

RAPID SCREENING METHOD FOR INHIBITORS OF
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Specific inhibitors of protein kinase C (PKC) were screened for with a unique detection system, named bleb forming assay. When K562, a human chronic myeloid leukemia cell, was treated with phorbol 12,13-dibutylate (PDBu) or teleocidin which are activators of PKC, many blebs appeared on the cell surface of K562 within 10 minutes. This appearance of blebs is inhibited by staurosporine and H7 which are known to be PKC inhibitors. Teleocidin and PDBu did not induce bleb formation of HL60, a human acute promyelocytic leukemia cell, and the mouse Friend leukemia cell, even though their morphology was changed 24 hours after treatment with teleocidin or PDBu. Many inducers of terminal differentiation of K562 have the same effect on HL60 and Friend cells. However, the bleb inducing activity of PKC activators seems to be specific for K562. The bleb forming assay satisfied the criteria (simplicity and specificity) required for preliminary screening of activators or inhibitors of PKC.

Teleocidins A and B, and tautomycin (a new antibiotic isolated in our laboratory) were identified as activators of PKC, and also staurosporine and isoflavones (daidzein and genistein) as inhibitors.

Previous studies^{1,2} revealed that protein kinase C (PKC) which requires Ca^{2+} and phospholipid for its activity is activated by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)³. Subsequently, PKC was characterized as the receptor for tumor promoting TPA^{4,5}. As PKC has pleiotropic effects in mammalian cells^{6,7}, it is worthwhile investigating its biological function. Out of several possible approaches for studying the function of PKC, we have chosen to look for specific inhibitors for PKC. We focused especially on low-molecular-weight inhibitors produced by actinomycetes.

A number of investigators have developed screening systems for activators or inhibitors of PKC^{8,9}, however, most of these procedures are time-consuming and laborious. In this paper, we describe our own screening method named bleb forming assay based on the unique characteristics of K562, a human chronic myeloid leukemia cell¹⁰. Several activators and inhibitors of PKC have been detected with this assay.

Materials and Methods

Cell Culture

K562¹⁰ was obtained from Dr. KENJI KOHNO (National Institute of Basic Biology, Okazaki, Japan). HL60¹¹, a human acute promyelocytic leukemia cell and mouse Friend leukemia cell¹² were provided by Dr. SHINTARO NOMURA (The University of Tokyo, Japan). These cells were grown in RPMI1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco, Grand Island, U.S.A.). All cells were negative for the presence of *Mycoplasma* by culture techniques.

Chemicals

Phorbol 12,13-dibutylate (PDBu), phorbol 12,13-didecanoate (PDD), 4 α -phorbol 12,13-didecanoate (4 α -PDD) were purchased from Sigma (St. Louis, U.S.A.). H7 is a product by Seikagaku

Kogyo Co., Ltd. (Tokyo, Japan). Olivoletins A and C were generously provided by Professor KOUICHI KOSHIMIZU, Kyoto University. Teleocidins A, B, isoflavones and staurosporine, which were detected by our bleb assay, were purified from the culture broth of unidentified streptomycetes.

Bleb Forming Assay

Cells were seeded at a density of 1×10^4 cells per well into 96-multiwell plates, which were then incubated with $2 \mu\text{M}$ of PDBu in the presence or absence of PKC-inhibitors. Cell morphology was observed under a microscope. 10 nM of staurosporine inhibited the bleb formation of K562 completely under these conditions.

Phosphorylation Assay of PKC

PKC was partially purified from a New Zealand white rabbit brain by KITANO's procedure¹³. The reaction mixture contained 25 mM Tris-HCl (pH 7.0), 10 mM MgCl_2 , 0.5 mM CaCl_2 , 50 $\mu\text{g}/\text{ml}$ phosphatidylserine, 10 mM β -mercaptoethanol, and 0.5 mg/ml histone as a substrate. 35 μl of reaction mixture, 5 μl of inhibitor, and 10 μl of PKC solution were preincubated at 37°C for 10 minutes, and then phosphorylation was started by adding 10 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($>50,000$ cpm). After a further 10 minutes incubation, the reaction was stopped by heating with LAEMMLI's sodium dodecyl sulfate (SDS) sample buffer¹⁴. Phosphorylated histone was detected by autoradiography after separation by SDS polyacrylamide gel (15.0%) electrophoresis.

