INDUCTION OF MORPHOLOGICAL CHANGE OF HUMAN MYELOID LEUKEMIA AND ACTIVATION OF PROTEIN KINASE C BY A NOVEL ANTIBIOTIC, TAUTOMYCIN

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(Received for publication December 4, 1987)

A novel antibiotic tautomycin induced many blebs on the surface of K562 human chronic myeloid leukemia cells, similar to the morphological change induced by phorbol esters. However, tautomycin did not induce nitroblue tetrazolium reducing activity, when HL60 human promyelocytic leukemia cells were caused to differentiate by quinomycin into mature granulocytes. It did not induce spread of HL60 cells, one of the phenotypes of mature macrophages. In addition, it did not compete with phorbol dibutyrate to bind to the cell surface of K562 cells. However, tautomycin significantly activated protein kinase C (PKC) extracted from K562 cells. These results indicate that tautomycin is a new activator of PKC, distinct from phorbol esters.

Phorbol esters are tumor promoters and have various biological activities which are attributed to the activation of protein kinase C (PKC), a high affinity receptor for these compounds[1,2]. Thus, PKC is assumed to play an important role in various biological reactions in eukaryotic cells.

We recently found that phorbol esters induced many blebs on the cell surface of K562 human chronic myeloid leukemia cells[3]. Bleb formation seemed to be associated with the activation of PKC because the inducing activity of phorbol esters was correlated with their tumor promoting activity and because it was prevented by specific inhibitors of PKC. Screening of many kinds of biologically active substances for inducing bleb formation, revealed that the cytochalasins but not colchicine induced a similar morphological change, indicating that the microfilament system may be involved. Since it is a rapid effect and easily observable microscopically, it is suitable for screening of modulators of PKC.

Here, we describe the induction of bleb formation by a novel antibiotic tautomycin, using the above screening system. Tautomycin is an antibiotic found in our laboratory[4]. It had strong toxicity against a variety of eukaryotic cells including fungi, yeasts and animal cells. It showed an excellent protective effect against cucumber gray mold in the pot test at the dose of 6 ppm. The structure is under investigation.

Materials and Methods

Cell Culture

The human leukemic cell lines, HL60 and K562, were cultured using RPMI1640 medium supplemented with antibiotics and 10% fetal bovine serum.

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Nitroblue Tetrazolium (NBT) Reducing Activity

HL60 cells were differentiated into mature granulocytes by culturing for 3 days at 37°C in the presence of 1 ng/ml quinomycin. Cells were washed three times to remove quinomycin and cultured with appropriate agents in the presence of 1 mg/ml NBT for 1 hour at 37°C. Cells with blue-black deposits of formazan were counted microscopically.

Binding Assay of [^{3}H]Phorbol Dibutyrate

Experiments were carried out according to the method described by SOLANKI *et al.*[5]. Each tube contained K562 cells (2×10^6 cells) and 10 ng/ml [^{3}H]phorbol dibutyrate (PDBu) and appropriate agents in 0.25 ml of RPMI1640 medium (without fetal bovine serum). The incubation was carried out for 45 minutes at 4°C and the reaction was terminated by adding 0.5 ml of cold phosphate-buffered saline (PBS, 0.8% NaCl, pH 7.4). The tubes were immediately centrifuged at $1,500\times g$ for 5 minutes at 25°C. The supernatant was carefully removed by aspiration and pelleted cells were further washed three times with 0.5 ml of ice-cold PBS. Finally cells were filtered on a glass filter. Radioactivity on filters was counted by liquid scintillation system.

Partial Purification of PKC

PKC was partially purified as described by FORSBECK *et al.*[6]. K562 cells ($1\sim3\times10^8$ cells) were harvested from late log cultures. They were then washed twice with PBS and centrifuged at $1,000\times g$ for 5 minutes. The washed cell pellet was suspended in 3 ml ice-cold 4 mM EDTA, 1 mM ethylene glycol bis(2-aminoethylether) tetraacetic acid (EGTA), 40 mM Tris-HCl (pH 7.5), homogenized with ten strokes in a Teflon-glass homogenizer, and treated for 20 seconds in a sonicator water bath. The lysed cell suspension was then mixed with an equal volume of 40 mM Tris-HCl (pH 7.5), 0.66 M sucrose, 0.1 M mercaptoethanol, 1% Triton X-100 and centrifuged at $100,000\times g$ for 60 minutes at 4°C. The resultant high speed supernatant was applied to a 2-ml bed volume DEAE-Sephacel column equilibrated in 20 mM Tris-HCl (pH 7.5) - 2 mM EDTA - 0.5 mM EGTA. The column was washed with 4 ml equilibrating buffer, then 4 ml equilibrating buffer containing 50 mM NaCl. PKC was eluted with 4 ml equilibrating buffer containing 150 mM NaCl. The elute was suspended in equal volume of glycerol and stored at −20°C until used.

