After intensive washing, alkaline phosphatase-conjugated goat anti-(human IgM) antibody (Sigma) was added and plates were incubated for another 1 h. The reactions were assayed with *p*-nitrophenyl phosphate (Sigma) as a substrate. The absorbance of each well was determined with a Titertek Multiscan Plus ELISA reader (ICN Flow, Thame, Oxon., UK) at a wavelength of 405 nm.

RNA isolation and hybridization analysis

BCLL cells (5×10^7) were harvested at various times after incubation with PMA, bryostatin or a combination of these two agents. In some experiments, the serine-threonine kinase inhibitor, 1-(5-isoquinolylsulphonyl)-2-methyl-piperazine hydrochloride (H7) was included at a final concentration of 100 µM [24]. Total cellular RNA was extracted with the RNAzol extraction technique (Biogenesis Ltd., Bournemouth, Hants., U.K.). Samples of total RNA were separated on 1% (w/v) agarose/0.8 M formaldehyde gels essentially as described previously [20] and stained with ethidium bromide to monitor the equivalence of loading by the fluorescence intensity of rRNA bands. After blotting on to GeneScreen Plus nylon membranes (NEN Research Products), prehybridization and hybridization of filters with ³²P-labelled probes were performed as described elsewhere [20]. Sources and details of the probes have been described previously [20,23]. Briefly, *c-myc*, *c-fos* *c-jun* and *junB* represent nuclear oncogene families that as ERGs are induced in response to mitogenic signalling in most cell types. The *c-myc* probe was a human 1.8 kb cDNA in pSP65 [24]. The *c-fos* probe was a human 1.5 kb cDNA probe in pUC9 (J. J. Murphy and J. D. Norton, unpublished work). The *c-jun* probe was a human 1.8 kb cDNA in pUC18 [25]. The *junB* probe was a human 1.2 kb cDNA clone in pGEM4 [26]. Probes for ERGs *1R19*, *1R20*, *1R21*, *3L3*, *4B1*, *10A* and *19A* were all human cDNAs in pUC 9 previously isolated from PMA-stimulated BCLL cells [20]. Filters were washed at room temperature with $2 \times$ SSC (four times for

Table 1 Surface markers of BCLL cells

BCLL cells were stained with monoclonal antibodies to the indicated antigens, together with FITC-labelled secondary antibody as described in the Experimental section, and the percentage of positive cells was analysed on a FACScan flow cytometer.

Patient	CD3	IgM	CD20	CD37	PCA1	FMC7	CD23	CD5
1	1.1	1.7	27.4	90.9	13.4	0.8	0.3	85.8
2	3.6	75.1	17.3	78.6	6.5	10.2	2.7	31.1
3	4.4	56.6	39.7	34.7	6.7	12.3	3.1	10.1
4	0.6	0.8	37.8	92.6	4.4	0.9	3.6	87.2
5	3.8	12.9	26.2	88.3	3.2	2.6	3.8	80.9
6	1.6	4.8	55.7	93.9	16.3	5.8	2.9	56.3
7	4.2	34.2	96.6	98.4	60.4	78.4	63.5	98.3
8	1.3	72.4	72.7	72.7	8.2	7.3	16.1	93.7

20 s), with $2 \times$ SSC (four times for 20 min), 1% (w/v) SDS in $2 \times$ SSC at 65°C for 20 min and finally at 65°C in $0.1 \times$ SSC (20 min). Autoradiography was performed at -70°C with Dupont Lightening Plus intensifying screens for 1–14 days. Normalization of RNA loading on Northern blots was further verified by rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe [27].

RESULTS

Bryostatin inhibits PMA-induced BCLL cell activation

The effects of different concentrations of bryostatin on PMA (30 nM)-induced BCLL cell (specimen from patient 6; see Table 1) RNA and DNA synthesis and IgM secretion were examined. As shown in Figure 1, bryostatin caused a significant inhibition of PMA-induced levels, in a dose-dependent manner, of all three parameters measured. The concentration range of bryostatin over which inhibitory effects were seen is comparable to that reported for this drug in previous studies [12]. Interestingly, the inhibitory effects of bryostatin, at all concentrations used, was markedly greater (by approx. 2-fold) for thymidine incorporation than for IgM secretion, implying selective inhibition of cell activation/DNA synthesis over differentiation.

