INHIBITION OF PROTEIN KINASE C ACTIVATION BY 8-(N,N-DIETHYLAMINO)-OCTYL-3,4,5-TRIMETHOXYBENZOATE (TMB-8), AN INTRACELLULAR CA2+ ANTAGONIST

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(Citation)
The Kobe journal of the medical sciences,31(6):221-232

(Issue Date)
1985-12

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100488771

Kobe J. Med. Sci. 31, 221/232, December 1985

INHIBITION OF PROTEIN KINASE C ACTIVATION BY 8-(N,N-DIETHYLAMINO)-OCTYL-3, 4, 5-TRIMETHOXYBENZOATE (TMB-8), AN INTRACELLULAR Ca^{2+} ANTAGONIST*

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INDEXING WORDS

TMB-8; protein kinase C; calcium mobilization

SYNOPSIS

8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8), known as an intracellular Ca^{2+} antagonist, inhibits thrombin-induced increase of cytoplasmic free Ca^{2+} in human platelets as measured directly by an intracellular Ca^{2+} indicator, quin 2, and indirectly by phosphorylation of the myosin light chain that is dependent on Ca^{2+}. This compound also inhibits thrombin-induced 47-kilodalton protein phosphorylation, that is catalyzed by protein kinase C. TMB-8 does not appear to inhibit inositol phospholipid hydrolysis, since neither thrombin-induced labelling of phosphatidic acid nor diacylglycerol formation is affected by this compound. Analysis with purified protein kinase C *in vitro* indicates that TMB-8 inhibits this enzyme directly in a manner competitive with phospholipid. It is concluded that TMB-8 is not

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* This article is the dissertation submitted by Makoto Sawamura for Kobe University School of Medicine for the requirement of Doctor of Medical Sciences.

Abbreviation used are : TMB-8, 8-(N,N-Diethylamino)-octyl-3, 4,5-trimethoxybenzoate; quin 2-AM, quin 2 acetoxymethyl ester; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate.

Received for publication : December 16, 1985

Author's name in Japanese : 澤 村　誠

only an intracellular Ca^{2+} antagonist but also an inhibitor of protein kinase C.

INTRODUCTION

TMB-8 is known to inhibit Ca^{2+}-dependent activation of various cellular functions, such as contraction of smooth and skeletal muscles,[18, 19] release reactions from neutrophils and platelets,[4, 26] and superoxide generation in neutrophils.[20] Although the mode of action is not clear, it is generally accepted that this compound inhibits intracellular Ca^{2+} mobilization from its internal stores.[5] Diacylglycerol derived from the receptor-mediated hydrolysis of inositol phospholipids plays a role in producing various cellular responses through the activation of protein kinase C (for a review, see Ref. 27). This enzyme cooperates with Ca^{2+} to induce the activation of various cellular functions. Such a role of protein kinase C in platelet activation has been extensively investigated.[10] In platelets, both activation of protein kinase C and mobilization of Ca^{2+} are induced by thrombin and collagen, as well as by platelet-activating factor. Protein kinase C then phosphorylates a 47-kilodalton protein, while Ca^{2+} stimulates the calmodulin-dependent phosphorylation of the myosin light chain. The phosphorylation of 47-kilodalton protein and myosin light chain appears to be essential to the release reactions of various constituents such as serotonin.[27] During studies on the role of protein kinase C and Ca^{2+} mobilization in platelets, it has been found that TMB-8 inhibits not only Ca^{2+} mobilization but also protein kinase C activation.

MATERIALS AND METHODS

Materials and chemicals

Thrombin, TMB-8, quin 2-AM, and TPA were obtained from Mochida Pharmaceutical Co., Aldrich Chemicals, Dojindo Laboratories, and P-L Biochemicals, respectively. [^{3}H]Arachidonic acid and ^{32}Pi (carrier free) were obtained from New England Nuclear and the Radiochemical Centre, respectively. Washed human platelets were prepared as described by Baenziger and Majerus.[1] A homogeneous preparation of protein kinase C was obtained from the soluble

fraction of rat brain as described previously.[14] Phospholipid was extracted from bovine brain and fractionated on a silicic acid column as described.[28] H1 histone used as phosphate acceptor was prepared as specified previously.[7] [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell.[6] Other chemicals and materials were obtained from commercial sources.

