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Bistratene A: A Novel Compound Causing Changes in Protein Phosphorylation Patterns in Human Leukemia Cells

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Abstract Bistratene A, a polyether toxin isolated from the colonial ascidian *Lissoclinum bistratum,* causes incomplete differentiation of human leukemia (HL-60) cells apparently through a mechanism not involving protein kinase C. In view of the importance of phosphorylation/dephosphorylation in cellular growth and differentiation we have investigated protein phosphorylation in these cells following exposure to bistratene A, using two-dimensional polyacrylamide gel electrophoresis. Marked increases in the phosphorylation of a protein of 20 kDa, pI 6.7, and a basic protein of 25 kDa were observed after incubation with bistratene A. A comparison was made with cells treated with 12-O-tetradecanoylphorbol 13-acetate and bryostatin 5. While changes in phosphorylation patterns were observed with these two compounds, the 20 kDa and 25 kDa proteins did not undergo phosphorylation changes. The 20 kDa protein was induced rapidly by very low concentrations of bistratene A reaching near maximal levels with 10 nM at 15 min exposure. This protein was found to be localised to the cytoplasm. Phosphoaminoacid analysis demonstrated that the majority of ^{32}P was present in serine and tyrosine residues. The increased phosphorylation of the 20 kDa protein appeared to be due to hyperphosphorylation of existing protein although there was some increase in the amount of the protein. These results suggest that bistratene A will be a useful tool with which to investigate cellular differentiation mechanisms.

Key words: bistratene A, phorbol ester, bryostatin, protein kinase C, differentiation, phosphorylation

We have previously described a marine toxin, bistratene A [Degnan et al., 1989], whose structure has recently been revised [Foster et al., 1992]. Bistratene A induces some properties indicative of differentiation in HL-60, human leukemia cells [Watters et al., 1990]. These cells have become an attractive model for studies of cellular differentiation [Collins, 1987]. Among the compounds which induce differentiation of HL-60 cells are the phorbol ester TPA (12-O-tetradecanoylphorbol 13-acetate) and marine animal antineoplastic constituents of the bryostatin series [Pettit et al., 1982]. The only known receptor for the phorbol esters is the enzyme protein kinase C (PKC). TPA activates the enzyme by substituting for the natural activator diacylglycerol [Castagna et al., 1982]. Bryostatin 1 is able to activate PKC in a similar manner [Smith et al., 1985]. However, the action of bryostatin 1 in HL-60 cells is quite complex: at low concentrations it induces monocytic differentiation, but at higher concentrations it antagonises the effect of TPA in a noncompetitive manner [Dale and Gescher, 1989]. Bryostatin 1 also induces the phosphorylation of a unique set of proteins in HL-60 cells [Warren et al., 1988]. Two proteins of molecular weight 70 kDa are

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid; IEF, isoelectric focussing; NEPHGE, nonequilibrium pH gradient eletrophoresis; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoylphorbol 13-acetate; 2D SDS-PAGE, two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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phosphorylated by low concentrations of bryostatin 1 but only by very high concentrations of TPA. These observations are consistent with the concept that bryostatin 1 interacts with either an isoform of PKC with low affinity for phorbol esters or with a protein distinct from PKC. The similarity of some of the effects of bistratene in HL-60 cells to those induced by TPA led us to investigate the possible interaction of the compound with PKC [Watters et al., 1990]. We found that bistratene could enhance the activation of the enzyme in vitro in a manner similar to TPA, but the concentration of bistratene required was in the micromolar range. This was in contrast to the low concentrations (nanomolar) required to elicit its biological effects. Thus it appears unlikely that PKC is directly involved in the action of bistratene.

In HL-60 cells the induction of granulocytic and monocytic differentiation is accompanied by increases in tyrosine kinase activity and even larger increases in protein phosphotyrosine phosphatase activity with a net reduction in cellular phosphotyrosine content [Frank and Sartorelli, 1986]. The regulation of biological processes such as growth and differentiation is thought to occur through a dynamic equilibrium between phosphorylation and dephosphorylation [Cohen and Cohen, 1989]. In this study we have investigated the changes in protein phosphorylation occurring in HL-60 cells after incubation with bistratene A, and compared them with the patterns induced by TPA and bryostatin 5. Bryostatin 5 has similar antineoplastic properties to bryostatin 1 [Pettit, 1991]. We show that bistratene induces a unique pattern of protein phosphorylation in these cells.