To evaluate the consistency of these inhibitory effects of bryostatin, we examined a further six BCLL cell populations by using 30 nM bryostatin, a concentration at which near-maximal inhibition was observed (see Figure 1). As shown in Figure 2, PMA-induced cell activation of all seven BCLLs was inhibited by bryostatin. Bryostatin on its own had a small stimulatory effect on RNA synthesis and IgM secretion, but its effects on DNA synthesis were minimal (Figure 2). As with the CLL population examined in Figure 1 (from patient 6), the inhibitory effect of bryostatin was greater for thymidine incorporation than for IgM secretion despite a wide range of surface immunophenotype markers of the CLLs studied (see Table 1). Interestingly, bryostatin was most effective at inhibiting IgM secretion in BCLL populations that showed the highest levels of secretion in response to PMA (from patients 1, 2 and 6).

Figure 1 Dose-dependent inhibitory effect of bryostatin on PMA-induced activation of BCLL cells

BCLL cells (patient 6) were cultured for 3 days with 30 nM PMA and different concentrations of bryostatin. The percentage inhibition of $[^3\text{H}]\text{TdR}$ incorporation, $[^3\text{H}]\text{UdR}$ incorporation and IgM secretion are shown. The results are expressed as the means $\pm$ S.E.M. for triplicate cultures and are representative of experiments performed with BCLL cells from three different experiments. 100% values for this experiment were 21067 ± 1235 c.p.m. for $[^3\text{H}]\text{TdR}$, 4459 ± 654 c.p.m. for $[^3\text{H}]\text{UdR}$ and 1.08 ± 0.09 absorbance for IgM.

Figure 2 Inhibitory effect of bryostatin on PMA-induced activation of different BCLL cell populations

BCLL cells from patients indicated were cultured for 3 days with medium alone, 30 nM PMA, 30 nM bryostatin or 30 nM PMA plus 30 nM bryostatin. $[^3\text{H}]\text{TdR}$ incorporation (A), $[^3\text{H}]\text{UdR}$ incorporation (B) and IgM secretion (C) were measured as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. for triplicate cultures.

ERG induction profile distinguishes PMA from bryostatin signalling

Bryostatin on its own is known to be an effective inducer of activation/differentiation in several cell types including BCLL [8–11]. To examine early-gene signalling events induced by bryostatin in these cells and to determine possible mechanisms underlying the antagonistic effects of bryostatin on PMA-induced activation and differentiation, we investigated the effect of this agent on the expression of a panel of ERGs whose induced expression precedes PMA-induced differentiation of BCLL cells, as we have previously shown [20,23]. Induced expression of these ERGs thus serves as a set of markers for activation of ‘third-messenger’ pathways coupled to PMA/PKC. In Figure 3, Northern blot time courses of PMA-induced and bryostatin-induced expression of a panel of 11 ERGs are compared. In addition, as part of the same experiment, we have compared the sensitivity of PMA and bryostatin-induced ERG expression with inhibition with the drug H7. This compound is a relatively non-specific inhibitor of PKC and other serine–threonine kinases [28].

Figure 3 Induction of ERG expression by PMA and bryostatin in BCLL cells

Cells from patient 8 were incubated with either PMA (30 nM) or bryostatin (30 nM) and harvested for RNA extraction at the time indicated (in hours) after induction. Identical time courses were also run in the presence of the serine–threonine kinase inhibitor H7 (+H7). Cells were pretreated with this drug for 3 h before the initiation of the time courses. Samples (5 µg) of total cellular RNA from the time points indicated were subjected to electrophoresis on replicate formaldehyde/agarose gels and analysed by Northern hybridization with each of the indicated probes. The equivalence of RNA loading was initially monitored from the fluorescence intensity of ethidium bromide-stained gels (results not shown) and subsequently verified by reprobing a representative filter (from the 3L3 blot) with a GAPDH probe. A residual signal from 3L3 hybridization appears as a more slowly migrating band at the 4 and 8 h time points on the GAPDH reprobe.

We reasoned that differences in second-messenger signalling pathways coupled to PMA and bryostatin might be manifested in qualitative or quantitative differences in the spectrum of ERGs induced, and also in the susceptibility of expression of individual ERGs to inhibition with H7. As shown in Figure 3, the spectrum of ERGs induced by PMA and bryostatin were, overall, very similar. This highlights the similarity in signalling between these two PKC agonists. Also, as shown in Figure 3, although different ERGs vary considerably in their susceptibilities to inhibition with H7 (consistent with previous studies [29]), the profile of inhibition for bryostatin was essentially indistinguishable from that seen with PMA. In addition, it should be noted that the concentrations of PMA and bryostatin used in the experiments were found to generate maximal biological responses in preliminary trial experiments (results not shown). Nonetheless, the magnitude of induction seen for several ERGs was noticeably lower with bryostatin than with PMA (Figure 3). The most extreme example of this was seen with the *c-myc* oncogene (and to a lesser extent with *c-jun* and *19A*), which was strongly induced in response to PMA treatment, but only marginally up-regulated by bryostatin.