Assay of Ca^{2+} mobilization

Cytoplasmic free Ca^{2+} concentration was measured with a fluorescent Ca^{2+} indicator, quin 2. Washed platelets (1 x 10^8 cells/ml) were incubated for 25 min at 37C with Hepes-buffered saline (145mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.5mM Na_2HPO_4, 10mM Hepes, 5mM glucose, pH 7.4) containing 5μM quin 2-AM. The suspension was washed twice with Hepes-buffered saline to remove any extraneous dye, and stimulated by thrombin (0.2unit/ml). Fluorescence was recorded at 37C in a Shimadzu fluorescence spectromonitor, Model RF-510LC. Excitation occurred at 339nm and emission at 500nm. The cytoplasmic free Ca^{2+} concentration was calculated from the fluorescence signal as described by Rink et al.[22]

Assay for platelet protein phosphorylation

Washed platelets (2 x 10^9cells/ml) were labelled with 0.5mCi ^{32}Pi for 60 min at 37C as described by Haslam et al.[8] The radioactive platelets (6 x 10^8cells/ml) were then stimulated by thrombin (0.2unit/ml) for 2 min at 37C. The radioactive proteins were subjected to SDS-polyacrylamide slab gel electrophoresis, stained, dried on a filter paper, and then exposed to an X-ray film to prepare an autoradiograph. Electrophoresis was performed under the conditions described by Laemmli.[16] The relative intensity was quantitated by densitometric tracing using a Shimadzu chromatogram scanner, Model CS-910.

Assay for ^{32}Pi-incorporation into phosphatidic acid

The radioactive platelets (6 x 10^8cells/ml, labelled with ^{32}Pi) were preincubated with various concentrations of TMB-8 for 2 min at 37C, and then stimulated by thrombin (0.5unit/ml). The incubation was carried out for 2 min at 37C, and was terminated by the addition of chloroform/methanol (1:2). The radioactive lipid was extracted by the method of Bligh and Dyer.[2] Individual

phospholipids were separated by silica gel G thin layer chromatography by the method of Skipski et al.[25] The area corresponding to phosphatidic acid was scraped into a vial and its radioactivity was determined.

Assay of diacylglycerol formation

Diacylglycerol formed was assayed as described previously.[10]

Assay of protein kinase C

Protein kinase C was assayed in the reaction mixture (0.25ml) containing 5μmol of Tris/HCL at PH 7.5, 1.25μmol of magnesium acetate, 50nmol of [γ-^{32}P]ATP (1-2 x 10^5cpm/nmol) and 4ng of homogeneous preparation of protein kinase C. Calcium chloride, phospholipid, TPA, and TMB-8 were added as indicated in each experiment. Phospholipid was suspended in 20mM Tris/HCL at pH 7.5 by sonication as described previously[28] and employed for the assay. The incubation was carried out for 3 min at 30C. The reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45μm).

Determinations

The radioactivity of ^{32}P- and ^{3}H-labelled samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 4640. Protein was determined by the method of Lowry et al.[17] with bovine serum albumin as a standard.

RESULTS

When platelets were stimulated with thrombin, the cytoplasmic free Ca^{2+} concentration was dramatically increased as monitored by the quin 2 method with the concomitant phosphorylation of the myosin light chain. This thrombin-induced increase of Ca^{2+} and phosphorylation of myosin light chain were both inhibited by TMB-8 as shown in Figs. 1 and 2. Presumably, in platelets, as was suggested for other cells,[5] TMB-8 inhibits intracellular mobilization of Ca^{2+} from its internal stores.