PKC Assay

PKC activity was determined by measuring the Ca^{2+} and phospholipid-dependent phosphotransferase reaction between [γ-^{32}P]ATP and histone III-S in the presence of PKC[7]. Each tube contained 20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), phosphatidylinositol (20 μg/ml) (or no phospholipid), 70 μM [γ-^{32}P]ATP, histone III-S (0.67 mg/ml), 15 μg partially purified PKC and where indicated, various agents, in 200 μl. Reaction was initiated by the addition of enzyme and incubated at 30°C for 5 minutes. Reaction was terminated by the 1 ml 10% TCA. 5 μl bovine serum was added as carrier protein, then the tubes were centrifuged at $1,000\times g$ for 5 minutes. The pellet was dissolved in 100 μl 1 M NaOH and reprecipitated by adding the 1 ml 10% TCA. The pellet was washed three times in this manner and finally filtered on glass filter paper. Radioactivity on filters was determined by liquid scintillation counting.

Chemicals

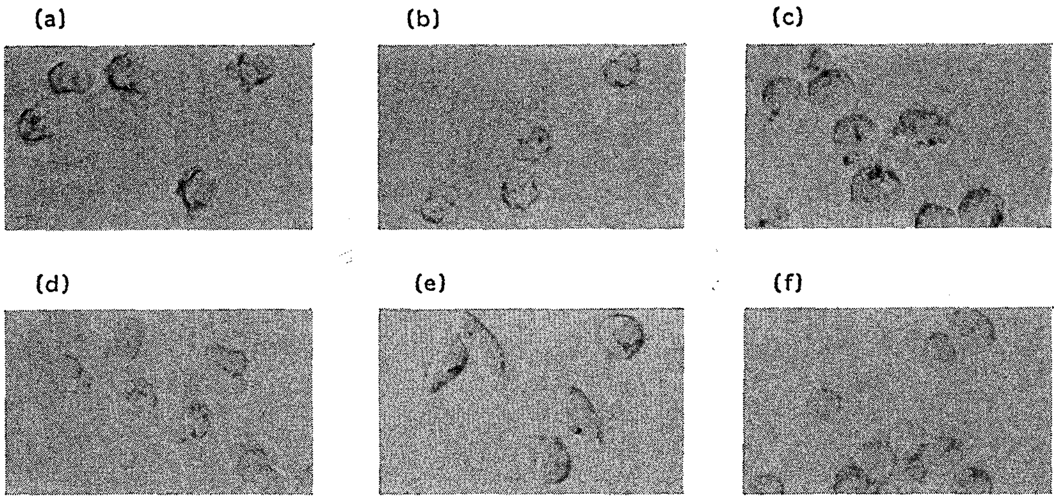
Radioactive compounds were obtained from New England Nuclear (Boston, MA, U.S.A.). PDBu, cytochalasin D, phosphatidylinositol and histone III-S were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). RPMI1640 medium was obtained from Nissui Seiyaku (Tokyo, Japan). Sera were obtained from Flow Laboratories (North Ryde, N.S.W., Australia). H7 was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). DEAE-Sephacel was obtained from Pharmacia (Uppsala, Sweden). Tautomycin and quinomycin were purified respectively, from the broth filtrate and the mycelium extract of unidentified actinomycetes.

