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# Inhibition by Diallyl Trisulfide, a Garlic Component, of Intracellular $Ca^{2+}$ Mobilization without Affecting Inositol-1,4,5-trisphosphate ($IP_3$) Formation in Activated Platelets

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**ABSTRACT.** Garlic has been used in herbal medicine for thousands of years. Some reports have shown that garlic has protective effects against atherosclerosis and inhibits platelet function. In this study, we investigated the mechanism by which diallyl trisulfide (DT), a component of garlic, inhibits platelet function. DT inhibited platelet aggregation and $Ca^{2+}$ mobilization in a concentration-dependent manner without increasing intracellular cyclic AMP and cyclic GMP. DT also had no inhibitory effects on thromboxane $A_2$ ($TXA_2$) production in cell-free systems. Collagen-related peptide (CRP)-induced $Ca^{2+}$ mobilization is regulated by phospholipase C-γ2 (PLC-γ2) activation. We evaluated the effect of DT on tyrosine phosphorylation of PLC-γ2 and the production of inositol-1,4,5-trisphosphate ($IP_3$). DT at concentrations that inhibited platelet aggregation and $Ca^{2+}$ mobilization had no effects on tyrosine phosphorylation of PLC-γ2 or on the formation of $IP_3$ induced by CRP. Similar results were obtained with thrombin-induced platelet activation. DT inhibited platelet aggregation and $Ca^{2+}$ mobilization induced by thrombin without affecting the production of $IP_3$. We then evaluated the effect of DT on the binding of $IP_3$ to its receptor. DT at high concentrations partially blocked the binding of $IP_3$ to its receptor. Taken together, our findings suggest that the agent suppresses $Ca^{2+}$ mobilization at a step distal to $IP_3$ formation. DT may provide a good tool for investigating $Ca^{2+}$ mobilization. BIOCHEM PHARMACOL **60**;10:1475–1483, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** diallyl trisulfide; platelet; calcium; $IP_3$; cyclic AMP and cyclic GMP; thromboxane $A_2$

Garlic has been used in herbal medicine for thousands of years, and it appears to contain a number of active compounds for medicinal use. Recently, sulfur compounds of garlic have been identified, which include allicin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, *trans*-ajoene, *cis*-ajoene, dimethyl trisulfide, and vinyl dithiins [1]. These sulfur-containing compounds appear to be responsible for the beneficial effects of garlic intake. Garlic has a wide range of therapeutic applications, and some reports have indicated that it has protective activity against atherosclerosis, and more specifically stroke and myocardial infarction [2]. Some investigations have shown that garlic prevents both the development and progression of atherosclerosis [3]. Dietary garlic increases fibrinolytic activity [4]. Garlic extracts can decrease the amount of low-density lipoprotein, serum triglyceride, and cholesterol and can prevent the oxidation of low-density lipoprotein [3, 5]. Furthermore, a recent report showed that atherosclerotic plaque volume can be reduced by supplying garlic extracts for several years [6]. With regard to platelet function, an animal *in vivo* experiment has shown that rabbits fed with garlic extracts were well protected against thrombus formation induced by a lethal dose of collagen or arachidonic acid [7]. Garlic can also inhibit the responses of platelets and reduce $TXB_2$§ formation [8–10]. However, most studies have employed garlic or its extracts, and studies on purified garlic components and on the precise mechanism of inhibition have been few. A recent study has indicated that ajoene, an extract of garlic, inhibits the protein tyrosine phosphatase activity in human platelets [11].

In this study, we used a component of garlic extracts, DT, which has been employed for the prevention of atherosclerosis in China for several years, and has proven to be useful

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§ *Abbreviations*: TX, thromboxane; DT, diallyl trisulfide; CRP, collagen-related peptide; EIA, enzyme immunoassay; $IP_3$, inositol-1,4,5-trisphosphate; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; pAb, polyclonal antibody; mAb, monoclonal antibody; PG, prostaglandin; TCA, trichloroacetic acid; fura 2-AM, fura 2 acetoxymethyl ester; and SNP, sodium nitroprusside.

in lowering the occurrence of cerebral infarction. DT is a sulfur-containing material with a MW of 178. In a preliminary study, we found that DT potently suppressed platelet aggregation induced by various agonists. In this study, we sought to explore the mechanism by which DT inhibits platelet functions.

## MATERIALS AND METHODS

### *Materials*

The following materials were obtained from the indicated suppliers: $PGI_2$ (Funakoshi); BSA (Sigma Chemical Co.); anti-PLC-γ2 pAb (Santa Cruz Biotechnology); anti-phosphotyrosine mAb (PY20) (Transduction Laboratories); anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Inc.); D-*myo*-$IP_3$ assay kit, $TXB_2$ EIA system, cyclic AMP and cyclic GMP EIA systems (Amersham Life Science); and fura 2-AM (Dojin Laboratories). CRP was a gift from Dr. M. Moroi (Department of Protein Biochemistry, Institute of Life Science, Kurume University). DT was obtained from the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine. DT was purified by HPLC, and the final product obtained was 98% pure.

### *Platelet Separation*

Platelets were obtained on the day of the experiment from volunteers who had taken no medication in the previous 2 weeks. The blood was centrifuged at 160 *g* for 15 min to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 600 *g* for 15 min and resuspended in HEPES–Tyrode's buffer (138 mM NaCl, 3.3 mM $NaH_2PO_4$, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/mL of glucose, and 20 mM HEPES, pH 7.4).