Fig. 2 shows that another platelet protein having a molecular weight of 47-kilodalton was heavily phosphorylated when stimulated

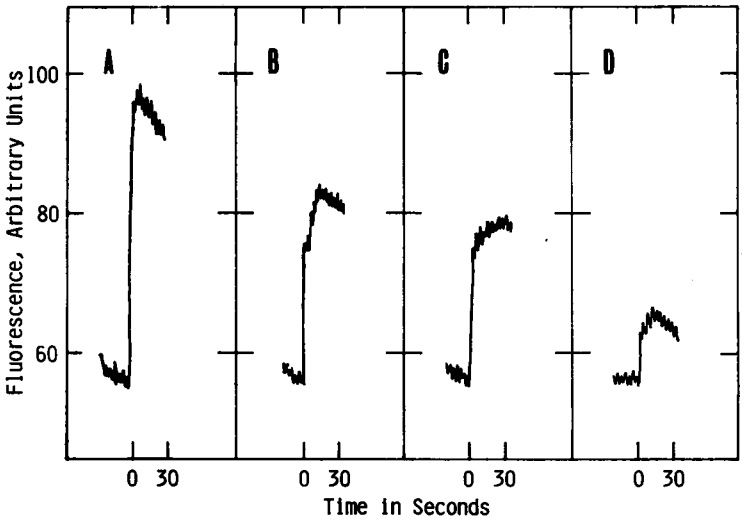

Fig. 1 Inhibition of thrombin-induced increase of cytoplasmic Ca^{2+} by TMB-8. The cytoplasmic free Ca^{2+} concentration was measured as described under "MATERIALS AND METHODS." (A) with thrombin (0.2unit/ml) alone; (B) with thrombin plus 1 x 10^{-4} M TMB-8; (C) with thrombin plus 2 x 10^{-4} M TMB-8; (D) with thrombin plus 4 x 10^{-4} M TMB-8.

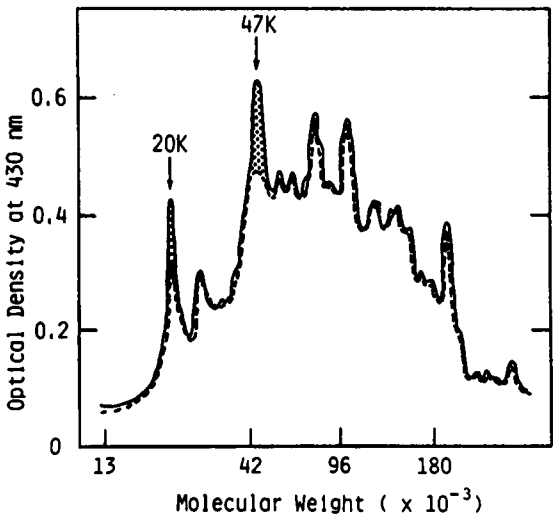

Fig. 2 Inhibition of thrombin-induced protein phosphorylation by TMB-8. ^{32}P-labelled platelets were preincubated with (-----) and without (——) 4 x 10^{-4} M TMB-8 for 2 min at 37C, and then stimulated by thrombin (0.2unit/ml) for 2 min at 37C. The analysis of radioactive proteins was made as described under "MATERIALS AND METHODS."

