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ARTICLES

The Possible Involvement of Protein Phosphatase 1 in Thrombin-Induced Ca^{2+} Influx of Human Platelets

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Abstract Protein phosphatase 1 is considered to be involved in thrombin-induced platelet activation (Murata et al., Biochem Int 26:327–334, 1992). To clarify the mechanism, we examined the effects of protein phosphatase 1 and 2A inhibitors (calyculin A, tautomycin, okadaic acid) on Ca^{2+} influx. In the presence of 1 mM Ca^{2+}, thrombin- (0.1 U/ml) induced platelet aggregation and ATP release were inhibited by calyculin A, while this inhibitory effect was abolished in the absence of Ca^{2+} (EGTA 1 mM). Furthermore, thrombin-induced Mn^{2+} influx but not intracellular Ca^{2+} mobilization was inhibited by calyculin A in a dose-related manner. Calyculin A also blocked the ongoing Ca^{2+} influx when added 3 min after thrombin stimulation. Similar inhibitory effects were observed with okadaic acid and tautomycin in the same potency sequence as the reported one for protein phosphatase 1 (calyculin A > tautomycin > okadaic acid). These results suggest that the anti-platelet effects of phosphatase inhibitors are due to the inhibition of Ca^{2+} influx and that protein phosphatase 1 plays a key role in the regulation of receptor operated Ca^{2+} channel of human platelets. © 1993 Wiley-Liss, Inc.

Key words: platelets, protein phosphatase, Ca^{2+} influx, calyculin A, tautomycin, okadaic acid, thrombin, receptor operated Ca^{2+} channel

It has been reported that thrombin-induced platelet aggregation, ATP release, and rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$) are inhibited by protein phosphatase 1 and 2A inhibitors, okadaic acid (OA) [1], and calyculin A (CLA) [2,3]. The potency sequence for these inhibitions is the same as the reported one for protein phosphatase 1: calyculin A > okadaic acid. Although these phosphatase inhibitors have no significant effects on the activity of cyclic AMP dependent kinase, myosin light chain kinase, or protein kinase C [4,5], they induce the phosphorylation of some specific proteins in non-stimulated platelets. These results suggest that protein phosphatase 1 may be involved in platelet activation via the dephosphorylation of specific phosphoproteins unidentified so far. To confirm further the involvement of protein phosphatase 1, we examined the effect of another protein phosphatase 1 and 2A inhibitor, tautomycin (TM) [6], on platelet reaction, in addition to calyculin A and okadaic acid. Furthermore, since the rise in $[Ca^{2+}]i$ is considered to play a pivotal role in platelet reaction, we also attempted to elucidate the possible involvement of protein phosphatase 1 in the regulation of $[Ca^{2+}]_i$.

Abbreviations used: ATP, adenosin tris phosphate; $[Ca^{2+}]_i$, cytosolic free calcium concentration; OA, okadaic acid; CLA, calyculin A; TM, tautomycin; EGTA, [ethylene bis(oxyethylene nitrilo)]tetra acetic acid; fura-2 AM, fura-2 acetoxy methyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; IP3, inositol-1,4,5-trisphosphate; MLCK, myosin light chain kinase; PRP, platelet rich plasma.

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MATERIALS AND METHODS

Materials

Calyculin A and okadaic acid were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Tautomycin was a kind gift of Drs. Hori and Karaki (Tokyo University, Tokyo). Fura-2 AM was purchased from Molecular Probes Inc. (Eugene, OR). Thrombin (bovine) was kindly given by Mochida Pharm. Co. (Osaka, Japan). A_{23187} was obtained from Sigma Chemical Co. (St. Louis, MO).

Washed Human Platelets

Platelet rich plasma (PRP) was prepared from fresh human blood anticoagulated with 0.1 vol

of 3.8% (w/v) trisodium citrate by centrifugation at 170*g* for 15 min. PRP was incubated with aspirin (1mM) and EGTA (4mM) for 15 min at 37°C. Platelets were then centrifuged at 850*g* for 10 min and washed by repeated centrifugation with HEPES-glucose buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 5 mM glucose) containing 2 mM EGTA. Finally, platelets were suspended with HEPES-glucose buffer at a concentration of 3.0×10^8/ml.

Platelet Aggregation and ATP Release

Platelet aggregation and ATP release were simultaneously measured by Lumi-Aggregometer (Chronolog, Havertown, PA) according to the method of Feinman [7]. Washed human platelets (3.0×10^8/ml) were incubated with a protein phosphatase inhibitor or 0.5% ethanol (vehicle) at 37°C for 3 min without stirring and subsequently for 2 min with stirring. Luciferin-Luciferase was added 1 min before platelet stimulation.

