COMPARISON OF THE EFFECT OF TAUTOMYCIN AND PHORBOL ESTER ON PROTEIN KINASE C IN A CELL-FREE SYSTEM

JUNJI MAGAE, HIROYUKI OSADA†, KAZUO NAGAI, MAKARI YAMASAKI and KIYOSHI ISONO*,†

Laboratory of Microbiology, Department of Agricultural Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan
†Antibiotics Laboratory, RIKEN, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

(Received for publication March 16, 1989)

The effect of tautomycin (TM) on protein kinase C (PKC) was studied in a cell-free system. TM, like phorbol dibutyrate (PDBu), enhanced both base-line and Ca^{2+}/phospholipids-dependent protein kinase activity. However, PDBu but not TM increased the affinity of the enzyme for calcium ions (Ca^{2+}), suggesting that TM is a new activator of PKC, distinct from PDBu. In the presence of 10 μg/ml phosphatidyl inositol, the activity of PKC reached maximum at 10^{-3} M Ca^{2+} concentration when the other co-factors were absent. Both TM and PDBu increased the maximum level of PKC activity at the optimum concentration of Ca^{2+}, suggesting that they interacted with the site of PKC which is distinct from the site where Ca^{2+} interacts. TM and PDBu did not activate the enzyme when protamine sulfate in place of histone III-S was used as a substrate, indicating that they activate PKC by affecting the regulatory domain of the enzyme.

Tautomycin (TM) is an antifungal antibiotic, which showed an excellent protective effect against cucumber gray mold in the pot test[1]. We have found that it induces a characteristic morphological change in human chronic myeloid leukemia cells (K562), which is similar to that induced by phorbol esters[2]. Biological activity of phorbol esters is attributed to their activation of protein kinase C (PKC)[3~5]. TM also enhanced PKC activity extracted from K562 cells, in a cell-free system. However, TM did not induce the differentiation of human promyelocytic leukemia cells (HL-60) and only weakly induced nitroblue-tetrazolium reducing activity in HL-60 cells which had differentiated to mature granulocytes[2]; both these are well-known biological responses of cells induced by phorbol esters[6,7]. These results suggest that the effect of TM on PKC is different from that of phorbol esters. In this paper, the mode of activation of PKC by TM was studied in comparison with that by phorbol esters in a cell-free system.

Materials and Methods

PKC was extracted from K562 cells and partially purified by DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column chromatography, as described previously[2]. PKC activity was assayed by measuring the phosphate transfer from [γ-^{32}P]ATP to histone III-S or protamine sulfate[8]. The standard reaction mixture (100 μl) contained Tris-HCl (pH 7.5) 20 mM, 2-mercaptoethanol 5 mM, $MgCl_2$ 10 mM, [γ-^{32}P]ATP (New England Nuclear, Boston, MA) 70 mM, histone III-S (Sigma, St. Louis, MO) or protamine sulfate (0.67 mg/ml, Sigma), 15 μg protein enzyme solution and various activators including $CaCl_2$, phosphatidyl-inositol (PI, Sigma), phorbol dibutyrate (PDBu, Sigma) and TM which was prepared in our laboratory[1]. Reactions were initiated by the addition of the

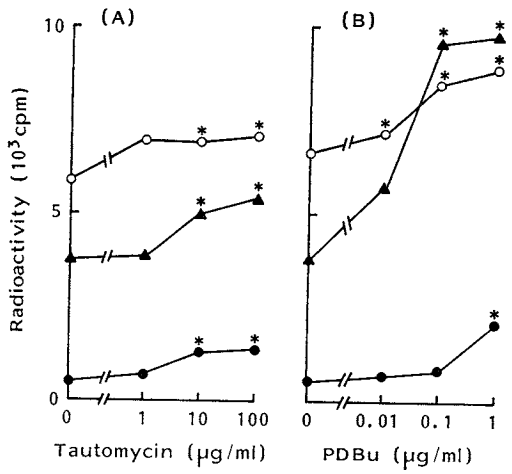
enzyme and the reaction mixture was incubated at 30°C for 15 minutes. Reactions were terminated by the addition of 200 μl of 20% TCA - 1% PPi to the reaction mixture. The precipitated solutions were filtered on Millipore HA filters (pore size 0.45 μm) and the filters were washed with additional 5 ml of TCA - PPi solution 5 times and counted by a liquid scintillation counter.