MATERIALS AND METHODS

Materials

Bistratene A was isolated from the ascidian *Lissoclinum bistratum* as previously described [Degnan et al., 1989]. 12-O-tetradecanoylphorbol 13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO). Bryostatin 5 was purified from the bryozoan *Bugula neritina* as described previously [Pettit et al., 1985]. [^{32}P] Ortho-phosphate was obtained from Amersham or Bresatec (Adelaide, Australia). CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was from Boehringer Mannheim. Nonidet P-40 was from Sigma. Ampholytes, pH range 3–10 and 5–8, were purchased from Pharmacia-LKB. Ultrapure electrophoresis materials were from Bio-Rad. All other reagents were analytical grade.

Cell Culture and Labelling

The human promyelocytic leukemia cell line HL-60 was obtained from Prof. P. Smith, Department of Pathology, University of Queensland. The cells were routinely cultured in RPMI-1640 medium containing 10% fetal calf serum. For labelling experiments, the cells (1.5×10^7/mL) were washed twice with phosphate-free MEM and incubated for 30 min at 37°C in the same medium. Carrier-free $^{32}P_i$ (0.5 mC_i/mL) was added and the cells equilibrated for 1.5–2 h. After the equilibration period aliquots containing approximately 3×10^6 cells were treated with TPA, bryostatin 5, or bistratene A for the times indicated in the Results section. The incubation was terminated by addition of 10 volumes of ice-cold stopping buffer (20 mM potassium phosphate (pH 7.4), 50 mM NaF, 5 mM sodium pyrophosphate, 100 μM sodium vanadate, 1 mM PMSF, 5 mM EDTA). The cells were then microfuged for 1 min and the supernatant discarded. The cellular pellet was washed twice with a further 1 mL of stopping buffer and then solubilised in 50 μL IEF sample buffer (9.2 M urea, 2% nonidet (v/v), 1% dithiothreitol (DTT) (w/v), 4% CHAPS (w/v), 1:16 dilution (6.25%) Ampholines [3-10]). Samples were stored at −70°C until use.

Cell Fractionation

At the end of the labelling procedure the cellular pellets were snap-frozen in liquid nitrogen and thawed in 5 volumes of extraction buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% (v/v) glycerol, 0.5 mM PMSF] and centrifuged for 10 sec in a microfuge to pellet the nuclei [Dale et al., 1989]. After washing twice with the same buffer, the nuclear pellet was solubilised in IEF sample buffer (50 μL) and stored at −70°C. The supernatant (cytoplasmic fraction) was subjected to ultracentrifugation at 100,000*g* for 50 min at 4°C in a Beckman TL100 Ultracentrifuge. The membrane pellet was solubilised in IEF sample buffer (50 μL) and stored at −70°C. The proteins in the soluble fraction were precipitated by the addition of 5 volumes of ice-cold acetone:NH_4OH (30:1.7 v/v) overnight at −20°C, followed by centrifugation in a microfuge for 15 min at 4°C [Feuerstein and Cooper, 1983]. After discarding the supernatant, the pellet was solubilised in

IEF sample buffer (50 μL) and stored at −70°C until electrophoresis.

Two-Dimensional Gel Electrophoresis

Two-dimensional SDS-PAGE was carried out according to Hochstrasser et al. [1988], a modification of the original method of O'Farrell [1975]. Isoelectric focussing in the first dimension was carried out for 15,700 V-h. Electrophoresis in the second dimension was performed using 12% gels. With care taken to add the equivalent amount of radioactivity to gels for comparative purposes, 100,000–200,000 TCA-precipitable counts were loaded on each gel. Nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension was carried out according to O'Farrell et al. [1977], to separate basic proteins. Labelled proteins were detected by autoradiography of dried gels using Kodak XAR-5 film. Accurate molecular weights were determined by using low molecular weight SDS-PAGE markers (Bio-Rad) in silver stained gels. Silver staining was carried out by the method of Rabilloud et al. [1988]. The isoelectric point of the 20 kDa protein was determined as follows. An IEF gel was run in parallel to the test gels and cut up into small pieces which were eluted into 1 mL of H_2O. After equilibration the pH of the solution was measured. This established a plot of pH vs. length of gel from which it was possible to estimate the pI of the protein. The value given is the average of 8 separate experiments (± 0.2 pH units).