### *Platelet Aggregation and $Ca^{2+}$ Measurement*

Fura 2-AM at a final concentration of 3 μM was added to platelet-rich plasma, and the mixture was incubated for 30 min. After washing, fura 2-loaded platelets were resuspended in HEPES–Tyrode's buffer at a concentration of $1 \times 10^8$ cells/mL. First, we used DT at a concentration of 10 μg/mL and evaluated the effects of DT on platelet aggregation at various time points including 30 sec and 1, 2, 5, and 10 min; there appeared to be no significant difference in its potency. Therefore, we set the incubation time to be 5 min throughout the study. Washed platelets were incubated with various concentrations of DT for 5 min; then, the agonist was added to the suspension for evaluation of $Ca^{2+}$ mobilization or platelet aggregation. Fura 2 fluorescence was measured with a Hitachi F 2000 fluorescence spectrophotometer with an excitation wavelength alternating every 0.5 sec from 340 to 380 nm; the emission wavelength was set at 510 nm. The $[Ca^{2+}]_i$ values were determined from the ratio of fura 2 fluorescence intensity at 340 and 380 nm excitation. Platelet aggregation was measured with an AA-100 platelet aggregation analyzer (Kowa).

### *Measurement of Cyclic AMP and Cyclic GMP*

Washed platelets were suspended in HEPES–Tyrode's buffer at a concentration of $3 \times 10^8$ cells/mL. Platelets were incubated with various concentrations of DT for 5 min, and then the samples were lysed by adding a lysis reagent, which was obtained from the supplier of the cyclic AMP and cyclic GMP EIA kits. The amount of cyclic AMP or cyclic GMP in supernatants was measured with a cyclic AMP or cyclic GMP EIA system.

### *Measurement of $TXB_2$*

Washed platelets were suspended in HEPES–Tyrode's buffer at a concentration of $2 \times 10^8$ cells/mL. The platelets were incubated with DT or an equal volume of PBS as vehicle for 5 min and then activated by 0.1 U/mL of thrombin or 0.5 μg/mL of CRP. Reactions were terminated after 1 min by adding 10 mM EDTA and 2 mM aspirin, and the samples were diluted 150-fold for $TXB_2$ measurement. The amount of $TXB_2$ was determined by using a $TXB_2$ EIA kit.

To determine the direct effects of DT on arachidonic acid metabolism, the cells were first sonicated to obtain cell lysates. The cell lysates were incubated with various concentrations of DT for 5 min, and then 2 μL of 10 μM arachidonic acid was added to 0.2 mL of the lysate. The mixture was incubated further for 10 min, and the amount of $TXB_2$ was determined as described above.

### *Immunoprecipitation of PLC-γ2*

Washed platelets were adjusted to a concentration of $1 \times 10^9$ cells/mL, and platelets pretreated with various concentrations of DT or with vehicle were stimulated with 0.5 μg/mL of CRP. Reactions were terminated by adding an equal volume of ice-cold lysis buffer [2% Triton X-100, 100 mM Tris–HCl (pH 7.2), 2 mM EGTA, 2 mM vanadate, 1 mM PMSF, and 100 μg/mL of leupeptin]. The lysates were sonicated and centrifuged at 16,000 *g* for 5 min. The soluble fraction was precleared with protein A–Sepharose beads for 30 min. The supernatant was incubated with a polyclonal anti-PLC-γ2 antibody for 1 hr, and the immune complex was precipitated with protein A–Sepharose beads. After the mixture was rotated for 1 hr at 4°, the Sepharose beads were washed three times with lysis buffer and once with 10 mM HEPES buffer. Finally, 50 μL of HEPES buffer and 25 μL of Laemmli buffer were added to the beads, and proteins were eluted by boiling for 3 min. Proteins were separated by SDS–PAGE (8%) under reducing conditions and transferred onto a nitrocellulose membrane. Tyrosine phosphorylation of PLC-γ2 was detected by western blotting, using an anti-phosphotyrosine mAb, 4G10.

### *Measurement of $IP_3$*

Platelets were suspended in HEPES–Tyrode's buffer at a concentration of $3 \times 10^9$ cells/mL. After platelets were activated by 2 U/mL of thrombin or 2 μg/mL of CRP, an equal volume of 15% TCA was added to the platelet suspension to terminate reactions, and the mixtures were kept on ice for 30 min. The mixtures were centrifuged at 2000 *g* for 15 min at 4°, and the resultant supernatant was treated five times with 5 mL of water-saturated diethyl ether to extract TCA. Residual ether was removed further *in vacuo* for 1 hr. The samples were neutralized by titration with 0.2 N NaOH, and the amount of $IP_3$ was measured with an $IP_3$ assay kit.

## RESULTS

### *Effects of DT on Platelet Aggregation Induced by Thrombin or CRP*

Platelets were activated by thrombin or CRP throughout this study. CRP is known to be a potent activator of GPVI, a receptor for collagen. Washed platelets were incubated with various concentrations of DT for 5 min, and then 0.1 U/mL of thrombin or 0.5 μg/mL of CRP was added to a platelet suspension. The magnitude of platelet aggregation was assessed by changes in the optical density of platelet suspensions. DT potently inhibited platelet aggregation induced by thrombin or CRP in a concentration-dependent manner. DT at a concentration of 10 μg/mL completely blocked CRP-induced platelet aggregation (Fig. 1B) and at 30 μg/mL fully suppressed thrombin-induced platelet aggregation (Fig. 1A). The $IC_{50}$ values were 10 μg/mL for thrombin and 4.3 μg/mL for CRP. Similarly, DT also blocked ADP- or 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619)-induced platelet aggregation in a concentration-dependent manner (results not shown).

