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# Enhancement of arachidonic acid liberation by protein kinase C activator is partially dependent on extracellular $Na^+$ in rabbit platelets

Satoshi Akiba, Takashi Sato and Tatsuzo Fujii

*Department of Biochemistry, Kyoto Pharmaceutical University, Kyoto 607, Japan*

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In [$^3$H]arachidonic acid-labeled rabbit platelets, pretreatment with phorbol 12-myristate 13-acetate (20 nM) or dioctanoylglycerol (20 $\mu$M) enhanced [$^3$H]arachidonic acid liberation induced by low concentration of A23187 (150 nM). When extracellular $Na^+$ was replaced with *N*-methyl-D-glucamine, the enhancement is reduced by about 50%. Similar synergistic enhancement of the liberation was obtained by using monensin (2–10 $\mu$M) or $NH_4Cl$ (5–20 mM) in place of protein kinase C activator in combination with A23187. The guanosine 5'-*O*-[3-thiotriphosphate] (100 $\mu$M)-induced liberation was also enhanced by a rise of extracellular pH (pH 7.0–7.8) in saponin-permeabilized platelets. These results suggest that the enhancement of arachidonic acid liberation by protein kinase C may partially be mediated by intracellular alkalinization in rabbit platelets.

Arachidonic acid liberation; Protein kinase C; $Na^+/H^+$ exchange; Intracellular alkalinization; (Rabbit platelet)

## 1. INTRODUCTION

Stimulation of platelets with agonist results in arachidonic acid liberation mainly through phospholipase $A_2$ activation [1,2]. Previous studies demonstrated that protein kinase C synergistically potentiated $Ca^{2+}$-dependent arachidonic acid liberation [3,4], while this enzyme has been shown to evoke intracellular alkalinization via acceleration of $Na^+/H^+$ exchange [5,6]. Furthermore, it has been reported that inhibition of $Na^+/H^+$ exchange suppresses agonist-induced lysophosphatidylinositol formation [7]. Although the mechanism by which protein kinase C enhances arachidonic acid liberation in platelets remains unelucidated, these observations led us to suppose that protein kinase C may facilitate phospholipase $A_2$ activation via acceleration of $Na^+/H^+$ exchange. Therefore, we examined here whether the enhancement of arachidonic acid liberation by protein kinase C activator is due to intracellular alkalinization in rabbit platelets. In addition, to confirm the contribution of intracellular alkalinization to activation of phospholipase $A_2$ associated with guanine-nucleotide-binding protein (G-protein) which was proposed in our recent work [8] and elsewhere [9], we investigated further the synergistic effect of artificial alkalinization of platelet interior on $Ca^{2+}$-ionophore- and GTP analogue-induced arachidonic acid liberation.

*Correspondence address:* T. Sato, Department of Biochemistry, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan

*Abbreviations:* PMA, 4$\beta$-phorbol 12-myristate 13-acetate; DOG, dioctanoylglycerol; GTP$\gamma$S, guanosine 5'-*O*-[3-thiotriphosphate]; G-protein, guanine-nucleotide-binding protein

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

A23187 was from Calbiochem (USA), PMA from LC Services Co. (USA), monensin from Sigma (USA), guanosine 5'-*O*-[3-thiotriphosphate] (GTP$\gamma$S) from Boehringer Mannheim (FRG) and [$^3$H]arachidonic acid (100 Ci/mmol) from New England Nuclear (USA). Other reagents were from Nakarai Tesque (Japan).

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2.2. *Preparation of labeled platelets*

Platelet-rich plasma from rabbit blood was incubated with [$^3$H]arachidonic acid (2 $\mu$Ci/ml) at 37°C for 1 h and then washed, as described recently [8]. The labeled platelets were suspended at $5 \times 10^8$ cells/ml in buffer (137 mM NaCl, 2.7 mM KCl, 3.3 mM $NaH_2PO_4$, 1 mM $MgCl_2$, 3.8 mM Hepes, 5.6 mM glucose and 0.35% bovine serum albumin, pH 7.4). In some experiments, NaCl and $NaH_2PO_4$ were replaced with *N*-methyl-D-glucamine [10] and $KH_2PO_4$, respectively.

