Differential Modulation of Protein Kinase C by Bryostatin 1 and Phorbol Ester

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SAKO, T., OHSHIMA, S., YOSHIZUMI, T. and SAKURAI, M. Differential Modulation of Protein Kinase C by Bryostatin 1 and Phorbol Ester. Tohoku J. exp. Med., 1988, 156 (3), 229-236 — Bryostatin 1, a macrocyclic lactone, activated protein kinase C purified from mouse brain in a dose-dependent fashion to the same degree as phorbol 12-myristate 13-acetate (PMA). There was no significant difference in calcium and phosphatidylserine requirements for activation of protein kinase C between bryostatin 1 and PMA. We also found no significant difference in the inhibitory effect between staurosporine and H-7 known to be potent inhibitors of protein kinase C. These data suggest that bryostatin 1 and PMA activate protein kinase C in a similar way. We found, however, that negative modulation of protein kinase C with bryostatin 1 was weaker than that with PMA. The reason of this difference was unclear. It may possibly suggest that there is some difference in configuration of protein kinase C after binding between these activators. ——— protein kinase C; bryostatin 1; cofactor requirement

The phospholipid-Ca\(^{2+}\)-dependent protein kinase, protein kinase C (PKC), has been postulated as an important regulatory element in signal transduction and tumor promotion (Nishizuka 1984). Considerable pieces of evidence suggest that tumor promotion by phorbol ester involves the activation of PKC (Blumberg et al. 1984). PKC was found to be copurified with the cellular phorbol ester receptor. Further, \([\beta\text{H}]\)-phorbol 12,13-dibutyrate (PDBu) binds to homogenous preparation of PKC isolated from mouse brain and the phorbol ester compete directory with 1,2-diacylglycerols, the natural activator of PKC, for binding to this enzyme (Leach et al. 1983; Niedel et al. 1983; Sando and Young 1983).

Bryostatin 1 is a macrocyclic lacton isolated from the marine bryozoa Bugula neritina (Pettit et al. 1982). Although structurally different from phorbol esters, it contains a 3-dimentional array of the functional group homologous to the postulated phorbol ester pharmacophore. Bryostatin 1 was initially isolated based on its anti-neoplastic activity against P-388 cells. A more recent investigation showed that bryostatin 1 induced a number of phorbol ester effects, including
mitogenesis and desensitization to phorbol esters in Swiss 3T3 cells (Smith et al. 1985), activation of human polymorphonuclear leukocytes (Berkow and Kraft 1985), epidermal growth factor (EGF) binding inhibition and ornithine decarboxylase (ODC) induction in mouse epidermal cells (Sako et al. 1987). Likewise, bryostatin 1 activated PKC in vitro and competitively inhibited PDBu binding (Berkow and Kraft 1985). Paradoxically, however, bryostatin 1 did not induce the differentiation and further blocked the phorbol ester-induced differentiation of the HL-60 cells (Kraft et al. 1986) and mouse epidermal cells (Sako et al. 1987). Bryostatin 1 also blocked the inhibitory effect of phorbol ester on differentiation of Friend erythroleukemia cells (Dell’aquila et al. 1987).

The objective of the present experiments was to investigate the molecular mechanism of bryostatin 1 for PKC activation by the mixed micellar assay. Here, we report that cofactor requirement for activation of PKC. We found that there was no significant difference for calcium ion and phosphatidylserine (PS) requirements between bryostatin 1 and phorbol 12-myristate 13-acetate (PMA). Also, there was no significant difference in inhibitory effects between staurosporine and 1-(5-isoquinolinylsulfonfyl)-2-methylpiperazine (H-7), but some difference in negative modulation of PKC.

### Materials and Methods

#### Materials

Bryostatin 1, isolated from *Bugula neritina*, was kindly provided by Dr. G.R. Pettit. PMA, PS, histone III-S, fatty acid free bovine serum albumin and H-7 were purchased from Sigma (St. Louis, MO, USA). ATP was obtained from Boehringer Manheim (Indianapolis, IN, USA). Staurosporine was purchased from Kyowa Medex (Tokyo).