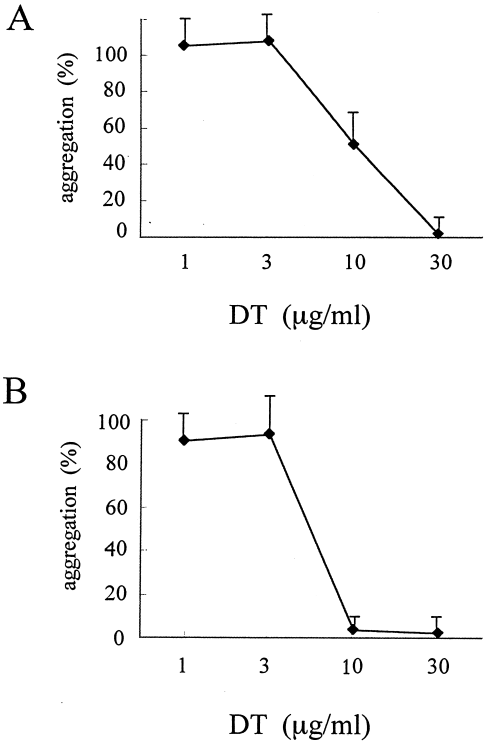


**FIG. 1. Effects of DT on platelet aggregation induced by thrombin or CRP. Washed platelets were incubated with various concentrations of DT for 5 min, and then 0.1 U/mL of thrombin or 0.5 μg/mL of CRP was added to the platelet suspensions to activate platelets. (A) Thrombin-induced platelet aggregation. (B) CRP-induced platelet aggregation. The changes in optical density induced by 0.1 U/mL of thrombin were in the range of 62–72%, and with 0.5 μg/mL of CRP, 71–77%. The data are the means ± SD of five experiments.**

### *Effects of DT on Cyclic AMP and Cyclic GMP*

Cyclic AMP and cyclic GMP are important negative regulators of platelet functions, and many agents exert their inhibitory effects by increasing the intracellular concentration of cyclic AMP or cyclic GMP. They appear to suppress the early process of platelet activation [12, 13]. To determine whether DT inhibits platelet activation by increasing the intracellular level of cyclic AMP or cyclic GMP, we examined the generation of intracellular cyclic AMP or cyclic GMP in resting platelets treated with DT. Platelets were incubated with various concentrations of DT for 5 min, and then the production of intracellular cyclic AMP or cyclic GMP was measured by using cyclic AMP or cyclic GMP EIA systems. As the positive controls, we simultaneously evaluated the effect of 0.1 μM $PGE_1$ on cyclic AMP generation or that of 100 μM SNP on cyclic GMP generation in resting platelets. There was no significant increase in the level of intracellular cyclic AMP or cyclic GMP in platelets treated with DT up to the concentration of 10 μg/mL. Although there was a slight increase in the level of cyclic AMP and cyclic GMP induced by 30 μg/mL of DT, it was far below the levels of intracellular cyclic AMP or cyclic GMP induced by $PGE_1$ or SNP (Fig. 2, A and B). In a preliminary experiment, we evaluated the effects of various concentrations of $PGE_1$ and SNP on the production of cyclic AMP or cyclic GMP in platelets, respectively. The level of cyclic GMP production induced by 30 μg/mL of DT was equal to that induced by 0.1 to 0.3 μM SNP, and the level of cyclic AMP production induced by 30 μg/mL of DT was equal to that induced by 2 nM $PGE_1$. At these concentrations, SNP and $PGE_1$ had no inhibitory effects on platelet aggregation induced by thrombin. These findings suggest that DT inhibits platelet aggregation independently of the production of cyclic AMP or cyclic GMP.

### *Effects of DT on $TXA_2$ Production Induced by Thrombin*

$TXA_2$ is a metabolic product of arachidonic acid. It is a potent endogenous agonist for platelet activation, which can induce full platelet aggregation. Inhibition of its production at any step may lead to the impairment of platelet

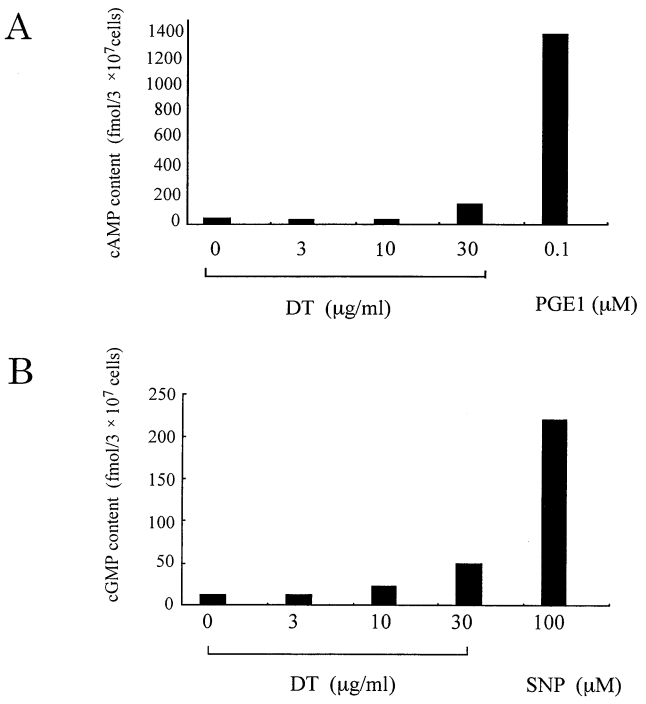


**FIG. 2. Effects of DT on the production of cyclic AMP or cyclic GMP in resting platelets. Platelets were treated with DT or vehicle for 5 min, and then reactions were terminated by adding lysis reagent. The amount of cyclic AMP or cyclic GMP was measured by using a cyclic AMP or cyclic GMP EIA assay kit. (A) Cyclic AMP. (B) Cyclic GMP. The data represent the means of two experiments.**