Results

PKC activity is dependent on the concentration of Ca^{2+} and phospholipid added to the reaction mixture[9]. In the absence of the co-factors, phosphate transfer was only at background level (Fig. 1). When TM was added to the reaction mixture, significant increase of the base-line activity, without cofactors, was observed. When 10^{-3} M Ca^{2+} and 20 μg/ml PI were added to the reaction mixture, 6,000 cpm of radioactivity was incorporated into the acid-insoluble fraction. The addition of TM over 10 μg/ml also enhanced the incorporation. PDBu modulated PKC in the similar fashion to TM. It enhanced both base-line and Ca^{2+}/phospholipid-dependent transfer of phosphate from ATP to histone III-S. Since TM and PDBu induced morphological change of K562 cells over 10 μg/ml and 100 ng/ml[2,10] respectively, the concentrations required for enhancing the PKC activity in the cell-free system were comparable to those required for the biological activity in the cellular level. However, a marked difference in the effects of the compounds was found when suboptimal Ca^{2+} concentration (10^{-5} M) was used. In this condition, PDBu (over 100 ng/ml) enhanced the incorporation more remarkably than that in the presence of 10^{-3} M Ca^{2+}. On the other hand, only an additive incorporation was observed in the case of TM. The result suggested that TM but not PDBu increased incorporation independent from Ca^{2+} concentration.

We, therefore, compared the enhancement of PKC activity by TM and PDBu as a function of

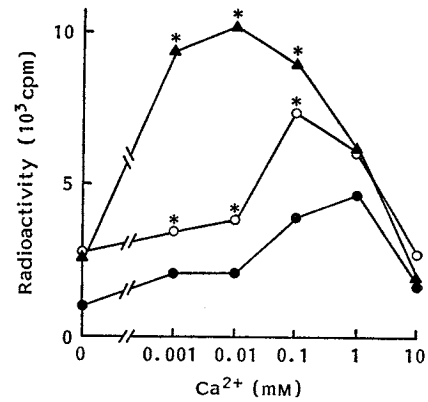
Fig. 1. Activation of PKC by TM and PDBu.



PKC activity was determined as described in Materials and Methods in triplicate assays. Reaction mixture contained TM (A) or PDBu (B), in the absence (●) or presence of Ca^{2+} 10^{-3} M (○) or 10^{-5} M (▲).

* Statistically significant as compared with the radioactivity incorporated in the absence of TM or PDBu ($P<0.05$).

Fig. 2. Comprison of the effect of TM and PDBu on the PKC reaction as a function of Ca^{2+} concentration.



PKC activity was determined as described in Materials and Methods in triplicate assays in the absence(●) or presence of TM (100 μg/ml, ○) or PDBu (100 ng/ml, ▲). The reaction mixture contained PI 10 μg/ml, histone as a substrate and Ca^{2+} as indicated.

* Statistically significant as compared with the radioactivity incorporated in the absence of TM or PDBu ($P<0.05$).

Ca^{2+} concentration (Fig. 2). In the presence of 10 μg/ml PI, maximal incorporation was obtained at 10^{-3} M Ca^{2+} whereas the optimal Ca^{2+} concentration was decreased to 10^{-6} M when 100 ng/ml of PDBu was added to the reaction mixture. Higher concentration of Ca^{2+} (10^{-2} M) rather inhibited the incorporation. The results were consistent with the conclusion of CASTAGNA *et al.*[4] that PDBu enhanced the affinity of the enzyme for Ca^{2+}. When TM was added to the reaction mixture, the Ca^{2+} concentration required for the maximal incorporation was only slightly affected although the incorporation was enhanced through all the Ca^{2+} concentrations studied. This suggested that TM increased the PKC activity without affecting affinity for Ca^{2+}. It should be noted that both PDBu and TM increased the maximal level of incorporation. The result suggests that TM, like PDBu, interacts with a site of PKC different from that for Ca^{2+}.

Table 1. Effect of TM on the PKC reaction using protamine sulfate as a substrate.

	Radioactivity (cpm)
Control	9,854±1,675
TM (100 μg/ml)	8,746±1,059
PDBu (100 ng/ml)	10,023±2,379
H-7 (0.1 mM)	4,005±1,092*
Staurosporine (100 ng/ml)	1,709±628*

PKC activity was determined as described in Materials and Methods in triplicate assays. Reaction mixture contained 1 mM EGTA and no PI.
* Statistically significant as compared with control ($P<0.01$).

PKC is composed of two domains; a catalytic domain and a regulatory domain[11]. PDBu is known to activate PKC by interacting with the regulatory domain[12,13]. It did not activate the enzyme reaction when protamine sulfate was used as a substrate, in place of histone III-S (Table 1), presumably because protamine sulfate can be phosphorylated in a regulatory domain-independent manner[8,9].