Results and Discussion

Time Course of Bleb Formation

K562 is known to undergo differentiation to erythrocytes under the influence of several inducers^{15,16} and can also be made to differentiate to granulocytes under specific conditions¹⁷. However, no report has described the adherence of K562 induced by tumor promoters, even though there have been many reports about morphological changes of HL60 induced by tumor promoters^{18,19}. We observed that a small population of K562 adhered to plastic dishes after 24 hours of incubation with PDBu or teleocidin (Fig. 1(a)). HL60 and Friend cells changed their morphology 24 hours after addition of PDBu, but there was no significant morphological change observed at shorter times (Figs. 1(b) and 1(c)). On the other hand, microscopic observation at appropriate times revealed that many blebs appeared on the K562 cell surface within 10 minutes of PDBu addition and that these blebs disappeared spontaneously after 12 hours (Fig. 2). Some chemical compounds (actinomycin D, hemin, retinoic acid and so on) induce the terminal differentiation of K562, HL60, and Friend cells²⁰. However, bleb formation by tumor promoters which are inducers of these three cells, only occurred with K562.

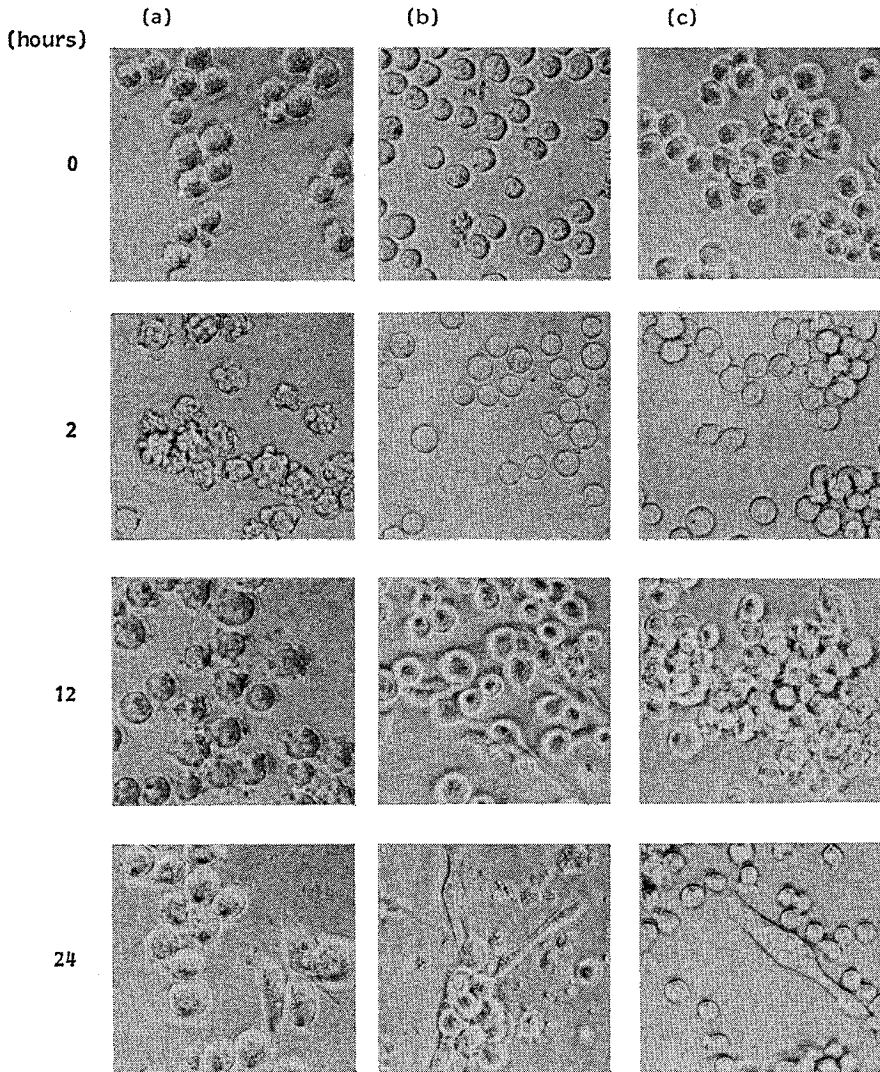
PKC Takes Part in Bleb Formation of K562

Two different groups of tumor promoters were tested for their bleb forming activity; plant terpenes (phorbol esters) and indole alkaloids (teleocidin related compounds). PDBu had the strongest activity among the three phorbol esters tested, PDD has medium and 4α -PDD has no bleb forming activity (Fig. 3(a)). This result is comparable to the order of the tumor promoting activity as assayed in the mouse skin system and in the HL60 cell adherence system²¹.