activation. We thus evaluated the effects of DT on $TXA_2$ production. We measured the formation of $TXB_2$, the final and stable metabolite of $TXA_2$, induced by thrombin or CRP, by using a $TXB_2$ EIA system. Platelets pretreated with DT or with vehicle were stimulated by 0.1 U/mL of thrombin or 0.5 μg/mL of CRP. Thrombin or CRP induced the $TXB_2$ production of 9.2 pg/6.5 × $10^4$ cells, and 30 pg/6.5 × $10^4$ cells in platelets, respectively. DT inhibited the formation of $TXB_2$ induced by thrombin or CRP in a concentration-dependent manner. At the concentration of 30 μg/mL, DT almost completely inhibited $TXB_2$ formation induced by thrombin or CRP. The $IC_{50}$ values were approximately 10 μg/mL for both thrombin and CRP (Fig. 3, A and B). To determine whether DT has a direct inhibitory effect on $TXA_2$ production (cyclooxygenase or $TXA_2$ synthase), platelets at a concentration of 2 × $10^8$ cells/mL were first lysed by sonication and incubated with various concentrations of DT for 5 min. Then, 100 pmol arachidonic acid was added to 0.2 mL of the cell lysate. After 10 min of incubation, the reaction was terminated by adding 10 mM EGTA and 2 mM aspirin (final concentrations); then the samples were diluted 150-fold with HEPES–Tyrode's buffer. $TXB_2$ production was measured with a $TXB_2$ EIA assay kit. DT up to the concentration of 10 μg/mL had no significant effect on $TXB_2$ production in this system (Fig. 4). $TXB_2$ production appeared to be slightly enhanced at 30 μg/mL. However, the difference was not statistically significant ($P = 0.05$). These findings taken together suggest that DT exerts its inhibitory effect on $TXA_2$ production by suppressing the signal transduction pathway leading to $TXA_2$ formation, but not by directly inhibiting cyclooxygenase or $TXA_2$ synthetase.

### *Effects of DT on $Ca^{2+}$ Mobilization Induced by Thrombin or CRP*

$Ca^{2+}$ mobilization is a critical step in various aspects of platelet activation. Thus, we investigated the effects of DT on $Ca^{2+}$ mobilization induced by thrombin or CRP. To exclude the secondary effect of $TXA_2$ on $Ca^{2+}$ mobilization, fura 2-loaded platelets were pretreated with 1 mM aspirin for 30 min. Platelets were incubated with various concentrations of DT for 5 min, and then thrombin or CRP was added to the suspension to induce $Ca^{2+}$ mobilization. $[Ca^{2+}]_i$ measurement was performed in the presence of 200 μM EGTA or 1 mM $Ca^{2+}$, and the peak $[Ca^{2+}]_i$ increase was used for evaluating the effects of DT. In both cases, DT suppressed $Ca^{2+}$ mobilization induced by thrombin or CRP, suggesting that $Ca^{2+}$ mobilization from intracellular $Ca^{2+}$ stores and $Ca^{2+}$ influx from the extracellular fluid

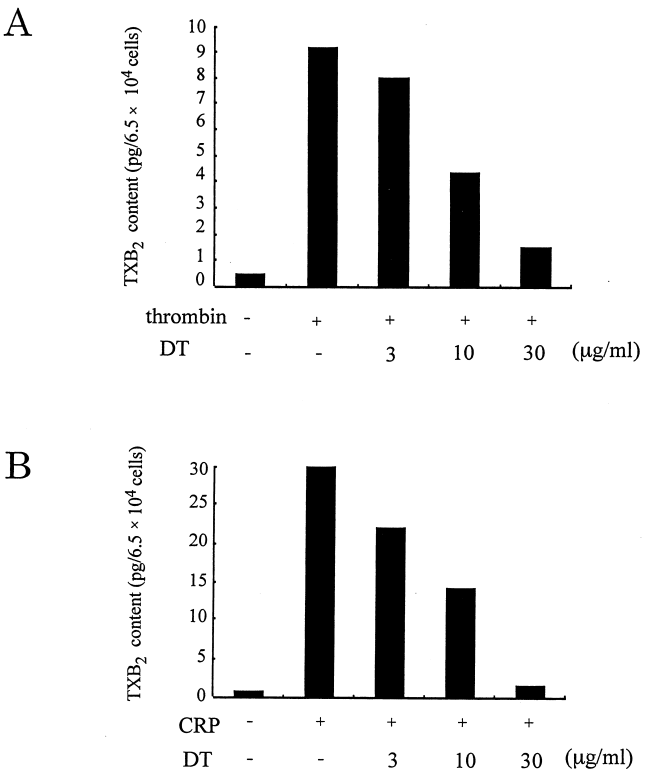


**FIG. 3. Effects of DT on the generation of $TXB_2$ induced by thrombin or CRP. Platelets were incubated with various concentrations of DT or with vehicle for 5 min, and then thrombin or CRP was added to the platelet suspension. Reactions were terminated by adding 10 mM EDTA and 2 mM aspirin. The amount of $TXB_2$ was measured by using a $TXB_2$ EIA kit. (A) Thrombin-induced formation of $TXB_2$. (B) CRP-induced formation of $TXB_2$. The data represent the means of two experiments.**

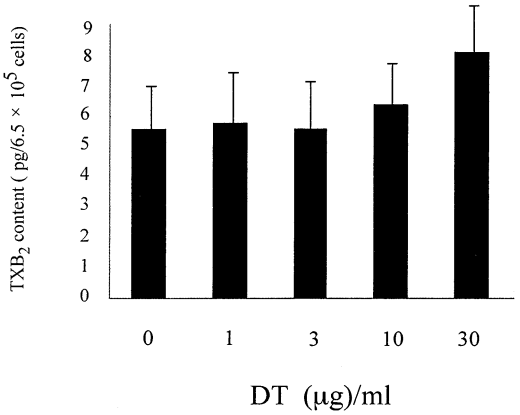


**FIG. 4. Effects of DT on $TXB_2$ production induced by arachidonic acid in a cell-free system. Washed platelets were sonicated three times for 5 sec, and DT was added to the lysates and incubated for 5 min. Then 100 pmol arachidonic acid was added to 0.2 mL of cell suspensions. After the mixture was incubated for 10 min, reactions were terminated by adding 10 mM EGTA and 2 mM aspirin. The samples were diluted 150-fold, and the amount of $TXB_2$ was measured by using an EIA system. The data represent the means ± SD of three experiments.**