To evaluate further the relationships between the signalling pathways coupled to PMA and bryostatin, we compared the ERG profile induced by PMA with that seen with a combination of PMA plus bryostatin in a separate experiment. As shown in Figure 4, PMA-responsive induction of most ERGs examined was either not affected or marginally potentiated by bryostatin. However, two ERGs, *c-myc* and to a lesser extent *10A*, were significantly inhibited by bryostatin. Bryostatin did not inhibit PMA-induction of either *c-jun* (Figure 4) or *19A* (results not shown) although both these ERGs, like *c-myc*, were differentially regulated by bryostatin and PMA (Figure 3). It should be noted that the concentration of bryostatin used in these experiments

Figure 4 Effect of bryostatin on the induction of ERG expression by PMA

Time courses of BCLL cells from patient 8 were run with either PMA (30 nM) alone or with a combination of PMA and bryostatin (30 nM each). RNA from the time points indicated was extracted and analysed by Northern hybridization on replicate filters with the probes indicated. To monitor the equivalence of RNA loading, the filter probed with *c-myc* was subsequently reprobed with a GAPDH probe.

(30 nM) was previously shown to inhibit PMA-induced thymidine incorporation by approx. 60% (see Figure 1). Thus the magnitude of PMA-responsive *c-myc* inhibition seen with bryostatin (Figure 4) is commensurate with its antagonistic effects seen on PMA responses at a biological level.

Figure 5 Dose-dependent effect of *c-myc* anti-sense oligonucleotide on PMA-induced proliferation and IgM secretion of BCLL cells

BCLL cells (patient 1) were cultured for 3 days with 30 nM PMA and different concentrations of sense (S) or anti-sense (AS) oligonucleotides, and the percentage inhibitions of [3 H]TdR incorporation and IgM secretion were determined. Results are expressed as means $\pm$ S.E.M. for triplicate cultures and are representative of three experiments performed with BCLL cells from four different patients. 100% values for this experiment were 23425 ± 823 cpm for [3 H]TdR, and 0.71 ± 0.08 absorbance for IgM.

Anti-sense *c-myc* oligonucleotide inhibits PMA-induced BCLL DNA synthesis but not IgM secretion

To evaluate further the possible coupling of ERG up-regulation to biological responses of BCLL cells in terms of proliferation and differentiation, we examined the effects of anti-sense oligonucleotides to several ERGs including *c-fos*, *c-jun*, *10A*, *1R21*, *1R20* and *c-myc*. With the exception of *c-myc*, however, no significant inhibition of either thymidine uptake or IgM secretion was observed compared with that seen with control sense oligonucleotides for these genes (results not shown). Figure 5 shows the effects of anti-sense and sense *c-myc* oligonucleotide on PMA-induced BCLL cell (patient 1) DNA synthesis and IgM secretion. Anti-sense *c-myc* inhibited PMA-induced BCLL DNA synthesis in a dose-dependent manner (Figure 5). However, PMA-induced IgM secretion, as a measure of differentiation, was not inhibited by any concentration of anti-sense *c-myc* tested. As expected, the control sense *c-myc* oligonucleotide had only a minimal effect on PMA-induced DNA synthesis and IgM secretion (Figure 5). Figure 6 shows that in seven different BCLL populations examined, anti-sense *c-myc* oligonucleotides (10 μ M) consistently inhibited PMA-induced DNA synthesis but not IgM secretion. Figure 7 shows that anti-sense *c-myc* oligonucleotides significantly reduced the level of *c-myc* mRNA (approx. 50% inhibition as measured by densitometry) in PMA-treated BCLL cells (3 h, patient 8) compared with the level seen in sense oligonucleotide-treated cells. This effect appeared to be specific to *c-myc* because mRNA levels of another ERG *junB* were similar for cells incubated in the presence of anti-sense or sense oligonucleotides (Figure 7).

DISCUSSION

In previous studies bryostatin has been reported to antagonize the actions of phorbol esters by inhibiting cell differentiation and/or proliferation [12–15]. In BCLL cells in particular it has been shown that this drug, although an effective stimulator on its own, significantly inhibits PMA-induced differentiation as

Figure 6 *c-myc* anti-sense oligonucleotide inhibits PMA-induced proliferation but not IgM secretion of BCLL cells

BCLL cells from the indicated patients were cultured for 3 days with medium alone, medium plus 30 nM PMA, medium plus 30 nM PMA plus 10 μ M sense (S) oligonucleotides or medium plus 30 nM PMA plus 10 μ M anti-sense (AS) oligonucleotides. [3 H]TdR incorporation (A) and IgM secretion (B) were measured as described in the Experimental section. The results are expressed as means $\pm$ S.E.M. for triplicate cultures.