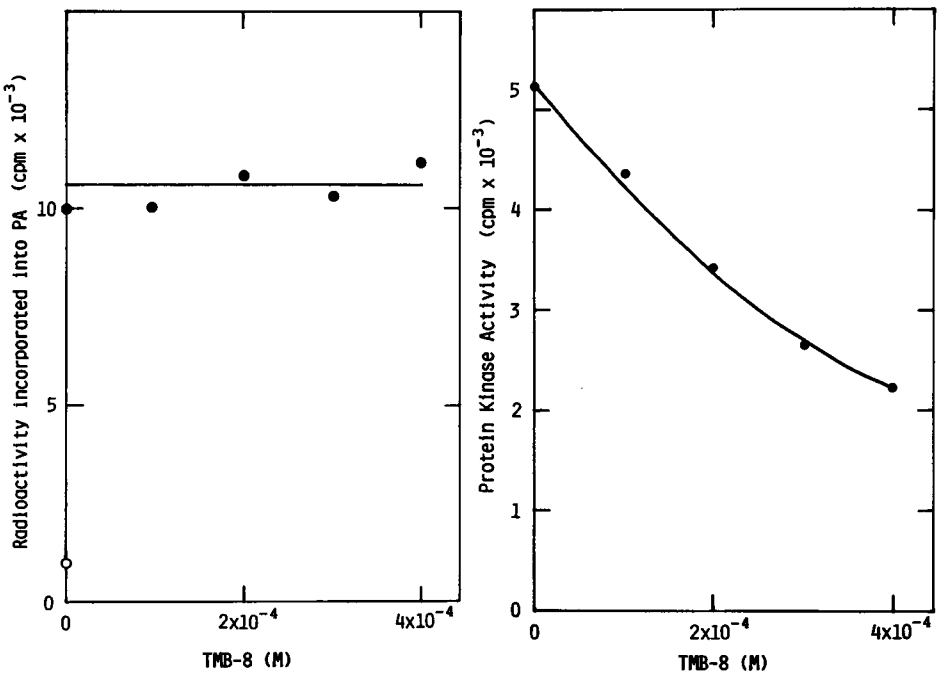

Fig. 3
Effect of TMB-8 on thrombin-induced ^{32}Pi-incorporation into phosphatidic afid. ^{32}P-labelled platelets were preincubated with various concentrations of TMB-8 as indicated for 2 min at 37C, and then stimulated with thrombin (0.5unit/ml). ^{32}Pi-incorporated into phosphatidic acid was analyzed as described under "MATERIALS AND METHODS." (●—●) and (○) indicate the radioactivity incorporated into phosphatidic acid in the presence and absence of thrombin, respectively.

Fig. 4
Inhibition of protein kinase C activation by TMB-8. Protein kinase C was assayed under the standard conditions in the presence of 1 x 10^{-5} M $CaCl_2$, 10ng/ml of TPA, 20μg/ml of phospholipid and the various concentrations of TMB-8 indicated. The enzymatic activity of protein kinase C in the absence of $CaCl_2$, TPA, phospholipid, and TMB-8 was about 850 cpm and this value was subtracted from each experimental value.

by thrombin. It was noted that this protein phosphorylation was also inhibited by the addition of TMB-8. It has been described that 47-kilodalton protein is phosphorylated by protein kinase C.[13, 23] TMB-8 did not affect thrombin-induced ^{32}P-labelling of phosphatidic acid as shown in Fig. 3 nor decrease diacylglycerol formation (data not shown), suggesting that this compound does not inhibit the thrombin-induced hydrolysis of inositol phospholipids. These results suggest that TMB-8 directly affects the activation of protein kinase C. In fact, this compound inhibited directly the activation of purified protein kinase C in a cell-free system as shown in Fig. 4. In this assay TPA was employed instead of diacylglycerol.[3] The concentration of TMB-8 needed to inhibit protein kinase C activation was nearly identical with that needed for the inhibition of Ca^{2+} increase. Kinetic studies indicated that TMB-8 inhibited the activation of protein kinase C in a manner competitive with phospholipid as shown in Fig. 5. This inhibition was not overcome by the addition of increasing amounts of Ca^{2+} and TPA as shown in Table 1, indicating that TMB-8 did not compete with Ca^{2+} or diacylglycerol for the activation of protein kinase C.

Table 1 Effect of TMB-8 on protein kinase C activation. Protein kinase C was assayed under the standard conditions except that Ca^{2+}, phospholipid, and TPA were added as indicated.

