Biochemical Pharmacology, Vol. 58, pp. 383–388, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00063-5

COMMENTARY

Critical Targets of Protein Kinase C in Differentiation of Tumour Cells

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ABSTRACT. The ultimate target of pharmacological research is to find new drugs for treating human diseases such as cancer. Agents causing differentiation and thus growth arrest should be particularly useful in this regard. A potential target for such anticancer therapy is the enzyme family protein kinase C (PKC), which is involved in the transduction of signals for cell proliferation, differentiation, and apoptosis. Our recent work showing the induction of differentiation in melanoma cells by an activator of one PKC isoform, PKCδ, touches on several important areas of investigation, which will form the basis of this review: the role of individual isoforms of PKC, their downstream targets and their specific substrates, the mechanism of activation of specific genes involved in the differentiation process, and the molecular basis for the morphological changes associated with differentiation. The central role that PKC plays in these processes points to the need for a greater understanding of the signalling pathways utilized by individual isoforms of this family of enzymes. BIOCHEM PHARMACOL **58**;3:383–388, 1999.

KEY WORDS. bistratene A; PKC isoforms; signal transduction; melanogenesis; transcription factors; stathmin; cell cycle

The different expression patterns, cofactor requirements, and substrate specificities of individual isoforms of PKC† suggest that each is involved in specific regulatory processes within the cell [1]. Information on the role of individual isoforms is limited. To determine the role of a PKC isoform in a particular cell function, a correlation with activation or down-regulation of the specific isoform is required. The identification of isoform-specific substrates will increase our understanding of how these enzymes mediate their effects. For such studies it is necessary to have isoform-specific activators and inhibitors. Some success has been achieved with inhibitors such as rottlerin [2], which is relatively specific for PKCδ. Calphostin C has been shown to be a more efficient inhibitor of PKCδ than chelerythrine chloride, with these efficiencies reversed for PKCγ [3]. Few isoform-specific substrates have been identified. In the case of PKCδ, there are the neuronal substrate GAP-43 [4], eukaryotic elongation factor 1α [5], and heat shock proteins [6].

The PKC isoforms are differentially involved in the regulation of proliferation, differentiation, and apoptosis. PKCε overexpression results in tumorigenesis, leading to its classification as an oncogene [7], whereas PKCδ appears to have a tumour suppressor role [2]. While there are likely to be variations between cell types, the current literature points to a role for PKCδ in the execution of the apoptotic programme, whereas PKCα and ζ are frequently associated with cell survival and suppression of apoptosis [8]. Other specific roles attributable to PKCδ are regulation of Na-K-2Cl co-transport [9] and inhibition of the Stat signalling pathway induced by Bmx kinase [10].

The potential of PKC as a target for anticancer drugs has been recognized for some time and has been the subject of several reviews [11–14]. Bryostatin 1 (Fig. 1), a macrocyclic lactone from the bryozoan *Bugula neritina* [15], has a wide range of activities including hematopoietic and immune stimulation and induction of differentiation of both myeloid and lymphoid cell lines [16]. It interacts with PKC and competes with the PKC activator and tumour promoter PMA in a complex manner [17]. Bryostatin is not, however, a tumour promoter, but an antineoplastic agent that has been used successfully to treat murine melanoma [18]. It is currently in clinical trials for several types of tumours [19–21]. Bryostatin 1, which inhibits the PMA-induced down-regulation of the PKCδ isoform specifically, blocks the tumour-promoting effects of PMA, implicating PKCδ as the target of the tumour promoting phorbol esters. This suggests that tumour promotion is due to the depletion of PKCδ, which has an apparent tumour suppressor function [2]. There is some evidence, however, using 26-epi-bryostatin 1, that the bryostatins inhibit the growth of B16/F10

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† *Abbreviations:* cAMP, cyclic AMP; CDK, cyclin-dependent kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MITF, microphthalmia associated transcription factor; α-MSH, α-melanocyte stimulating hormone; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; and TRP, tyrosinase-related protein.