Quantitation of ^{32}P Label in Specific Proteins

The protein of interest was located on fixed and stained gels by autoradiography, excised, and the radioactivity determined in a liquid scintillation spectrometer. A gel piece of identical size was excised from an area in the gel where no radioactivity was detected by autoradiography and used as a control.

Phosphoaminoacid Analysis

The 20 kDa protein spot was cut out and subjected to phosphoaminoacid analysis. Gel pieces were pooled from several experiments for both control and bistratene-treated cells and hydrolysed according to the method of Cooper et al. [1983]. The resulting phosphoaminoacids were separated by thin layer chromatography and autoradiographed as described by Munoz and Marshall [1990].

RESULTS

The dramatic effects of bistratene A at low concentrations on HL-60 cells and inability to activate protein kinase C in vitro except at high concentrations prompted us to look for changes in the phosphorylation patterns of proteins in these cells. Figure 1 shows a comparison of phosphoproteins separated by 2D SDS-PAGE from untreated cells and cells treated with three agents: bistratene A, TPA, and bryostatin 5 (Fig. 1A–D). The most obvious difference between these patterns was the marked increase in phosphorylation of a protein of molecular weight approximately 20 kDa, pI 6.7, after treatment with bistratene A (Fig. 1B, arrow). The region of the gel corresponding to this protein was cut out for quantitation of the radioactive label. On average the amount of ^{32}P incorporated was 3–7 times that of control cells. The intensity of the spot corresponding to the 20 kDa protein was also compared in cells treated with the three agents using computing densitometry of the autoradiographs in Figure 1. The results demonstrate that bistratene A causes a sixfold increase in phosphorylation, whereas TPA and bryostatin 5 had negligible effect. Silver staining of gels from control and bistratene-treated cells (Fig. 2) showed that there was a twofold increase in the amount of the 20 kDa protein as determined by computing densitometry. The gels were routinely silver stained and dried prior to autoradiography. Under these conditions it was possible to demonstrate that the labelled and stained protein were exactly superimposable. Figure 3 shows the patterns obtained when gels are run under nonequilibrium conditions. There is a dramatic increase in the phosphorylation of a 25 kDa protein induced by bistratene. This protein is not affected by TPA and bryostatin 5. In addition dephosphorylation of another protein of slower mobility was markedly dephosphorylated after all treatments. This experiment has been performed five times with similar results.

The concentration dependence and time course of the increase in phosphorylation of the 20 kDa protein was studied. Cells were exposed to varying concentrations of bistratene A for 15 min or to 100 nM bistratene for various lengths of time. The 20 kD spot was cut out of each gel for counting of the radioactivity and the results are shown in Figure 4A,B. The incorporation of ^{32}P increased rapidly over the first 15 min and then