Results

Induction of Bleb Formation by Tautomycin

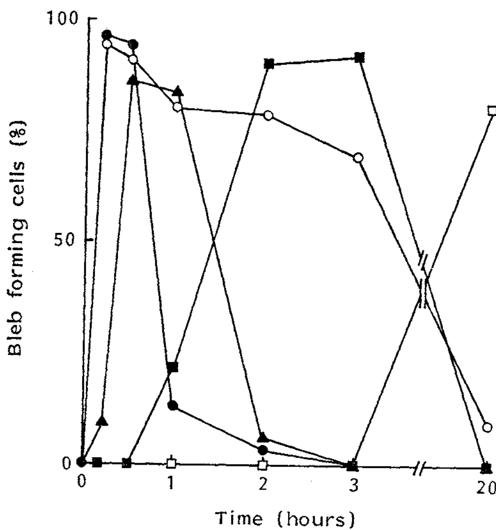
An active tumor promotor, PDBu, induced many blebs on the surface of K562 human chronic

Fig. 1. Morphological change of K562 cells.



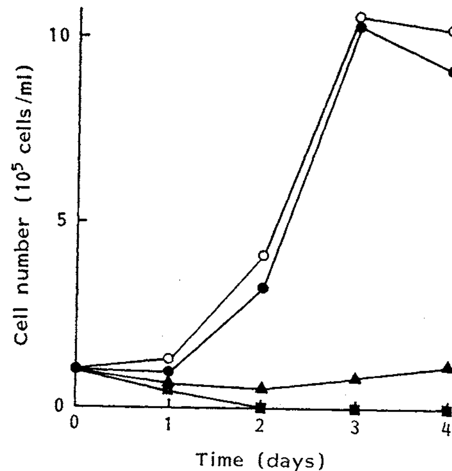
K562 cells (10^5 cells/ml) were treated with 1 μg/ml PDBu for 60 minutes (a), 30 μg/ml tautomycin for 10 minutes (b) or 60 minutes (c) and 1 μg/ml cytochalasin D for 10 minutes (d) or 60 minutes (e). Untreated control (f).

Fig. 2. Kinetic study of bleb formation by tautomycin.



K562 cells (10^5 cells/ml) were incubated with tautomycin 30 (●), 3 (▲), 1 (■) and 0.3 (□) μg/ml or PDBu 1 μg/ml (○) for indicated time. Cells with blebs were counted microscopically. Each point represents average value of triplicate cultures.

Fig. 3. Growth inhibition of K562 by tautomycin.



K562 cells (10^5 cells/ml) were cultured with tautomycin 1 (■), 0.3 (▲) and 0.1 (●) μg/ml or without the antibiotic (○). Viable cells were counted by dye-exclusion test using trypan blue.

myeloid leukemia cells. Bleb formation was also induced by cytochalasin D, a potent inhibitor of polymerization of actin filaments[8,9]. Tautomycin induced a similar morphological change (Fig. 1). It was transient and blebs on the cell surface disappeared on further exposure to the antibiotic.

Time required to induce bleb formation depended on the concentration of tautomycin. 30 μg/ml

Table 1. Effect of tautomycin on NBT reducing activity of HL60 cells differentiated by quinomycin.

Addition		NBT positive cells (%)
None		<5
Tautomycin	10 μg/ml	<5
	20 μg/ml	7.5±0.6[a]
	30 μg/ml	5.9±2.9
Cytochalasin D	1 μg/ml	8.4±5.2
PDBu	100 ng/ml	50.0±5.0

[a] Mean±SD ($n=3$).

Table 2. Effect of tautomycin on the differentiation of HL60 cells.

Addition		Spreading cells (%)	
		Without PDBu	With PDBu
None		0	25.8±0.1
Tautomycin	0.3 μg/ml	0	0
	0.1 μg/ml	0	9.9±2.4
Cytochalasin D	1 μg/ml	0	0

HL60 cells (10^5 cells/ml) were incubated with appropriate agents in the presence or absence of PDBu (100 ng/ml) for 24 hours. Spreading cells on the bottom of the plate were counted microscopically. Values represent mean±SD of triplicate cultures.

tautomycin induced it within 10 minutes whereas 1 μg/ml required 2 hours. At 0.3 μg/ml, it was only observed after 20 hour-exposure, concentrations less than 0.3 μg/ml have no inducing activity (Fig. 2). PDBu also induced bleb formation within 10 minutes but it was maintained for more than 2 hours. Cytochalasin D (1 μg/ml) also induced bleb formation within 10 minutes and, like tautomycin, these blebs disappeared within 60 minutes (Fig. 1). Fig. 3 shows growth inhibitory effect of tautomycin on K562 cells. The MIC was 0.3 μg/ml and lower than this concentration it did not affect the growth, this correlated with the bleb forming activity.