#### Methods

**Protein kinase.** Protein kinase C was purified from mouse brain cytosol according to the method of Jeng et al. (1986) through the mono-Q/ATP step. Purity was greater than 95% by silver staining following SDS polyacrylamide gel electrophoresis.

**Protein kinase assay.** Protein kinase activity was assayed by the mixed micellar method (Hannun et al. 1985). The required amount of PS in chloroform was dried under a stream of nitrogen in a glass tube and solubilized in 1.5% Triton X-100 by vortexing and incubating at 27°C for 5 min.

The reaction mixture (100 μl) contained 20 mM tris HCl, 200 mg/ml of histone III-S, 10 μM (5-10 × 10⁶ cpm) [γ-³²P]ATP, 10 mM MgCl₂ and CaCl₂ (at the concentration indicated in figures) or 1 mM ethylene glycol bis (2-aminoethyl)ether)-N, N', N'-tetraacetic acid (EGTA). The Triton X-100, PS mixed micellar solution was added to the reaction mixture. The reaction was started by addition of enzyme and terminated after 5 min at 25°C by cooling in ice and 25 μl of aliquot was put on the Whatmann P81 ion exchange chromatography paper. The paper was washed with ice cold 20% TCA and water by 3 times. The paper was placed in a scintillation vial and ³²P was counted.

Kinase activity in the absence of PS or Ca²⁺ (in the presence of 2 mM EGTA) was subtracted as background. PKC activity was expressed as nmol/mg protein/min.

Data were analyzed by using Student's *t* test. Points in figures represent the means of three different experiments (n = 9).
RESULTS

Protein kinase activation. Fig. 1 shows the activation of PKC by bryostatin 1 or PMA in the presence of Ca^{2+} at 100 \mu M and 6 mole% PS. These compounds activated PKC in a dose-dependent fashion to the same degree.

Phosphatidylserine requirement. Fig. 2 shows the dependence of PKC activation by bryostatin 1 or PMA on PS concentration in the presence of Ca^{2+} (100 nM) and 6 mole% PS.

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**Fig. 1.** Activation of mouse brain PKC by bryostatin 1 or PMA. The assay was carried out as described in "Methods" in the presence of Ca^{2+} at 100 \mu M and PS at 6 mole% with varying concentrations of bryostatin 1 (○—○) or PMA (●—●).

**Fig. 2.** PS dependence of PKC activation by bryostatin 1 at 100 nM (○—○) or PMA at 100 nM (●—●). The assay was carried out in the presence of Ca^{2+} at 100 \mu M. One mole% of PS corresponds to 43 \mu M.
Fig. 3. Calcium ion dependence of PKC activation by bryostatin 1 at 100 nM (---○) or PMA at 100 nM (●-●). The assay was carried out in the presence of PS at 6 mole% with varying concentration of Calcium ion or 2 mM EGTA. Greater than 3 mole% PS was required before activation became apparent and saturation was achieved at 6-8 mole%. There was also no significant difference (p > 0.05) between bryostatin 1 and PMA.

Calcium ion requirement. Bryostatin 1 and PMA activated PKC in calcium ion dependent manner (Fig. 3). Greater than 100 μM of calcium ion was required

Fig. 4. Dose-response for the effect of H-7 on PKC activity. The assay was done in the presence of bryostatin 1 at 100 nM (---○) or PMA at 100 nM (●-●) and absence of activator (□-□). Ca²⁺ (100 μM) and PS (6 mole%) was present in the reaction mixture.
before activation and saturation was found over 1000 μM. No significant difference ($p > 0.05$) was found in calcium ion requirement between bryostatin 1 and PMA.