Figure 7 *c-myc* anti-sense oligonucleotides inhibit PMA-induced *c-myc* expression

Northern blot of RNA extracted from BCLL cells (patient 8) treated with 30 nM PMA for 3 h in the presence of 10 μ M *c-myc* anti-sense (A) or sense (B) oligonucleotides. Levels of expression of GAPDH are shown as a control for RNA loading.

assessed by IgM secretion [9]. In extending these studies we have now shown that bryostatin also elicits a dose-dependent inhibition of PMA-induced BCLL cell activation as assessed by the incorporation of thymidine and uridine. Moreover this inhibitory effect was consistently more pronounced than that seen for IgM secretion, suggesting that the antagonistic effects of bryostatin are to some extent selective for cell proliferation over differentiation.

To explore further the relationships between the regulatory pathways coupled to PMA and bryostatin, we examined the spectrum of ERGs seen with each agent alone or with a combination of both. The overall induction profiles for each agent used in isolation were remarkably similar, reinforcing earlier studies invoking PKC as a common receptor for these two drugs [30]. Moreover, use of the serine–threonine kinase inhibitor, H7, as a ‘probe’ failed to discriminate between bryostatin and PMA-coupled ERG signalling pathways. As expected, however, this ‘PKC’ inhibitor variably abrogated bryostatin-induced expression of different ERGs as reported previously for PMA [29].

Although the overall ERG profiles activated by PMA and bryostatin were broadly similar, they did show some quantitative differences. The magnitude of induction of several of the ERG transcripts was consistently lower with bryostatin than with PMA, presumably reflecting the lower potency of bryostatin in mimicking many of the effects of phorbol esters [9], and results in the present paper). The most extreme example of this was seen with the oncogene *c-myc* and to a lesser extent with *c-jun* and *19A*, which were all induced to markedly high levels with PMA than when bryostatin was used. Therefore, as with all other ligand–receptor pathways of B-cells that we have studied [21, 22, 29], the coupled ERG responses of these B-cell stimulators allows discrimination between their cellular signalling pathways. In principle this could in turn facilitate the elucidation of those second-messenger signalling determinants unique to PMA/bryostatin that underlie differences in their biological effects. Although we have not addressed this issue, a prime candidate might be a distinct isoform of PKC, activated preferentially by PMA/bryostatin [31]. Alternatively, it might be that up-regulation of *c-myc* expression in BCLL cells, which occurs with relatively delayed kinetics, requires prolonged PKC-coupled signalling. The failure of bryostatin to induce this gene significantly might then be explained by preferential degradation of PKC by bryostatin, as has recently been shown to occur in primary T-cells [13]. In contrast, the observation that other ‘delayed’ ERGs were induced by a similar magnitude by both PMA and bryostatin is not obviously compatible with this explanation.

In contrast with PMA, which as well as functioning as a tumour promoter induces the proliferation of T-lymphocytes [13, 32] and to a limited degree of BCLL cells [29], bryostatin alone does not significantly stimulate T-cell proliferation and indeed has been reported to antagonize PMA-induced proliferation of this cell type [13]. Also, bryostatin has previously been reported to elicit only a minimal effect on DNA synthesis in BCLL cells [9], and results in the present paper) while being an effective inducer of differentiation. Because the *c-myc* oncoprotein has been widely implicated in the regulation of cell growth/tumorigenesis from numerous studies [33], we reasoned that this nuclear oncoprotein might be a candidate principle effector molecule coupled specifically to PMA signalling in BCLL in association with proliferation. In further exploring this issue we found that the preferential inhibition of PMA-induced DNA synthesis over differentiation by bryostatin was mirrored by a significant inhibition of some PMA-induced ERG responses; this was most pronounced for *c-myc*. These results, however, do not exclude other possible mechanisms besides ablation of *c-myc* expression to account for the anti-proliferative effects of bryostatin. Finally, we showed directly that *c-myc* is coupled to the proliferative response induced by PMA in several BCLL cell populations by the observation that anti-sense oligonucleotides to this oncogene inhibit DNA synthesis, but not IgM secretion. As *c-myc* suppression does not block PMA-induced BCLL

differentiation, this implies that one or more additional/alternative mechanisms must be responsible for inhibition of PMA-induced BCLL differentiation by bryostatin. Thus elevated *c-myc* expression does not seem to be a requirement for terminal B-cell differentiation and the accompanying up-regulation of immunoglobulin gene expression [34]. Such a conclusion is entirely in accord with known transcription regulatory mechanisms associated with immunoglobulin genes that do not invoke a *c-myc* function [35]. Taken together, our results therefore implicate *c-myc* expression both as an important functional determinant of PMA action in potentiating proliferation and as a third-messenger effector target for the antagonistic effects of bryostatin on malignant B-cells.

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