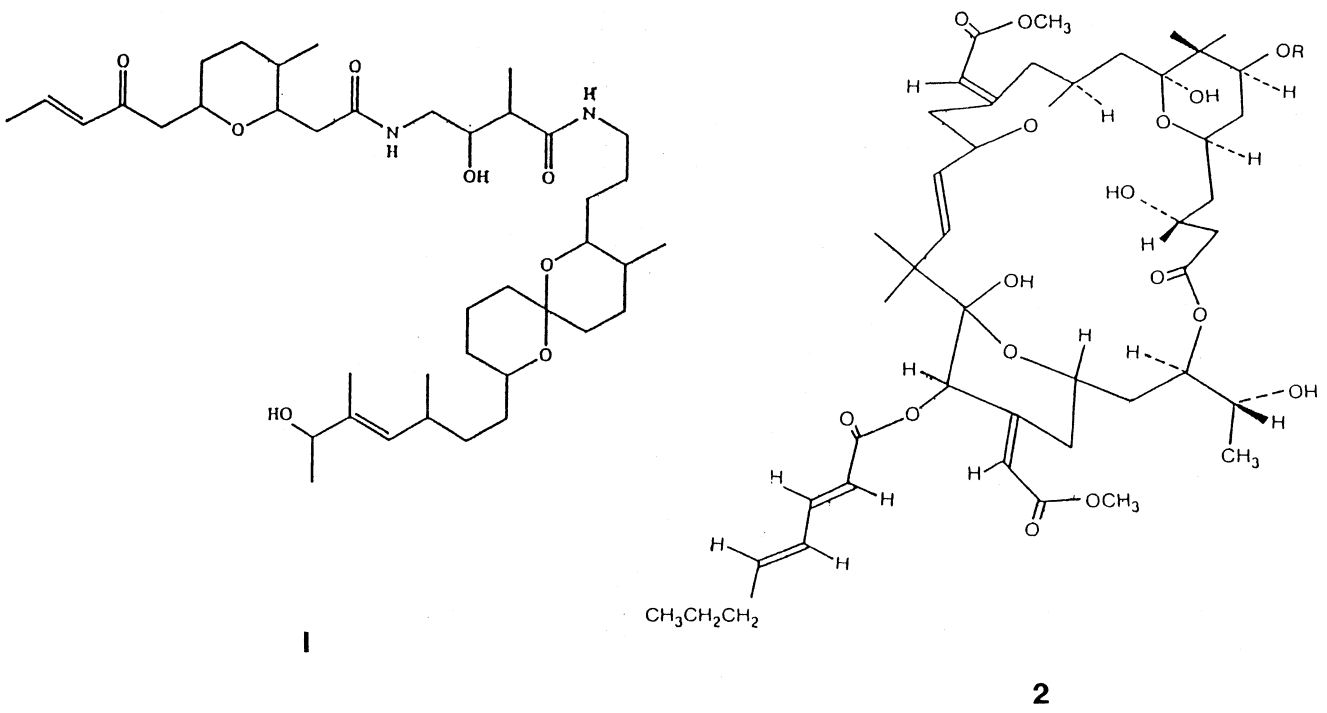

FIG. 1. Structures of (1) bistratene A and (2) bryostatin 1.

melanoma cells *in vitro* through a PKC-independent mechanism [22]. In addition, 26-succinylbryostatin 1 shares some but not all of the biological properties of bryostatin 1 [23], suggesting that analogues with reduced toxicity and greater selectivity for tumour cells may be designed.

Bistratene A (bistramide A, Fig. 1) is a polyether toxin isolated from the colonial ascidian *Lissoclinum bistratum* [reviewed in Ref. 24]. It displays potent antitumour activity *in vitro* and, being lipophilic, is cell permeable. It is currently the only described specific activator of protein kinase Cδ and thus is extremely useful for determining the specific role of this isoform [25]. Bistratene A does not compete with phorbol esters for binding to PKC and is a less efficient activator of PKCδ *in vitro* than PMA (Watters DJ, unpublished results). The mode of interaction and the basis for the PKC isoform selectivity of this compound await further investigation. A number of derivatives of bistratene A have been isolated by Riou *et al.* [26], and some of these appear to be less toxic than the parent compound. As such they may provide a basis for the design of effective antineoplastic agents for clinical trials, since bistratene A itself is too toxic for administration to humans due to neurological side-effects [27]. Attempts to chemically synthesize this compound are underway (Kitching W, unpublished results; cited with permission); however, this is no easy task, due to the number of asymmetric carbon atoms in the molecule. In the meantime, its use for cell biological studies *in vitro* should increase our understanding of the differentiation process.