were suppressed. In the absence of extracellular $Ca^{2+}$, DT at 10 μg/mL inhibited $Ca^{2+}$ mobilization almost completely (Fig. 5, A and B). The $IC_{50}$ values of DT were 4.5 μg/mL for thrombin and 3.7 μg/mL for CRP, which were similar to those required for blocking platelet aggregation (Fig. 6, A and B). To characterize the effects of DT on $Ca^{2+}$ mobilization, we employed thapsigargin, an inhibitor of $Ca^{2+}$-ATPase of $Ca^{2+}$ stores, which induces $Ca^{2+}$ influx by emptying internal $Ca^{2+}$ stores. DT did not inhibit thapsigargin-induced $Ca^{2+}$ influx (Fig. 5C), implying that DT had no direct effect on $Ca^{2+}$ store-regulated $Ca^{2+}$ entry.

### *Effects of DT on Tyrosine Phosphorylation of PLC-γ2 Induced by CRP*

Although DT potently inhibited $Ca^{2+}$ mobilization, the findings hitherto suggest that it does not exert its inhibitory effect by elevating the intracellular level of cyclic AMP and cyclic GMP, or by directly suppressing $TXA_2$ production. We next sought to evaluate the signal transduction pathway leading to $Ca^{2+}$ mobilization. $Ca^{2+}$ mobilization from internal $Ca^{2+}$ stores is mediated by $IP_3$, which is produced by PLC. PLC-β is activated in thrombin stimulation, and PLC-γ2 is activated in CRP-mediated platelet activation. Whereas the activation of PLC-β can be assessed only by the level of $IP_3$ production, PLC-γ2 activation also can be evaluated by the level of its tyrosine phosphorylation. Therefore, we sought to determine whether DT modified PLC-γ2 tyrosine phosphorylation induced by CRP. First, we evaluated the time course of tyrosine phosphorylation of PLC-γ2 induced by CRP. Thirty seconds after stimulation with CRP, PLC-γ2 appeared to be tyrosine-phosphorylated, with the maximum level of tyrosine phosphorylation occurring 1 min after platelet activation (Fig. 7A). Thus, the effect of DT on tyrosine phosphorylation of PLC-γ2 was determined 1 min after CRP stimulation. Even at the highest concentration of 30 μg/mL, which almost completely inhibits $Ca^{2+}$ mobilization or platelet aggregation, DT had no inhibitory effect on tyrosine phosphorylation of PLC-γ2 induced by CRP (Fig. 7B).

### *Effects of DT on $IP_3$ Formation Induced by Thrombin or CRP*

The results with PLC-γ2 suggest that DT does not affect PLC-γ2 activation induced by CRP, whereas it inhibits $Ca^{2+}$ mobilization induced by CRP. We therefore examined the effects of DT on the production of $IP_3$ induced by CRP and by thrombin. First, we determined the time course of $IP_3$ production induced by CRP. Fifteen seconds after stimulation with 2 μg/mL of CRP, $IP_3$ formation could be detected, with the maximum level of $IP_3$ generated at 30 sec. After 5 min of stimulation, $IP_3$ formation decreased by

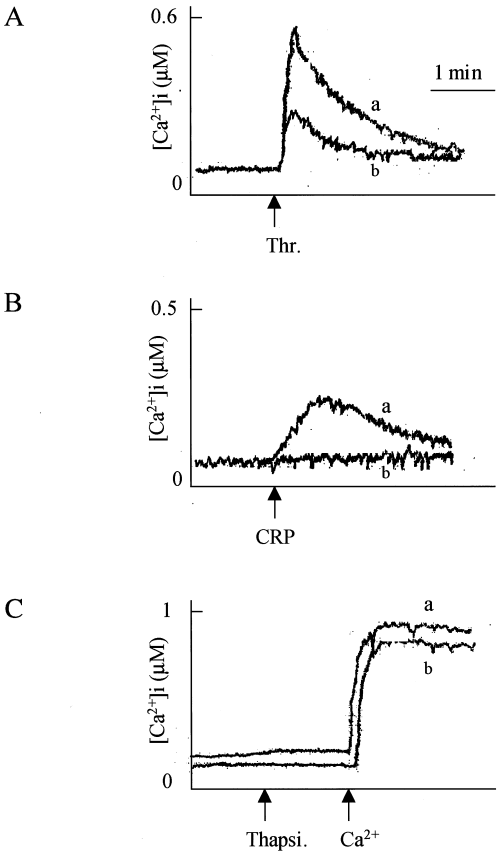


**FIG. 5. Differential effects of DT on thrombin-, CRP-, or thapsigargin-induced $Ca^{2+}$ mobilization. Platelets were suspended in a buffer containing 200 μM EGTA and incubated with various concentrations of DT for 5 min; then agonists were added to the samples at the time indicated by an arrow. With thapsigargin-induced $Ca^{2+}$ influx, 5 mM $Ca^{2+}$ was added to the platelet suspension 2 min after the addition of 1 μM thapsigargin for evaluating $Ca^{2+}$ influx. (A) Thrombin-induced $Ca^{2+}$ mobilization. (B) CRP-induced $Ca^{2+}$ mobilization. (C) Thapsigargin-induced $Ca^{2+}$ mobilization. Key: (a) without DT; (b) with 10 μg/mL of DT.**