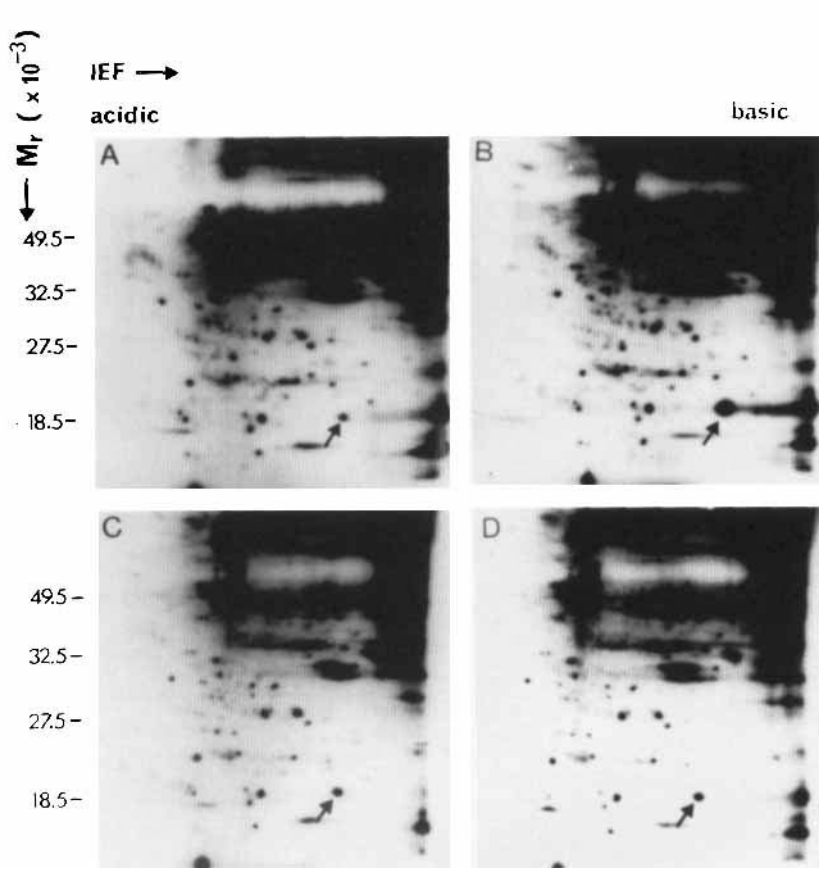

Fig. 1. Autoradiographs of phosphorylated proteins in HL-60 cells (whole cell lysates) separated by 2D SDS-PAGE. The cells were exposed to 100 nM of either bistratene A (**B**), TPA (**C**), or bryostatin 5 (**D**), after pre-equilibration with [^{32}P] orthophosphate. Control cells (**A**) received no compound. The arrows show the position of the 20 kDa protein. The dashes indicate the positions of prestained molecular weight markers from Bio-Rad which have the apparent molecular weights indicated. Accurate molecular weight and isoelectric point were determined as described in the Materials and Methods section. This experiment has been performed 12 times with the same result each time.

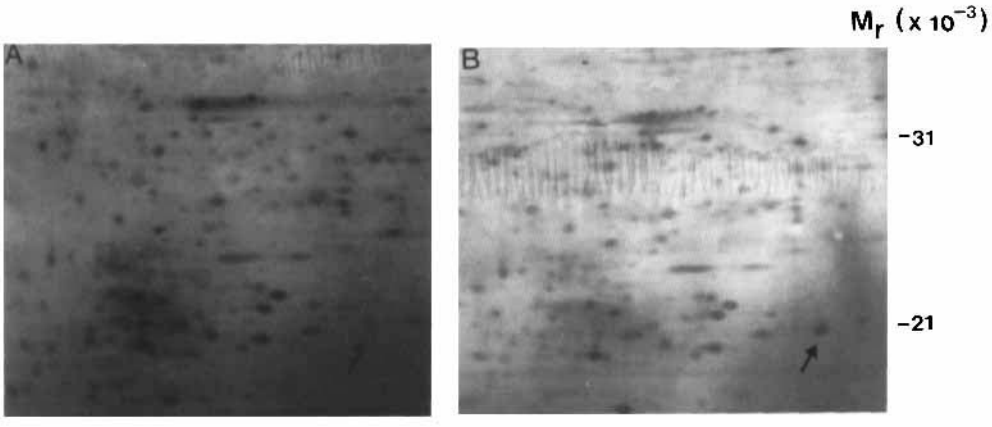

Fig. 2. Silver staining of a small region of the 2D gels from control (**A**) and bistratene-treated cells (**B**). The arrows show the position of the 20 kDa protein and the positions of low molecular weight SDS marker proteins are shown.