HL-60 human leukemia cells have been a useful model for the study of differentiation *in vitro* since they mature along either the granulocytic or monocytic pathway in response to a variety of agents. PMA and bryostatin induce a monocyte phenotype [28]. Bistratene A induces a partial differentiation along this same pathway [29]. We have evidence that in HL-60 cells induced to undergo monocytic differentiation in response to bistratene A, the activity of the AP-1 transcription factor is increased, most likely through phosphorylation by JNK, which is also elevated after bistratene A treatment (Fig. 2).

Efficient terminal differentiation of human melanoma cells along the pigmentation pathway has been difficult to achieve. In our hands, bistratene A has proven to be more effective in this respect than differentiation agents such as butyrate [31, 32]. Identification of the essential targets of bistratene A in melanocytic cells, therefore, may provide a pharmacological basis for the control of pigmentation in the context of melanoma therapy or, in normal melanocytes, protection from solar UV radiation.

The control of tyrosinase activity appears to be the pivotal factor in melanogenesis regulation. Melanin content has also been shown to correlate with tyrosinase activity but not protein levels [33], and PKCβ is able to phosphorylate and activate tyrosinase directly in human melanocytes [34]. In normal human melanocytes, melanogenesis in response to UVB radiation proceeds via cAMP formation resulting from the release of α-MSH [35]. However, α-MSH also stimulates PKC activity in murine B16 melanoma cells [36], and α-MSH-induced pigmentation is blocked by depletion of PKC [37]. The transcription factor AP-1 has been shown to be activated through the MAPK pathway during cAMP-induced melanogenesis in B16 mel-

anoma cells [38]. PKC is known to stimulate AP-1 and AP-2 transcription factors [39, 40], and consensus sequences for these transcription factors are present in the tyrosinase gene promoter [41].

A variety of genes in addition to tyrosinase play a role in humans [42]. The melanosomal P protein is an integral component of the melanosomal membrane and part of a high molecular weight complex that includes tyrosinase, TRP-1, and TRP-2 [43]. The *Brn-2* gene, which encodes the N-Oct-3 transcription factor, is required for transcription of a number of melanocyte-specific genes including tyrosinase, TRP-1, TRP-2, and MITF [44]. Melanoma cells lacking Brn-2 lose the ability to form tumours when injected into mice [44], and *Brn-2* gene expression is elevated in malignant melanoma [45]. Ablation of the neural-specific transcription factor Brn-2 was associated with loss of MITF and pigmentation gene expression in human melanoma cells [44]. MITF has also been shown to regulate transcription of tyrosinase and tyrosinase-related proteins [46–48]. The activity of MITF is itself regulated by PKC [49]. Long-term treatment of B16 melanoma cells with PMA, which down-regulates PKC conventional and novel isoforms, has been shown to negatively regulate reporter gene expression from the tyrosinase promoter, whereas short-term treatment activates PKC and gene expression. Thus, MITF activity in response to PKCδ activation by bistratene A needs investigation using reporter genes coupled to the promoters of tyrosinase and related genes. Expression of *Brn-2* also is activated by the transcription factor AP-2* which, interestingly, is activated by bistratene A treatment in HL-60 cells (Fig. 3).