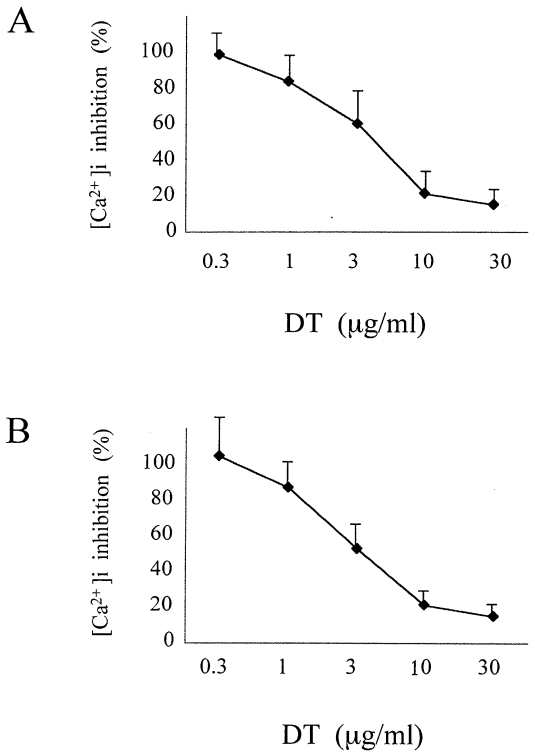


**FIG. 6. Effects of DT on $Ca^{2+}$ mobilization induced by thrombin or CRP. Fura 2-loaded platelets treated with aspirin were incubated with various concentrations of DT for 5 min in the presence of 200 μM EGTA, and then 0.1 U/mL of thrombin or 0.5 μg/mL of CRP was added to the suspension. (A) Thrombin-induced $Ca^{2+}$ mobilization. (B) CRP-induced $Ca^{2+}$ mobilization. The peak intracellular calcium concentrations induced by 0.1 U/mL of thrombin were in the range of 350–710 nM, and with 0.5 μg/mL of CRP, 280–460 nM. The data are the means ± SD of five experiments.**

approximately 70% of the peak value (Fig. 8A). Next, we evaluated the effect of DT on $IP_3$ production induced by CRP. Platelets were pretreated with various concentrations of DT, and then stimulated with 2 μg/mL of CRP for 30 sec. DT up to the concentration of 30 μg/mL had no inhibitory effects on $IP_3$ formation (Fig. 8B). Similar observations were obtained with thrombin-induced production of $IP_3$ (Fig. 9). These findings suggest that DT at concentrations that potently block $Ca^{2+}$ mobilization does not affect $IP_3$ formation. Then, we asked whether DT modifies the binding between $IP_3$ and the $IP_3$ receptor. Various concentrations of DT were added to the membrane fractions of rat cerebellum, which contains abundant $IP_3$ receptors (preparations contained in an Amersham $IP_3$ assay kit), and incubated for 15 min; then, the $IP_3$ binding to the $IP_3$ receptor was determined, using the $IP_3$ assay kit. DT at 30 μg/mL inhibited $IP_3$ binding to its receptor by approximately 57%, whereas $Ca^{2+}$ mobilization was almost completely suppressed at the same concentration of DT. Even at the concentration of 100 μg/mL, DT failed to completely block the association between $IP_3$ and its receptor (Fig. 10).

## DISCUSSION

A number of studies have reported on the inhibitory effects of garlic on platelet aggregation, release response, and metabolism of arachidonic acid *in vitro* [14–17]. Although different preparations of garlic have been tested for their anti-platelet effects, there have been few studies using purified components of garlic. In this study, we sought to probe the mechanism by which DT, a component of garlic, inhibits platelet activation. First, we examined the effects of DT on platelet aggregation induced by various agonists. DT potently inhibited platelet aggregation induced by thrombin, CRP, U46619 (a $TXA_2$ mimetic), ADP, or SFLL (a thrombin receptor agonist peptide). These findings suggest that DT acts at a certain step of the signal transduction pathway common to these agonists.

Cyclic AMP and cyclic GMP are potent endogenous inhibitors of platelet function. A number of reports have

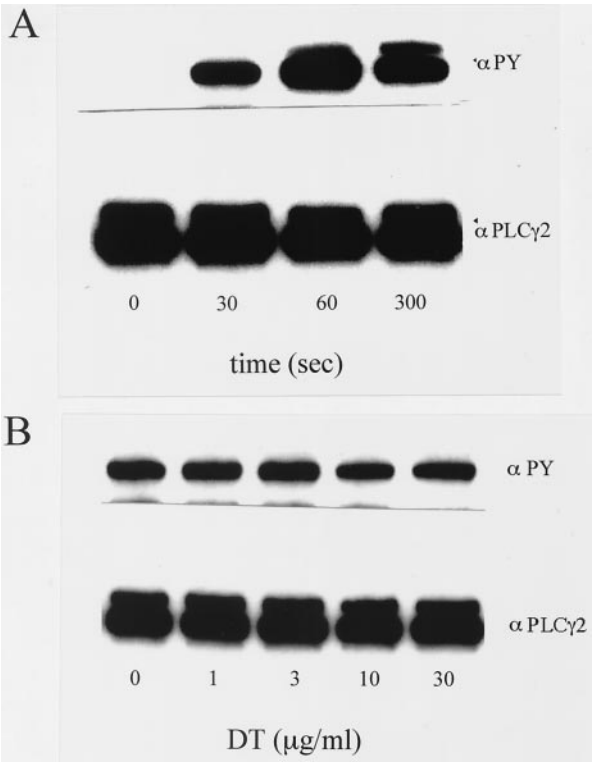


**FIG. 7. Effects of DT on tyrosine phosphorylation of PLC-γ2 induced by CRP. Platelets were incubated with various concentrations of DT for 5 min, and then 0.5 μg/mL of CRP was added to the platelet suspensions. Reactions were terminated by adding lysis buffer. After immunoprecipitation with anti-PLC-γ2 pAb, the samples were subjected to western blotting with anti-phosphotyrosine mAb or anti-PLC-γ2 pAb. (A) Time course of tyrosine phosphorylation of PLC-γ2 induced by CRP. The upper bands represent western blotting with anti-phosphotyrosine mAb; the lower bands represent western blotting with anti-PLC-γ2 pAb to confirm the recovery of PLC-γ2. (B) Effects of DT on tyrosine phosphorylation of PLC-γ2 induced by CRP. The upper bands represent western blotting with anti-phosphotyrosine mAb; the lower bands represent western blotting with anti-PLC-γ2 pAb to confirm the recovery of PLC-γ2. The data are representative of three experiments.**