**Effects of PKC inhibitors.** Staurosporine and H-7 have been to known was

![Fig. 5. Dose-response for the effect of staurosporine on PKC activation. The activity was measured in the presence of bryostatin 1 at 100 nM (○→○) or PMA at 100 nM (●→●), and in the absence of activator (□→□). Ca$^{2+}$ (100 μM) and PS (6 mole%) was present in the reaction mixture.](image)

![Fig. 6. Negative modulation of PKC by bryostatin 1 or PMA. After preincubation of the reaction mixture containing PKC with bryostatin 1 at 100 nM (○→○) or PMA at 100 nM (●→●) for indicated time at 25°C. Histone phosphorylation was measured upon the addition of [$\gamma$-32P] ATP. Ca$^{2+}$ (100 μM) and PS (6 mole%) was present in the reaction mixture.](image)
potent PKC inhibitors. H-7 inhibits PKC by competing with ATP (Hidaka et al. 1984), and staurosporine does without competing with ATP, Ca$^{2+}$, PS or histone (Tamaoki et al. 1986). Activation of PKC by bryostatin 1 or PMA was inhibited by H-7 (Fig. 4) and staurosporine (Fig. 5) in a dose-dependent fashion. We did not find any difference ($p>0.05$) between bryostatin 1 and PMA in the effects of inhibitors.

Negative modulation of PKC. Pretreatment of PKC with PMA and PS has been known to result in complete inhibition of ATP/phosphotransferase activity, irreversibly (Inagaki et al. 1986). In the presence of PMA, the inactivation of PKC was rapid and greatly enhanced. However, in the presence of bryostatin 1, inactivation was slower and was less than with PMA (Fig. 6).

**DISCUSSION**

Bryostatin 1 was originally detected by Pettit et al. in the marine bryozoan *Bugula neritina* (Pettit et al. 1982). Bryostatin 1 competed effectively for PDBu binding (Berkow and Kraft 1985), activate PKC purified from HL-60 cells (Kraft et al. 1986) and from GH$_4$C$_5$ cells (Ramsdell et al. 1986). In the present paper, we also showed that bryostatin 1 activated PKC purified from mouse brain as PMA did. We also found no significant difference for calcium ion and PS requirements in protein kinase activation between bryostatin 1 and PMA. These findings suggest that bryostatin 1 may activate PKC in similar manner to PMA.

Biologically, bryostatin 1 is known to mimic the effects of phorbol ester on DNA synthesis in Swiss 3T3 cells (Smith et al. 1985), activation of human polymorphonuclear cells (Berkow and Kraft 1985), protein synthesis and prolactin release in GH$_4$C$_5$ cells (Ramsdell et al. 1986). Sako et al. (1987) also reported that bryostatin 1 mimicked the effects of PDBu on EGF binding and ODC induction in primary mouse epidermal cells. Bryostatin 1, however, does not necessarily mimic all actions of phorbol ester, such as on HL-60 cell differentiation (Kraft et al. 1986), terminal differentiation in the primary mouse epidermal cells (Sako et al. 1987), and cell stretching of GH$_4$C$_5$ cells (Ramsdell et al. 1986). Furthermore, bryostatin 1 blocked the effects of phorbol ester on differentiation in these cells and human colon cancer cells (McBain et al. 1987). The reason of these differences is unclear. Recent evidence focused on the PKC degradation induced by bryostatin. Bryostatin 1 has been reported to stimulate more rapid degradation of PKC than phorbol ester (Reeves et al. 1987). Edgar Rivedal, one of our collaborators, also demonstrated the rapid rate of PKC degradation induced by bryostatin 1 in mouse epidermal cells and fibroblast (unpublished). In the present paper, we showed the difference for PS-induced inactivation of PKC between bryostatin 1 and PMA. These findings suggest that the alteration in configuration of PKC after binding of bryostatin 1 might cause the difference between bryostatin 1 and PMA on negative modulation and break-down of PKC.

We conclude that bryostatin 1 and PMA activate PKC by a similar mecha-
Differential Modulation of PKC between TPA and Bryostatin 1

nism, although the precise configurational difference of PKC may be induced after binding and activation by bryostatin 1 compared to PMA.

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