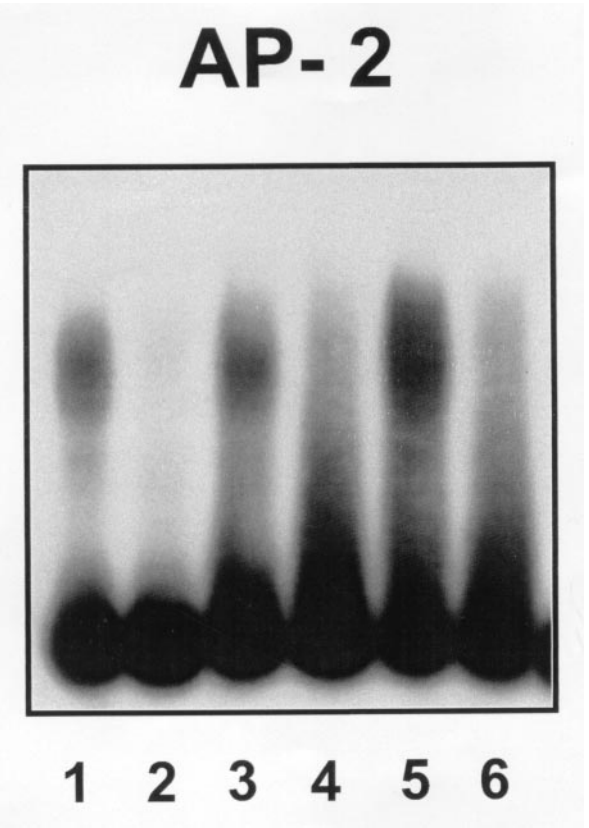

FIG. 3. Activation of AP-2 in HL-60 cells. The same method as described in Fig. 2B was used with a consensus oligonucleotide for the transcription factor AP-2 (Promega Corp.). Lane 1, untreated; lane 3, PMA; lane 5, bistratene A; lanes 2, 4, and 6 contain excess unlabelled oligonucleotide probe.

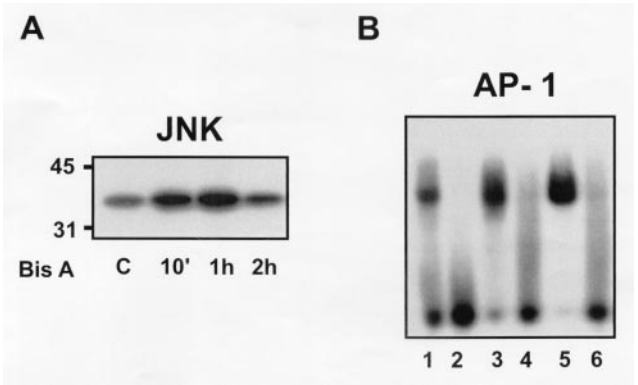

FIG. 2. Activation of JNK and AP-1 in HL-60 cells after bistratene A treatment. Cell lysates [untreated (C), and bistratene A-treated (10 min, 1 hr, and 2 hr)] were immunoprecipitated with antibody to JNK1 (Santa Cruz Biotechnology Inc.), and the immunoprecipitates were bound to protein A beads. The washed beads were incubated in kinase buffer with recombinant GST-jun (40 kDa) and [^{32}P]ATP, and the mixture was analysed by SDS–PAGE followed by autoradiography as described in Ref. 30. The numbers on the left side of the blot show the molecular mass in kDa of marker proteins. (B) Nuclear extracts from untreated and treated (100 nM bistratene A or PMA, 1 hr) cells were used in a gel shift assay with a ^{32}P-labelled consensus oligonucleotide for the transcription factor AP-1, according to the Promega technical bulletin. Lane 1, untreated; lane 3, PMA; lane 5, bistratene A; lanes 2, 4, and 6 contain excess unlabelled oligonucleotide probe.

Further elucidation of signalling pathways leading to differentiation requires the identification of additional components of the pathway, for example, the specific substrates and downstream targets of PKCδ. Two-dimensional SDS–PAGE is a very powerful tool to compare treated and untreated cell lysates, or lysates from normal and malignant cells. Coupled with protein sequencing by mass spectrometry [50] this provides a means to analyse several components simultaneously. Using the more tedious and less sensitive traditional approach of Edman degradation from a large number of collected two-dimensional spots, we were able to identify stathmin as a downstream target for PKCδ activation in HL-60 cells [25] and in MM96E cells [32]. Stathmin is absolutely required for nerve growth factor-induced differentiation of PC12 cells, as shown by antisense experiments [51]. MAPK activation is also essential, and these authors showed that MAPK was responsible for nerve growth factor-induced stathmin phosphorylation by using the MAPK inhibitor PD 098059. Stathmin phosphorylation was not required, however, for

* Smit DJ, Smit SE, Brightwell G, Smith AG, Parsons PG, Muscat GEO and Sturm RA, Manuscript submitted for publication.