10 nM of teleocidins A and B induced blebs on 100% of K562 cells and their activity was similar to PDBu. Olivoletins A and C are known to have slight tumor promoting activity although they have closely related structures to the teleocidins²². Fig. 3(b) demonstrates that even high concentrations of olivoletins did not induce blebs at all.

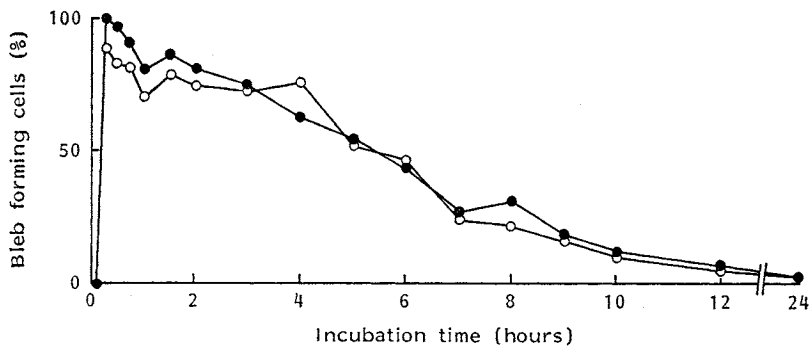
These results suggested that our bleb forming assay could be useful to screen for TPA-type pro-

Fig. 1. Microscopic observation of morphological changes.



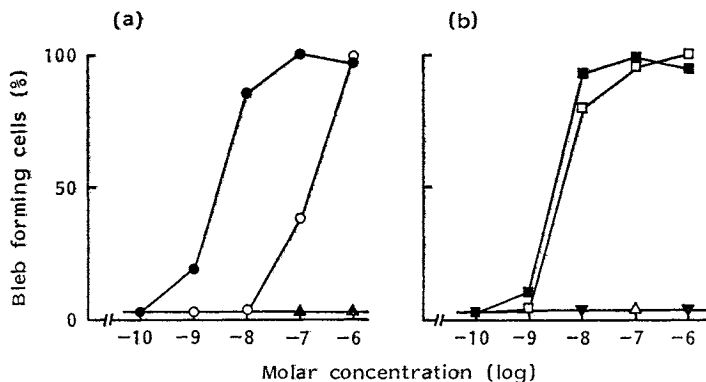
K562 (a), HL60 (b), and Friend leukemia cells (c) were treated with 1 μM of PDBu for indicated time.

Fig. 2. Time course of bleb formation of K562 cells.



PDBu (●) and teleocidin B (○) were added to K562 at a concentration of 1 μM .

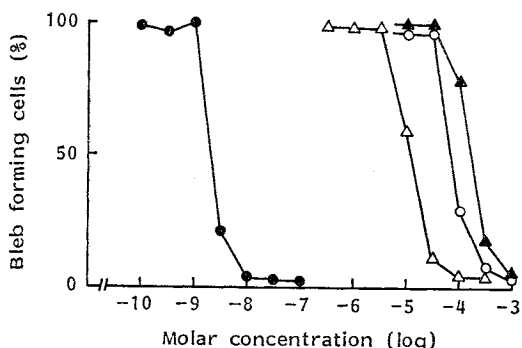
Fig. 3. Bleb forming activity of tumor promoters.



The following compounds were assayed; (a) PDBu (●), 4 α -PDD (▲), PDD (○), (b) teleocidin A (■), teleocidin B (□), olivetolet A (▼), olivetolet C (△).

moters. It was reasoned that the tumor promoting PDBu and teleocidins enhanced the activity of PKC and caused bleb on cell membrane of K562, because when cells were treated with staurosporine and H7 (known inhibitors of PKC) bleb formation by PDBu and teleocidin was completely suppressed (Fig. 4). Bleb formation was observed only at a physiological temperature and not at 4°C. The mechanism of bleb formation is still not known but our data suggest that the bleb forming activity may have a close relationship to the induction of adherence of HL60, and that the bleb forming assay satisfied the criteria for a routine screening system *i.e.*, simplicity, quantitiveness, and specificity.

Fig. 4. Suppression of bleb formation.