2.3. *Measurement of arachidonic acid liberation*

[$^3$H]Arachidonic acid-labeled platelets were pretreated with 3-amino-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline (BW755C, 50 $\mu$M) [11] at 37°C for 2 min in the presence of $CaCl_2$ (1 mM), and further treated with various reagents. After lipid extraction, [$^3$H]arachidonic acid liberated was analyzed by thin-layer chromatography with a developing solvent of petroleum ether/diethyl ether/acetic acid (80:70:1.5, v/v), and the radioactivity was determined by liquid scintillation counting.

2.4. *Preparation and treatment of saponin-permeabilized platelets*

[$^3$H]Arachidonic acid-labeled platelets ($2.5 \times 10^9$ cells/ml, pH 7.0) were diluted 5-fold with KCl-buffer (160 mM KCl, 2.3 mM $MgCl_2$ and 12 mM Hepes, pH 7.0), just before use. The platelets were pretreated with BW755C (50 $\mu$M) at 37°C for 2 min and then incubated with saponin (18 $\mu$g/ml) in the presence of GTP$\gamma$S (200 $\mu$M) for 2 min. The platelet suspension was adjusted to pH 7.4 or 7.8 with an addition of KCl-buffer (pH 10.0) and further incubated at 37°C for 10 min (final concentration of GTP$\gamma$s 100 $\mu$M).

## 3. RESULTS

As shown in fig.1, pretreatment of [$^3$H]-arachidonic acid-labeled platelets with PMA (20 nM) or dioctanoylglycerol (DOG, 20 $\mu$M) enhanced markedly A23187 (150 nM)-induced [$^3$H]arachidonic acid liberation in the presence of extracellular $Na^+$, as shown by other authors [3,4]. When extracellular $Na^+$ was gradually replaced with *N*-methyl-D-glucamine, the PMA- and DOG-enhanced liberation was reduced with decrease in concentration of extracellular $Na^+$. In the labeled platelets suspended in $Na^+$-free buffer, the attenuation was about 50%.

When [$^3$H]arachidonic acid-labeled platelets were stimulated with A23187 (150 nM) and the $Na^+$-ionophore monensin (2–10 $\mu$M) in the presence of extracellular $Na^+$, [$^3$H]arachidonic acid liberation was increased as a function of monensin concentration (fig.2A). The potentiating effect of monensin was eliminated by exclusion of extracellular $Na^+$. Similarly, $NH_4Cl$ (5–20 mM) also enhanced dose-dependently the A23187-induced liberation (fig.2B).

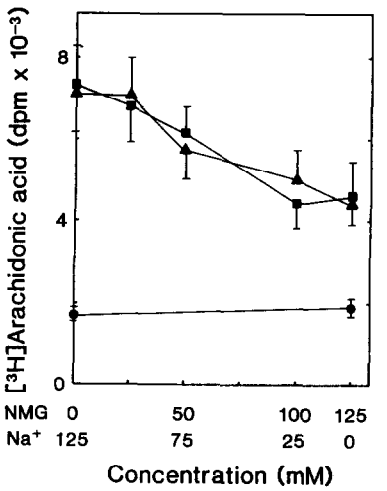


Fig.1. Effect of extracellular $Na^+$ on enhancement by PMA or DOG of A23187-induced arachidonic acid liberation in rabbit platelets. [$^3$H]Arachidonic acid-labeled platelets, suspended in a mixture of various proportions of $Na^+$ and *N*-methyl-D-glucamine (NMG), were pretreated with dimethyl sulfoxide (solvent control, ●), PMA (20 nM, ■) or DOG (20 $\mu$M, ▲) at 37°C for 1 min, and then stimulated with A23187 (150 nM) for 4 min. Each point represents the mean ± SD of three determinations performed in duplicate.