H-7 and staurosporine, which inhibit PKC activity by competing with ATP[14,15], significantly inhibited the reaction using protamine sulfate as a substrate. TM did not significantly affect the incorporation in this experiment, suggesting that TM like PDBu interact with a regulatory domain of PKC.

Discussion

Present results suggested that TM enhanced the PKC activity in a cell-free system by interacting with the regulatory domain. This indicated that TM is a novel activator of PKC with a mechanism of action distinct from that of phorbol esters. TM modulates the function of the regulatory domain by interacting with the site other than that for PDBu, because TM did not activate PKC when using protamine sulfate as a substrate and because TM did not increase the affinity of the enzyme for calcium ions.

Although TM activated PKC in the cell-free system, it failed to induce the differentiation of promyelocytic leukemia cells. Our experiments revealed that the most marked difference between PDBu and TM was their effect on the affinity of the enzyme for Ca^{2+}; PDBu enhanced the affinity while TM did not affect significantly. The results suggest that the increase of affinity of the enzyme for Ca^{2+} is important for the induction of the biological activity of phorbol esters. It has been reported that the differentiation of HL-60 cells is induced by phorbol esters but not by 1-oleoyl-2-acetylglycerol (OAG), although OAG can activate PKC both *in vitro* and *in vivo*[16,17]. Therefore, it is possible that some biological activity of phorbol esters, other than activation of PKC, is necessary for the differentiation of HL-60 cells.

On the other hand, the morphological change of K562 cells was fully induced by TM[2], indicating that only the quantitative activation of PKC is enough for the induction. Further analysis of the mode of protein phosphorylation at molecular level is required for understanding the relationship between induction of the morphological change and PKC activation. However, it seems likely that

TM, a new type of modulator of PKC, provides a new basis for explaining biological reactions involving PKC.

References

1) 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
2) MAGAE, J.; C. WATANABE, H. OSADA, X.-C. CHENG & K. ISONO: Induction of morphological change of human myeloid leukeimia and activation of protein kinase C by a novel antibiotic, tautomycin. J. Antibiotics 41: 932~937, 1988
3) KIKKAWA, U.; Y. TAKAI, Y. TANAKA, R. MIYAKE & Y. NISHIZUKA: Protein kinase C as a possible recepor protein of tumor-promoting phorbol ester receptor. J. Biol. Chem. 258: 11442~11445, 1983
4) CASTAGNA, M.; Y. TAKAI, K. KAIBUCHI, K. SANO, U. KIKKAWA & Y. NISHIZUKA: Direct activation of calcium-activated, phospholipid dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847~7851, 1982
5) SAKATA, A.; E. IDA, M. TOMINAGA & K. ONOUE: Arachidonic acid as an intracellular activator of NADPH-oxidase in Fc receptor-mediated superoxide generation in macrophages. J. Immunol. 138: 4353~4359, 1987
6) HUBERMAN, E. & M. F. CALLAHAM: Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agent. Proc. Natl. Acad. Sci. U.S.A. 76: 1293~1297, 1979
7) 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
8) 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
9) TAKAI, Y.; A. KISHIMOTO, Y. IWASE, Y. KAWAHARA, T. MORI & Y. NISHIZUKA: Calcium-dependent Activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254: 3692~3695, 1979
10) OSADA, H.; J. MAGAE, C. WATANABE & K. ISONO: Rapid screening method for inhibitors of protein kinase C. J. Antibiotics 41: 925~931, 1988
11) KISHIMOTO, A.; N. KAJIKAWA, M. SHIOTA & Y. NISHIZUKA: Proteolytic activation of calcium-activated phospholipid-dependent protein kinase by calcium-dependent neutral protease. J. Biol. Chem. 258: 1156~1164, 1983
12) HUANG, K.-P. & F.-L. HUANG: Immunochemical characterization of rat brain protein kinase C. J. Biol. Chem. 261: 14781~14787, 1986
13) LEE, M.-H. & R. M. BELL: The lipid binding, regulatory domain of protein kinase C. A 32-Kda fragment contains the calcium- and phosphatidylserine-dependent phorbol diester binding activity. J. Biol. Chem. 261: 14867~14870, 1986
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) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1986
16) KREUTTER, D.; A. B. CALDWELL & M. J. MORIN: Dissociation of protein kinase C activation from phorbol ester induced maturation of HL-60 leukemia cells. J. Biol. Chem. 260: 5979~5984, 1985
17) YAMAMOTO, S.; H. GOTOH, E. AIZU & K. KATO: Failure of 1-Oleoyl-2-acetylglycerol to mimic the cell-differentiating action of 12-*O*-tetradecanoyl phorbol 13-acetate in HL-60 cells. J. Biol. Chem. 260: 14230~14234, 1985