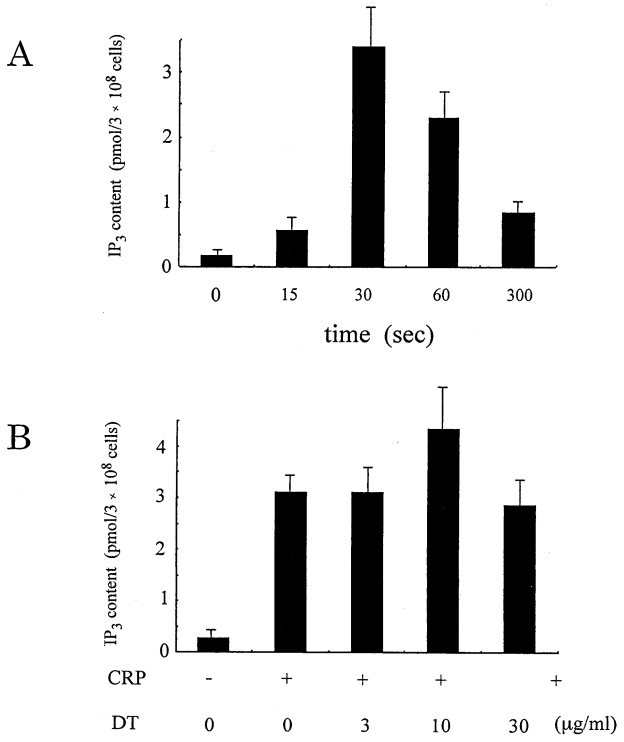


**FIG. 8. Effects of DT on $IP_3$ generation induced by CRP. Platelets were incubated with various concentrations of DT for 5 min, and then 2 μg/mL of CRP was added to the platelet suspensions. Reactions were terminated by adding an equal volume of ice-cold 15% TCA. $IP_3$ was measured by using an Amersham $IP_3$ assay kit. (A) Time course of $IP_3$ production induced by CRP. (B) Effects of various concentrations of DT on $IP_3$ production induced by CRP. The data are the means ± SD of three experiments.**

shown that agents that increase the intracellular level of cyclic AMP or cyclic GMP suppress platelet aggregation as well as $Ca^{2+}$ mobilization [18, 19]. Therefore, we asked whether DT inhibits platelet aggregation or $Ca^{2+}$ mobilization by increasing the intracellular level of cyclic AMP or cyclic GMP. In this study, platelets pretreated with various concentrations of DT failed to elevate intracellular cyclic AMP or cyclic GMP to a level that could suppress platelet function. Based on these results, we suggest that DT inhibits platelet aggregation and $Ca^{2+}$ mobilization, independently of the cyclic AMP or cyclic GMP pathway.

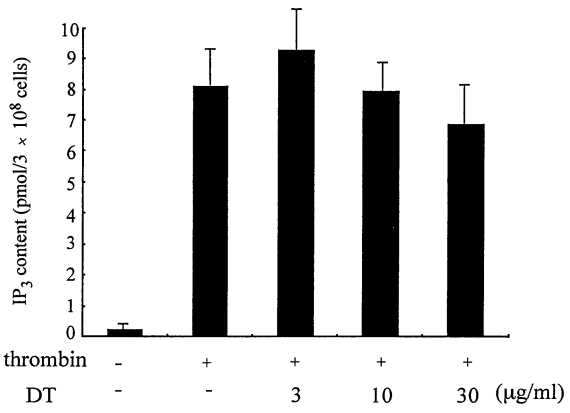


**FIG. 9. Effects of DT on $IP_3$ generation induced by thrombin. Platelets were incubated with various concentrations of DT for 5 min, and then 2 U/mL of thrombin was added to the platelet suspensions. Reactions were terminated 5 sec after stimulation by adding 15% TCA. $IP_3$ was measured by using an Amersham $IP_3$ assay kit. The data are the means ± SD of three experiments.**

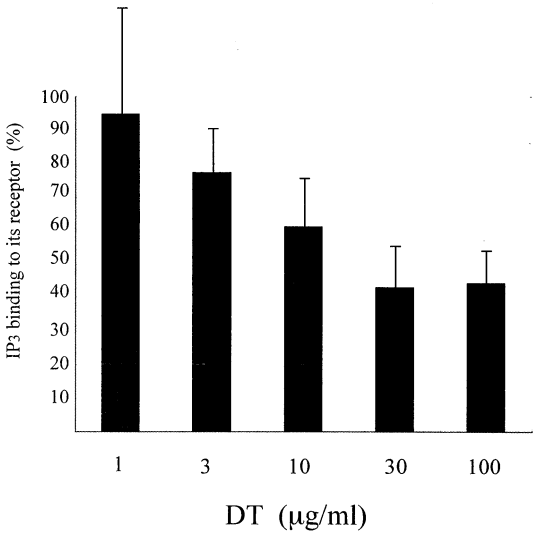


**FIG. 10. Effects of DT on $IP_3$ binding to its receptor. Various concentrations of DT were incubated first with membrane fractions of rat cerebellum, and then the $IP_3$ binding to the $IP_3$ receptors was determined. The $IP_3$ binding to its receptor was evaluated by the $IP_3$ tracer and the tubes containing the membrane preparations provided by the supplier of the kit. The tracer bound to the membranes in the test tubes was in the range of 2200–2550 cpm/tube. The data are the means ± SD of four experiments.**