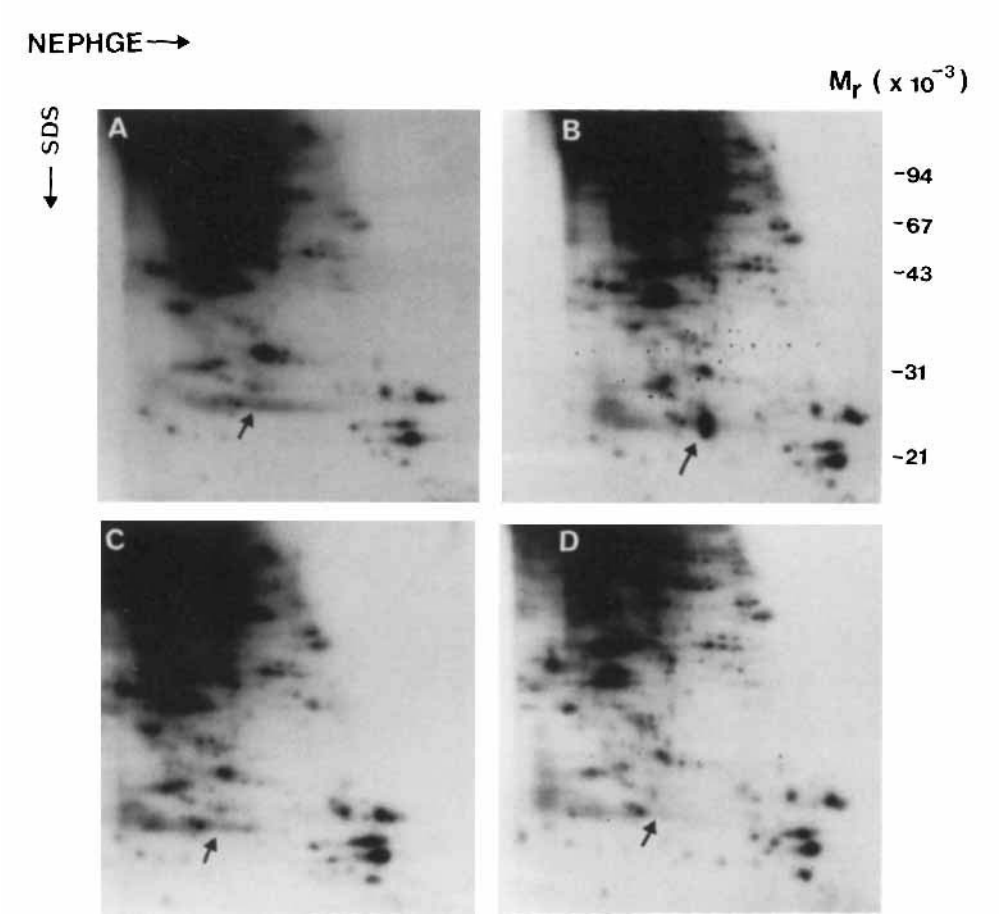

Fig. 3. Autoradiographs of phosphorylated proteins in HL-60 cells separated by NEPHGE-SDS gels. **A:** Control. **B:** Bistratene A. **C:** TPA. **D:** Bryostatin 5. The arrows indicate the position of the 25 kDa phosphoprotein induced by bistratene A.

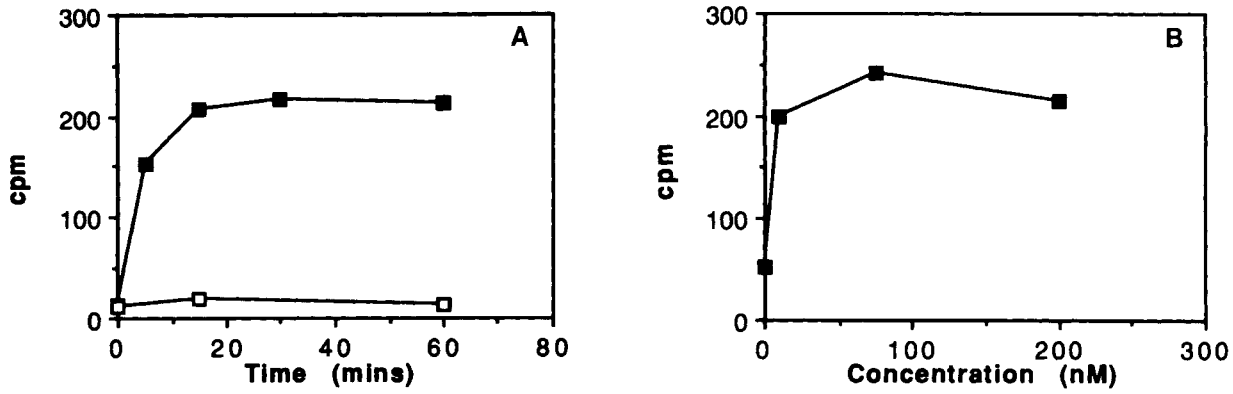

Fig. 4. **A:** Time course of increased phosphorylation of the 20 kDa protein. HL-60 cells were prelabelled with ^{32}P, then exposed to 100 nM bistratene A for the indicated times. Radioactivity in the excised gel piece was determined as described in the Materials and Methods section. Open squares represent control (untreated) cell extracts and closed squares represent extracts from bistratene treated cells. **B:** Concentration dependence of the increased phosphorylation of the 20 kDa protein. Cells were exposed to the indicated concentrations of bistratene A for 15 min.