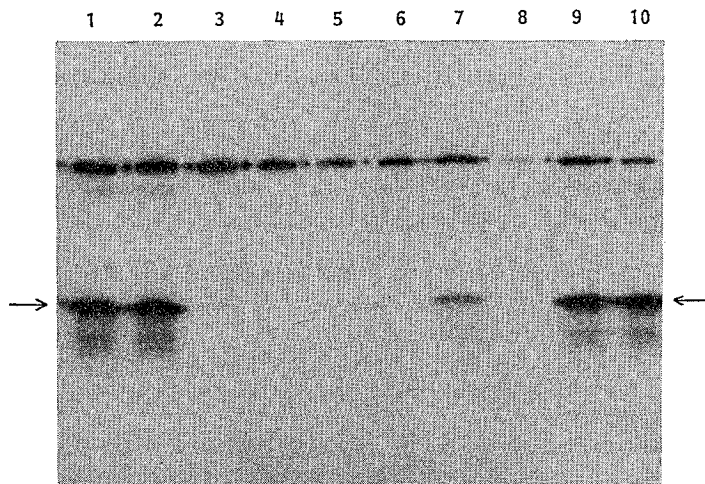


Bleb forming cells were counted under a microscope 15 minutes after addition of 1 μ M of PDBu. K562 cells were pretreated with following compounds for 10 minutes before addition of PDBu; staurosporine (●), H7 (△), daidzein (○), and genistein (▲).

Screening of Culture Broths of Streptomycetes

We have screened about 1,000 strains of soil actinomycetes using the bleb forming assay, and found 9 (0.9%) different strains that were active. Active substances were extracted with ethyl acetate and identified by UV spectra. All of them gave the same UV absorption spectrum as teleocidin A or B. The results indicate that this assay method is useful as a specific and sensitive screening test for TPA-type tumor promoters.

Bleb suppressing substances were produced by several strains. Two strains produced staurosporine, which suppressed bleb formation by PDBu completely at low concentrations. Three strains produced isoflavones (daidzein and genistein) which inhibited bleb formation at relatively high concentrations (Fig. 4). We used H7, which is a synthetic inhibitor of PKC, as a control in the bleb suppression assay. H7 inhibited bleb formation completely at a concentration of 0.1 mM. Isoflavones were 10 times less active than H7. OGAWARA *et al.* reported²³⁾ that genistein caused a 50%-inhibition of the tyrosine kinase activity at a concentration of 5~50 μ M. However, 50 μ M of daidzein and

Fig. 5. Inhibition of *in vitro* kinase activity of PKC.

The band indicated by an arrow is a phosphorylated histone, a substrate of PKC. 1 μM of PDBu was added to PKC of all lanes except lane 2. A basal activity of PKC was measured without inhibitor as described in the text (lane 2). PKC activity was completely inhibited by 1 mM of H7 (lane 3) and 10 nM of staurosporine (lane 4). PKC in lanes 5, 6, and 7 was treated with 300 μM , 100 μM , and 30 μM of daidzein, respectively. Genistein was added to lanes 8, 9, and 10 at the same concentrations as daidzein.

200 μM of genistein were required for inhibition of bleb formation.

As shown in Fig. 5, H7 and staurosporine inhibited phosphorylation of histone (lower band in gel) but not an unidentified upper band. Daidzein which is a 5-dehydroxy form of genistein also inhibited the phosphorylation of histone specifically (lanes 5 and 6), although daidzein inhibited the tyrosine kinase carried on the receptor of epidermal growth factor (data not shown). On the other hand, a high concentration of genistein inhibited phosphorylation of both bands, presumably because it inhibits a number of kinases.

Isoflavones might be generated by hydrolysis of glycosylated precursors contained in soy bean meal which is used as a fermentation medium for actinomycetes. However, the inhibition of PKC activity can not be detected in fresh media without bacterial culture.

Inhibition of bleb formation is rather specific so far, but some non-specific inhibitors were detected and identified as nystatin and amphotericin B which are tetraene antibiotics. In these cases, however, the concentration which showed the bleb suppressing activity was almost toxic to the cells. Moreover, such a high dose of antibiotic could neither suppress bleb formation completely nor inhibit PKC activity *in vitro*. Therefore, this inhibition by tetraene antibiotics was thought to be a non-specific effect mediated through membrane damage.

It is noteworthy that cytochalasins B and D, mold metabolites, showed bleb forming activity. However, this bleb formation is thought to be independent of PKC activity.

Tautomycin was originally isolated as an antifungal antibiotic in our laboratory²⁴⁾. We found this antibiotic induced bleb formation at a concentration of 1~10 ng/ml and also stimulated activity of PKC in a different manner to the TPA-type promoters. Details will be discussed in a subsequent paper²⁵⁾.

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