$TXA_2$ is an endogenous product of arachidonic acid that potently activates platelets. Many agents are known to inhibit platelet aggregation by blocking the synthesis of $TXA_2$. Since several studies have reported that garlic extracts can block the generation of $TXA_2$ by inhibiting cyclooxygenase or $TXA_2$ synthetase [1, 10, 16], we examined whether DT modifies arachidonic acid metabolism in human platelets. DT effectively inhibited the generation of $TXB_2$, a stable metabolite of $TXA_2$, concentration-dependently in platelets activated by thrombin or CRP. However, since DT had no effect on $TXB_2$ generation in a cell-free system, DT appears to have no direct effect on cyclooxygenase or $TXA_2$ synthetase. The inhibitory effect of DT on $TXA_2$ production in activated platelets suggests that DT acts at a certain step of the signal transduction pathway, leading to the release of arachidonic acid.

We then evaluated the effects of DT on $Ca^{2+}$ mobilization induced by thrombin or CRP. Since we found that DT suppresses $TXA_2$ production, platelets were first incubated with aspirin to exclude the secondary effects of $TXA_2$. DT suppressed $Ca^{2+}$ influx as well as $Ca^{2+}$ mobilization from internal $Ca^{2+}$ stores induced by thrombin or CRP. The $Ca^{2+}$ mobilization induced by these agonists requires the production of $IP_3$, which is an important second messenger

for $Ca^{2+}$ mobilization. We first checked whether DT might have a direct inhibitory effect on $Ca^{2+}$ influx. Thapsigargin, a $Ca^{2+}$-ATPase inhibitor, induces $Ca^{2+}$ influx by depleting internal $Ca^{2+}$ stores [20–22]. $Ca^{2+}$ mobilization induced by thapsigargin is regulated by store-regulated $Ca^{2+}$ entry, which is distinct from $IP_3$-induced $Ca^{2+}$ mobilization. Whereas DT inhibited $IP_3$-mediated $Ca^{2+}$ mobilization, it had no effect on thapsigargin-induced $Ca^{2+}$ entry. These findings suggest that the inhibitory effects of DT on $Ca^{2+}$ mobilization are specific for the $IP_3$-mediated signal pathway. Thrombin-induced $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores involves PLCβ with resultant formation of $IP_3$ [23–25]. Since we have no suitable method to assay the activity of PLCβ except for the measurement of $IP_3$, we first assessed the production of $IP_3$ induced by thrombin. We found that DT had no effect on the formation of $IP_3$, whereas DT potently inhibited $Ca^{2+}$ mobilization in platelets activated by thrombin. To further clarify the site of DT action, we investigated the signal transduction pathways involved in CRP-induced platelet activation. Recent findings suggest that, upon the binding of CRP with the collagen receptor, Syk, a tyrosine kinase, is activated and undergoes tyrosine phosphorylation. Syk lies upstream of PLC-γ2, which catalyzes the production of $IP_3$ [26–28]. We first checked for Syk tyrosine phosphorylation and found that DT had no effect on tyrosine phosphorylation of Syk (results not shown). We next examined tyrosine phosphorylation of PLC-γ2 and $IP_3$ production, the indices of PLC-γ2 activation, induced by CRP. DT, even at a high concentration of 30 μg/mL, had no significant effects on tyrosine phosphorylation of PLC-γ2 or $IP_3$ production induced by CRP. Finally, we evaluated the binding of $IP_3$ to the $IP_3$ receptor in the presence or absence of DT by using rat cerebellum membranes, which are rich in $IP_3$ receptors. DT showed a partial inhibition of the binding of $IP_3$ to its receptor, even at a concentration of 30 μg/mL, which almost completely suppressed $Ca^{2+}$ mobilization. Thus, the inhibitory effect of DT on $Ca^{2+}$ mobilization may be partially attributed to the step of $IP_3$ binding to its receptor. However, this incomplete effect points to the presence of an additional mechanism by which DT inhibits $Ca^{2+}$ mobilization in human platelets. We suggest that DT also inhibits $Ca^{2+}$ release at a site distal to $IP_3$ binding to its receptor. Whereas the sulfur-containing structure of DT may react with the $IP_3$ receptor, the precise biochemical property that renders DT reactive with the $IP_3$ receptor remains elusive. Our findings taken together suggest that DT is a unique, membrane-permeable inhibitor of $Ca^{2+}$ mobilization, which acts on the $IP_3$ receptor. It is of interest that an antibody against the $IP_3$ receptor also inhibits $IP_3$-induced $Ca^{2+}$ release, but does not interfere with the binding of $IP_3$ to its receptor [29]. Since this is an antibody, it should be administered to cells whose membranes have been permeabilized by detergents or electroporation. The inhibitory mechanism of DT appears to be similar to those of xestospongin [30, 31] and 2-aminoethoxydiphenyl borate [32, 33], which are membrane-permeable blockers of $IP_3$-induced $Ca^{2+}$ release. They also do not block the binding of $IP_3$ to the $IP_3$ receptor. However, it should be noted that xestospongin at high concentrations also inhibits thapsigargin-induced $Ca^{2+}$ release [30]. DT, 2-aminoethoxydiphenyl borate, and xestospongin constitute a family of membrane-permeable agents that should be useful for investigating $IP_3$-induced $Ca^{2+}$ release.

A previous report has suggested that ajoene, a component of garlic, might modify platelet function by inhibiting the activity of tyrosine phosphatases [11]. We found that DT at 30 μg/mL slightly elevated the level of tyrosine phosphorylation of Syk and PLC-γ2 in the resting state (data not shown). However, as described in Results, DT even at the highest concentration tested had no effects on PLC-γ2 tyrosine phosphorylation in the activated platelets. Hence, we assume that the effect of DT is unrelated to the activity of tyrosine phosphatases.

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