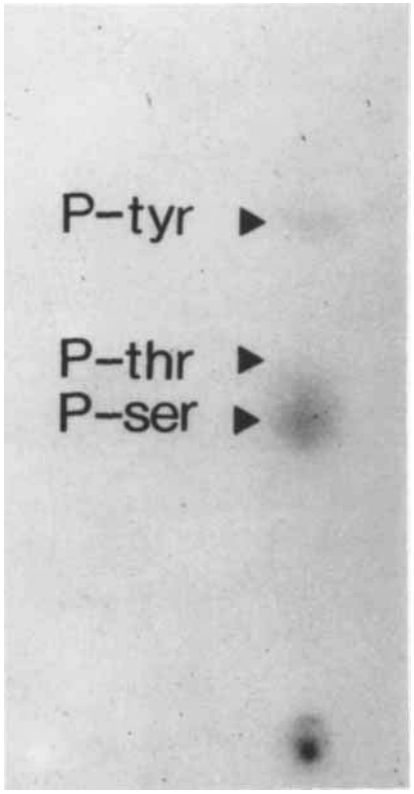

Fig. 5. Phosphoamino acid analysis of the 20 kDa protein. The labelled protein was excised from several gels, eluted, and subjected to acid hydrolysis. The phosphoamino acids were separated by two cycles of one-dimensional thin layer chromatography using absolute ethanol:25% ammonia (3.5:1.6). The positions of phosphoamino acid standards are indicated. P-ser, phosphoserine; P-Thr, phosphothreonine; P-tyr, phosphotyrosine.

reached a plateau with no further increases up to 60 min, the last time point studied. The effects of bistratene A are clearly rapid, with a fivefold increase occurring in the first 5 min. The concentration dependence is shown in Figure 4B. Near-maximal incorporation is seen at approximately 10 nM, the lowest concentration studied, with only slightly higher values occurring at 200 nM bistratene. Thus bistratene A is active in inducing these changes at very low concentrations, in the range that caused the incomplete differentiation of HL-60 cells [Watters et al., 1990].

The region of gel containing the 20 kDa protein was cut out and subjected to phosphoaminoacid analysis. The results are shown in Figure 5. The majority of the ^{32}P in the protein from bistratene-treated cells was found in the phosphoserine and phosphotyrosine peaks. A similar distribution albeit with fewer counts was seen in samples from control cells. From these experiments we conclude that the phosphorylation of the 20 kDa, pI 6.7 protein is occurring mainly on serine and tyrosine residues. In order to localise the cellular compartment which contains the 20 kDa protein, HL-60 cells were treated with bistratene A (100 nM) for 15 min. The nuclear and cytoplasmic fractions were then prepared and each run on separate 2D gels. The resulting autoradiographs are shown in Figure 6. These experiments demonstrate that the 20 kDa phosphoprotein is located in the cytoplasm.

DISCUSSION

Bistratene A is a unique compound with some similarities in action to the phorbol ester TPA

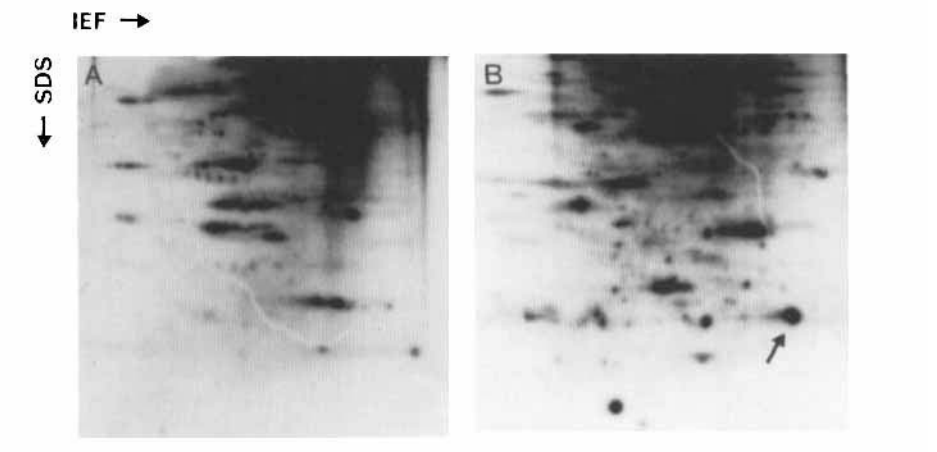

Fig. 6. Autoradiographs of phosphoproteins in nuclear (**A**) and cytoplasmic (**B**) fractions of HL-60 cell lysate after treatment with bistratene A (100 nM) for 15 min. The arrow indicates the position of the 20 kDa protein.

and to bryostatin. It induces HL-60 cells to differentiate although the extent of differentiation is not as complete as that seen with TPA and bryostatin [Watters et al., 1990]. It is also potent in causing growth inhibition of the A549 human lung carcinoma cell line [Stanwell et al., 1991].