Ca^{2+}	phospholipid	TPA	Protein kinase activity (cpm)	
(M)	(μg/ml)	(ng/ml)	none	TMB-8 4×10^{-4} M
1×10^{-5}	20	10	5,364	3,068
1×10^{-5}	20	50	6,577	4,353
1×10^{-4}	20	10	7,259	2,196
1×10^{-5}	100	10	10,210	9,222

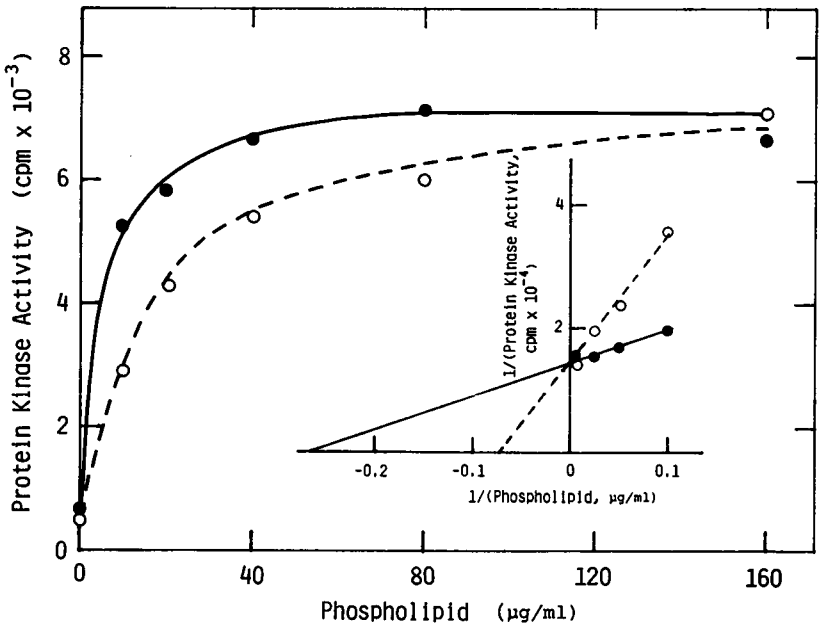

Fig. 5 Effect of TMB-8 on protein kinase C activation in the presence of various amounts of phospholipid. Protein kinase C was assayed under the standard conditions in the presence of 1 x 10^{-5} M $CaCl_2$, 10ng/ml of TPA, the various amounts of phospholipid indicated, and 4 x 10^{-4} M TMB-8. (o---o) and (●——●) indicate the enzymatic activity in the presence and absence of TMB-8, respectively. The inset shows the double reciprocal plots of the data.

DISCUSSION

The results presented above indicate that the inhibitory effect of TMB-8 is not confined to the intracellular Ca^{2+} mobilization from its internal store but extends to the activation of protein kinase C. Since TMB-8 inhibits protein kinase C activation in a manner competitive with phospholipid, it is conceivable that the compound may interact with the phospholipid. Previous studies [11, 12, 15, 21, 24, 29] have shown that many phospholipid-interacting compounds inhibit the activation of protein kinase C, and most of these are known as calmodulin antagonists.[9, 11, 24, 29, 30] These include psychotic drugs such

as trifluoperazine, chlorpromazine, fluphenazine, haloperidol, imipramine, and local anesthetics such as dibucaine and tetracaine. TMB-8 may exert its inhibitory effect on protein kinase C in a manner similar to that of these phospholipid-interacting compounds. Although it is not known at present whether TMB-8 interacts also with calmodulin directly, TMB-8 may counteract both protein kinase C activation and Ca^{2+}-mediated processes, thereby inhibiting the activation of cellular functions.

ACKNOWLEDGMENTS

I wish to thank Professor Yasutomi Nishizuka for valuable discussion, encouragement and support in this work. Thanks are also due to Mrs. Sachiko Nishiyama and Miss Sachiko Fukase for their skillful secretarial assistance.

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