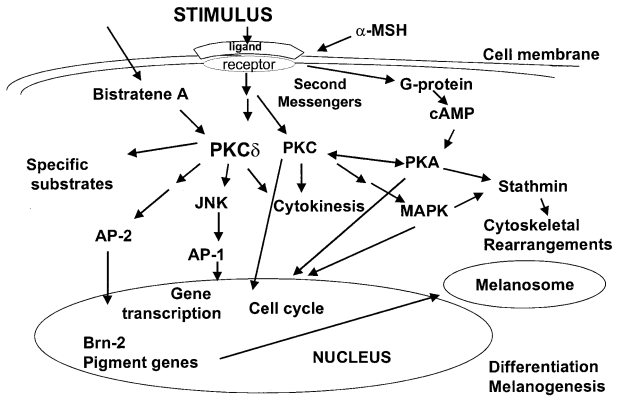

FIG. 4. Schematic diagram of possible signalling pathways involved in differentiation and melanogenesis.

induction of the late-induced genes, indicating that it is responsible for only specific aspects of the differentiation programme, for example, the morphological changes. Several recent papers have shown that the microtubule regulating ability of stathmin is controlled by phosphorylation and that the primary physiological role of stathmin is most likely to regulate microtubule dynamics in response to external signals during interphase of the cell cycle. PKA phosphorylation of Ser-16 and Ser-63 is sufficient to switch off the microtubule destabilizing activity of stathmin [52, 53], resulting in an increase in microtubule polymers. Dual phosphorylation on the CDK sites Ser-25 and Ser-38 is required to allow phosphorylation of Ser-16 and Ser-63. Curmi *et al.* [54] have shown that the stathmin destabilizing activity is related to the ability of stathmin to directly bind to and sequester tubulin. Phosphorylation blocks tubulin complex formation [53]. During mitosis, stathmin is phosphorylated on all four sites to high stoichiometry; thus, the data demonstrate that it is essential to switch off the activity of stathmin during mitosis to allow formation of the mitotic spindle, and the constitutive activities of all the kinase target-site deficient mutants cause a cell cycle block prior to metaphase [52]. In light of the new results, it seems unlikely that increased phosphorylation of stathmin in the presence of bistratene A is related to its cell cycle effects; rather, it is more probably related to the morphological changes. A close relative of stathmin with all four phosphorylation sites conserved is the neuronal SCG10 protein, which is highly concentrated in growth cones. The microtubule destabilizing activity of this protein is also regulated by phosphorylation; thus, it may link signal transduction to alterations of microtubule dynamics in the growth cone [55].

Since PKA has been identified as an important regulator of stathmin phosphorylation and also of melanogenesis, it follows that there must be some cross-talk between PKA and PKC signalling pathways. Many examples of such cross-talking can be found, for example, the signalling pathways activated by substance P and vasoactive intestinal peptide in lactotrophs [56], and in PC12 cells where cAMP leads to phosphorylation and activation of PKCζ [57]. The identification of multienzyme signalling complexes containing both PKC and PKA associated with anchoring proteins [58] makes it easy to envisage how such cross-talking is established.

PKC may be a conserved regulator of cell cycle events that links signal transduction pathways to the cell cycle machinery [59], and it appears to have a role in both the G_1 and G_2/M phases of the cell cycle. At the G_1 phase, the overall effect of PKC activation is inhibition at mid- to late G_1, correlating with inhibition of phosphorylation of the tumour suppressor retinoblastoma (Rb) protein. At the G_2/M transition, recent evidence suggests that PKC is involved in the regulation of cdc2 activity as well as being a regulator of lamin B phosphorylation and nuclear lamina disassembly [60]. PKC has been shown to associate with and/or phosphorylate a number of cytoskeletal components [61] and is thus an important regulator of cytoskeletal function. This would serve to link cell shape changes to the cell cycle and to differentiation. In addition, PKC has a major role in regulating transcription. The central role of PKC in relation to melanogenesis has been illustrated in Fig. 4.

Much has been learned about the distribution and targets of PKC isoforms in mammalian cells, in association with phenotypic outcomes that might be exploited for pharmacological purposes. However, even specific activators, such as bistratene A, may cause a vast number of changes, which for differentiating agents may involve up to 2% of all expressed genes [62]. Unravelling the complex and interacting signalling pathways that are relevant to pharmacological endpoints may require intensive protein and gene expression scanning. The latter will be facilitated by the use of microarrays for comparison of differential gene expression after drug treatment [63].

The authors wish to thank Bernadette Garrone for expert research assistance. This work was supported by funds from the National Health and Medical Research Council of Australia, and the Queensland Cancer Fund.

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