Treatment of HL-60 cells with TPA has been shown to give rise to change in the phosphorylation status of a variety of proteins [Feuerstein and Cooper, 1983; Mita et al., 1984; Zylber-Katz and Glazer, 1985; Faille et al., 1986; Anderson et al., 1985]. Anderson et al. [1985] have shown that 100 nM TPA led to the phosphorylation of at least ten different proteins in HL-60 cells. Amongst these they identified a major cytoplasmic protein 64 kDa in size, a 56 kDa cytoskeletal protein, and three proteins of 60 and 64 kDa localised to the cell nucleus. In this study using HL-60, the major changes in protein phosphorylation were observed in the size range 20–25 kDa. A rapid increase in the phosphorylation of a 17 kDa protein was detected in the same cells using 2D SDS-PAGE by Feuerstein and Cooper [1983]. While this protein reached a maximum at approximately the same time as the 20 kDa protein induced by bistratene it is unlikely to be the same. The isoelectric point is 5.5 compared with 6.7 for the bistratene-induced protein and it was only phosphorylated on serine residues, whereas the bistratene-induced protein is phosphorylated on both serine and tyrosine.

Bryostatin 1 enhanced the phosphorylation of the same set of proteins as did TPA [Warren et al., 1988]. In addition this compound caused the appearance of two phosphorylated protein spots of molecular weight 70 kDa with pI's of 6.3–6.4 within 30 min. These proteins were localised to the cytoskeleton. TPA was also able to induce their phosphorylation but only at very high concentrations. Fields et al. [1988] have shown that the nuclear envelope polypeptide, lamin B, is a major target for bryostatin-induced phosphorylation. This protein has a molecular weight of 67 kDa and an isoelectric point of 6.0. In all cases the only amino acid shown to be phosphorylated was serine. On the other hand in this study, phosphorylation of the 20 kDa protein was at both serine and tyrosine. Since TPA and bryostatin 1 both appear to function by activation of protein kinase C it is not surprising to see similarities in the protein phosphorylation patterns induced, and in the amino acids phosphorylated. We have previously shown that the concentration of bistratene needed for activation of PKC in vitro is 10 μM which is much higher than that required to cause differentiation of HL-60. On this basis we concluded that PKC was not directly involved in bistratene action. The present study showing a different pattern of protein phosphorylation and the presence of phosphotyrosine residues in addition to phosphoserine in a major altered protein, provide further evidence that bistratene functions through a mechanism which is distinct from that of either TPA or bryostatin 5. Retinoic acid which also causes differentiation of HL-60 cells along the granulocytic pathway did not cause an increase in the phosphorylation of the 20 kDa protein (results not shown). While phosphorylation is the major effect observed, a twofold increase in the amount of the 20 kDa protein also occurs. This suggests that in addition to post-translational effects, the action of bistratene may be mediated at the level of control of transcription.

Gouiffes et al. [1988], who independently isolated bistratene A which they named bistramide A, performed electrophysiological studies and found an effect of the compound on cardiac action potentials consistent with a block of sodium conductance [Roussakis et al., 1991]. Since the concentration of bistratene A used in their studies was in the micromolar range it is possible that the effects on sodium channels are secondary to effects on protein phosphorylation. Numann et al. [1991] have recently shown that phosphorylation of the α-subunit of the rat brain sodium channel results in modulation of the sodium current.

In summary, an early event of bistratene A action in HL-60 cells is the induction of changes in protein phosphorylation patterns. This occurs at very low concentrations (nM) and very rapidly (within 5 min). Bistratene A does not inhibit protein phosphatases type 1 and 2A as does okadaic acid [A. Sim and D. Watters, unpublished observations] and does not appear to function by activation of protein kinase C [Watters et al., 1990]. The nature of the signal transduction system affected by this ascidian toxin remains to be determined, and bistratene A promises to be useful tool for studying cellular growth regulation. Work is in progress to isolate and identify the phosphoproteins altered by bistratene A in order to obtain further information on this system.

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