Effect on NBT Reducing Activity of Mature Granulocytes and on the Differentiation of HL60 Human Promyelocytic Leukemia Cells

HL60 can be induced to differentiate into mature granulocytes by various agents and tumor promoters induce the activity to reduce NBT in these differentiated cells[10,11]. In the experiment shown in Table 1, HL60 cells were differentiated by incubating with quinomycin for 3 days. Resultant cells exhibited granulocyte-like phenotypes and 50% of them reduced NBT in response to PDBu. In contrast, tautomycin up to 100 μg/ml and cytochalasin D had only negligible effect.

Tumor promoters induce differentiation of HL60 cells to mature macrophages and the differentiated cells spread and adhere to the bottom of plastic dishes[5,12,13]. Table 2 shows the number of spreading cells after a 24 hour-incubation. 25.8% of cells cultured with PDBu spread on the bottom whereas no cells exhibited a morphological change in response to tautomycin and cytochalasin D. Both agents inhibited PDBu-induced spread of HL60 cells.

Effect of Binding of [^{3}H]PDBu to K562 Cells

Above results suggest that tautomycin has a different biological activity on intact cells from PDBu. This was further demonstrated by the effect on the binding of PDBu to the surface receptor of K562. K562 cells were incubated in the presence of 10 ng/ml PDBu and the antibiotic and specific binding of [^{3}H]PDBu determined. 10 μg/ml tautomycin, although inducing bleb formation within 10 minutes, did not cause a significant inhibition (Table 3), suggesting that tautomycin binds to the cells at a distinct site from PDBu.

Effect on PKC

A kinetic study of bleb formation (Fig. 1) and the results of Tables 1 and 2 suggest the similarity of tautomycin and cytochalasin D. Thus, a direct effect of these agents on PKC extracted from K562

Table 3. Effect of tautomycin on the binding of [^{3}H]PDBu on to K562 cells.

Addition		[^{3}H]PDBu bound (cpm)	Specific binding[b] (%)
None		3,548±448[a]	
Tautomycin	1 μg/ml	3,132±770	
	10 μg/ml	2,901±424	81
			88
PDBu	10 μg/ml	224±91	

[a] Mean±SD (n=3).

[b] Specific binding = 100× (test cpm − PDBu cpm)/(control cpm−PDBu cpm).

cells, was investigated (Table 4). Tautomycin but not cytochalasin D activated PKC in a dose-dependent manner (Experiment 1). Tautomycin also activated Ca^{2+} independent kinase activity although PKC was preferentially activated. Comparison to known modulators of PKC was evaluated in Experiment 2. Activation by PDBu (0.1 μg/ml) and tautomycin (10 μg/ml) was comparable. H7, a specific inhibitor of PKC[14], significantly inhibited Ca^{2+} and phospholipid-dependent phosphotransferase activity.

Table 4. Activation of PKC extracted from K562 cells by tautomycin.

Addition		Protein kinase activity (cpm)	
		Ca^{2+} independent	Ca^{2+} dependent
Expt 1			
None		87±24	395±34
Tautomycin	0.1 μg/ml	117±31	413±127
	1 μg/ml	127±53	501±62*
	10 μg/ml	187±23*	617±71***
Cytochalasin D			
	0.1 μg/ml	69±18	423±51
	1 μg/ml	146±26	362±82
Expt 2			
None		327±35	878±168
Tautomycin	10 μg/ml	544±194	1,185±19**
PDBu	0.1 μg/ml	628±230	1,335±17**
H7	0.1 mM	135±109**	294±23**

Ca^{2+} independent kinase activity is protein kinase activity in the presence of EGTA instead of Ca^{2+} and phosphatidyl inositol. Radioactivity when TCA was added immediately after the addition of enzyme is subtracted. Ca^{2+} dependent protein kinase activity is the difference between protein kinase activity in the presence of Ca^{2+} and phosphatidyl inositol and that in the presence of EGTA. Values represent mean±SD of triplicate assays.

Statistically significant compared to untreated control; * P<0.05, ** P<0.01, *** P<0.001.