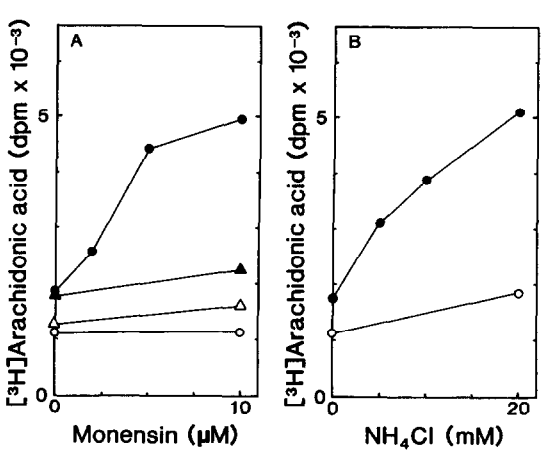


Fig.2. Effect of monensin and $NH_4Cl$ on A23187-induced arachidonic acid liberation in rabbit platelets. [$^3$H]Arachidonic acid-labeled platelets, suspended in the buffer containing $Na^+$ (circle) or *N*-methyl-D-glucamine (triangle), were stimulated with (closed symbol) or without (open symbol) A23187 (150 nM) at 37°C for 4 min in the presence of various concentrations of monensin (A) or $NH_4Cl$ (B). Each point represents the mean of two separate experiments performed in duplicate.

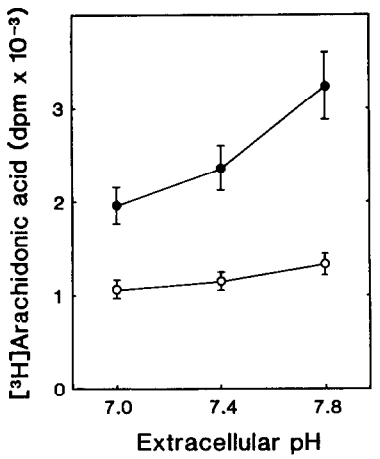


Fig.3. Effect of extracellular pH on GTPγS-induced arachidonic acid liberation in saponin-permeabilized rabbit platelets. [$^3$H]Arachidonic acid-labeled platelets were treated with saponin (18 μg/ml) in the presence (●) or absence (○) of GTPγS (200 μM) for 2 min. The platelet suspension was adjusted to pH 7.0, 7.4 and 7.8, and further incubated at 37°C for 10 min. Each point represents the mean ± SD of three determinations performed in duplicate.

Incubation of [$^3$H]arachidonic acid-labeled, saponin (18 μg/ml)-permeabilized platelets with GTPγS (100 μM) caused an increase in [$^3$H]-arachidonic acid liberation at pH 7.0 (fig.3). The GTPγS-induced liberation is increased further by a rise in extracellular pH (pH 7.4, 7.8).

## 4. DISCUSSION

Although some previous reports showed that protein kinase C activator potentiates $Ca^{2+}$-dependent arachidonic acid liberation [3,4], the mechanism was not elucidated. Here, we demonstrated that potentiation of A23187-induced arachidonic acid liberation by PMA or DOG is partially reduced by exclusion of extracellular $Na^+$. Since it has been shown that protein kinase C activator failed to raise intracellular pH in $Na^+$-free medium [5,6], our results suggest that arachidonic acid liberation enhanced by protein kinase C may be due to intracellular alkalinization via the kinase-accelerated $Na^+/H^+$ exchange. This suggestion is supported by the present observation that monensin ($Na^+$-ionophore) and $NH_4Cl$, which evoke intracellular alkalinization [12,13], enhanced A23187-induced arachidonic acid liberation. In the present experiment, stimulation with a combination of A23187 and a protein kinase C activator did not generate diacylglycerol which is known to be a source of free arachidonic acid liberated under an action of diacylglycerol-lipase (not shown). Therefore, these results indicate that the enhanced liberation of arachidonic acid must arise from $Ca^{2+}$-dependent phospholipase $A_2$ activation which is potentiated by protein kinase C-evoked intracellular alkalinization. In fact, a recent report has shown that $Ca^{2+}$ sensitivity of phospholipase $A_2$ in particulate membrane preparation is increased by a rise of pH within physiological range [14].

The exclusion of extracellular $Na^+$, however, did not completely inhibit the PMA- and DOG-enhanced arachidonic acid liberation. Furthermore, our recent study provides a possibility that protein kinase C may affect G-protein coupled to phospholipase $A_2$ to facilitate arachidonic acid liberation [8]. In the present study, we also showed that GTPγS-induced arachidonic acid liberation was enhanced by a rise of extracellular pH in saponin-permeabilized platelets. Accordingly, it is thought that protein kinase C concurrently stimulates $Na^+/H^+$ exchange and G-protein, and the resulting intracellular alkalinization synergistically activates phospholipase $A_2$ with the potentiated G-protein. Thus, the present results suggest that protein kinase C-accelerated $Na^+/H^+$ exchange may modulate, at least partially, phospholipase $A_2$ activation in rabbit platelets.

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