Discussion

In this paper, we have shown the induction of morphological change by tautomycin, similar to that induced by tumor promoters of the phorbol ester-type. Our foregoing studies suggested two possible mechanism for bleb formation, activation of PKC and inhibition of polymerization of actin filaments[3]. Kinetic studies indicated that tautomycin was similar to cytochalasin D. In addition neither tautomycin nor cytochalasin D induced NBT reducing activity of mature granulocytes and differentiation of HL60 cells. However, tautomycin but not cytochalasin D directly activated PKC extracted from K562 cells. Because tautomycin did not inhibit binding of [^{3}H]PDBu, the mechanism of activation of PKC by tautomycin must be different from that by phorbol esters. Since PKC used in this study was only partially purified, interaction with other unknown substances which affected the PKC activity, cannot be excluded.

The result that tautomycin activated PKC in a cell-free system but did not induce NBT reducing activity and differentiation of HL60 cells, suggest that a quantitative activation of PKC alone is not sufficient to explain the multifunctional biological activities of phorbol esters. Qualitative modulation, for example, of phosphorylation of specific substrates by phorbol ester-activated PKC or a PKC-independent pathway may exist. In this respect, translocation of PKC by phorbol esters[6,15] is noteworthy. Tautomycin could be a useful tool to distinguish dependency on phorbol esters of the biological reactions involving the function of PKC.

References

1) Kikkawa, U.; Y. Takai, Y. Tanaka, R. Miyake & Y. Nishizuka: Protein kinase C as a possible re-

ceptor protein of tumor-promoting phorbol esters. J. Biol. Chem. 258: 11442~11445, 1983

2) PARKER, P. J.; S. STABEL & M. D. WATERFIELD: Purification to homogeneity of protein kinase C from bovine brain—identity with the phorbol ester receptor. EMBO J. 3: 953~959, 1984
3) OSADA, H.; J. MAGAE, C. WATANABE & K. ISONO: Rapid screening method for inhibitors of protein kinase C. J. Antibiotics 41: 925~931, 1988
4) CHENG, X.-C.; T. KIHARA, H. KUSAKABE, J. MAGAE, Y. KOBAYASHI, R.-P. FANG, Z.-F. NI, Y.-C. SHEN, K. KO, I. YAMAGUCHI & K. ISONO: A new antibiotic, tautomycin. J. Antibiotics 40: 907~909, 1987
5) SOLANKI, V.; T. J. SLAGA, M. CALLAHAM & E. HUBERMAN: Down regulation of specific binding of [20-^{3}H]phorbol 12,13-dibutyrate and phorbol ester-induced differentiation of human promyelocytic leukemia cells. Proc. Natl. Acad. Sci. U.S.A. 78: 1722~1725, 1981
6) FORSBECK, K.; K. NILSSON, A. HANSSON, G. SKOGLUND & M. INGELMAN-SUNDBERG: Phorbol ester-induced alteration of differentiation and proliferation in human hematopoietic tumor cell lines: Relationship to the presence and subcellular distribution of protein kinase C. Cancer Res. 45: 6194~6199, 1985
7) O'BRIAN, C. A.; R. M. LISKAMP, D. H. SOLOMON & I. B. WEINSTEIN: Inhibition of protein kinase C by tamoxifen. Cancer Res. 45: 2462~2465, 1985
8) FLANAGAN, D. M. & S. LIN: Cytochalasins block actin filament elongation by bind to high affinity sites associated with F-actin. J. Biol. Chem. 255: 835~838, 1980
9) BRENNER, S. L. & E. D. KORN: The effects of cytochalasins on actin polymerization and actin ATPase provide insights into the mechanism of polymerization. J. Biol. Chem. 255: 841~844, 1980
10) COLLINS, S. J.; F. W. RUSCETTI, R. E. GALLAGHER & R. C. GALLO: Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. U.S.A. 75: 2458~2462, 1978
11) BREITMAN, T. R.; S. E. SELONICK & S. J. COLLINS: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. U.S.A. 77: 2936~2940, 1980
12) HUBERMAN, E. & M. F. CALLAHAM: Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. U.S.A. 76: 1293~1297, 1979
13) ROVERA, G.; T. G. O'BRIEN & L. DIAMOND: Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. Science 204: 868~870, 1979
14) HIDAKA, H.; M. INAGAKI, S. KAWAMOTO & Y. SASAKI: Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23: 5036~5041, 1984
15) KRAFT, A. S. & W. B. ANDERSON: Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 301: 621~623, 1983