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} Influx

PRP was incubated with fura-2 AM (2 μM) at 37°C for 30 min and with aspirin (1 mM) for 15 min. Cells were then washed with HEPES-glucose buffer containing 2 mM EGTA and suspended with the same buffer (1.0×10^8 cells/ml). After 5 min incubation with a protein phosphatase inhibitor, platelets were stimulated by thrombin (0.1 U/ml) and fura-2 fluorescence (emission at 500 nm) was measured by Hitachi F-3000 spectrofluorometer according to the method of Hallam et al. [8,9]. The rise in $[Ca^{2+}]_i$ was measured at 340 nm excitation wavelength. The influx of Mn^{2+} was evaluated by quenching of fura-2 fluorescence at 360 nm excitation, which is sensitive to Mn^{2+} but insensitive to Ca^{2+}. Both Mn^{2+} influx and Ca^{2+} mobilization from internal storage pool could be determined at 380 nm excitation as fura-2 fluorescence at 380 nm excitation is sensitive to both cations [9].

RESULTS

Calyculin A inhibited thrombin- (0.1 U/ml) induced platelet aggregation and ATP release in the presence of 1 mM Ca^{2+} but did not affect them in the absence of extracellular Ca^{2+} (1 mM EGTA) (Fig. 1A,B). On the other hand, it had no significant effect on calcium ionophore (A_{23187}) induced platelet aggregation and ATP release

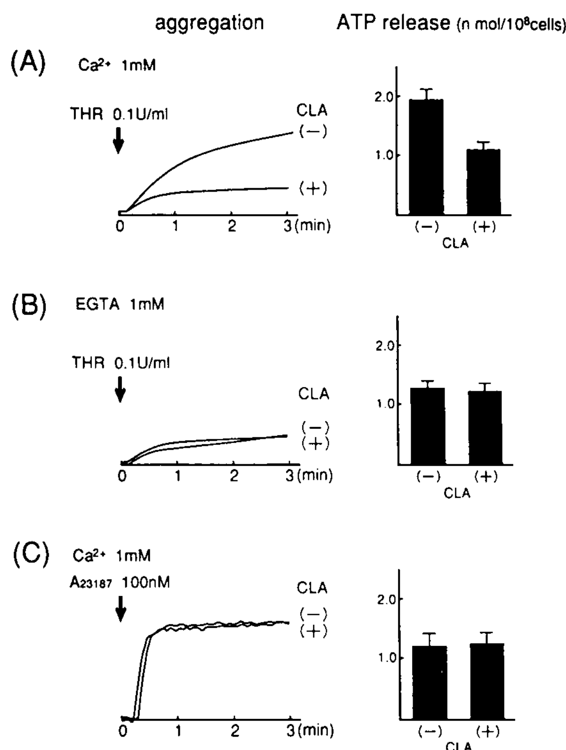

Fig. 1. Effects of calyculin A on thrombin- or A_{23187}-induced platelet aggregation and ATP release. Washed human platelets (3.0×10^8/ml) were incubated with 20 nM calyculin A (CLA) or 0.5% ethanol (vehicle) at 37°C for 5 min in the presence or absence of 1 mM $CaCl_2$. Then thrombin (THR) (0.1 U/ml) or A_{23187} (100 nM) was added to induce platelet aggregation and ATP release. **A:** Thrombin (0.1 U/ml) stimulation in the presence of 1 mM $CaCl_2$. **B:** Thrombin (0.1 U/ml) stimulation in the presence of 1 mM EGTA. **C:** A_{23187} (100 nM) stimulation in the presence of 1 mM $CaCl_2$. Representative patterns of platelet aggregation from at least three independent experiments are traced. ATP release is expressed as mean ± SE (n = 3).

TABLE I. Effects of Protein Phosphatase Inhibitors on Thrombin-(0.1 U/ml) Induced Platelet Reactions in the Presence of 1 mM $CaCl_2$

	IC_{50} (nM)		
	CLA	TM	OA
Aggregation	4.8	320	900
ATP release	4.2	400	1,300

(Fig. 1C). Similar inhibitory effects on platelet reactions were also observed with tautomycin and okadaic acid (Table I). These results indicate that protein phosphatase 1 and 2A inhibitors suppress thrombin-induced Ca^{2+} influx but not intracellular Ca^{2+} mobilization.

To confirm this possibility more directly, the effect of calyculin A on Mn^{2+} influx was exam-

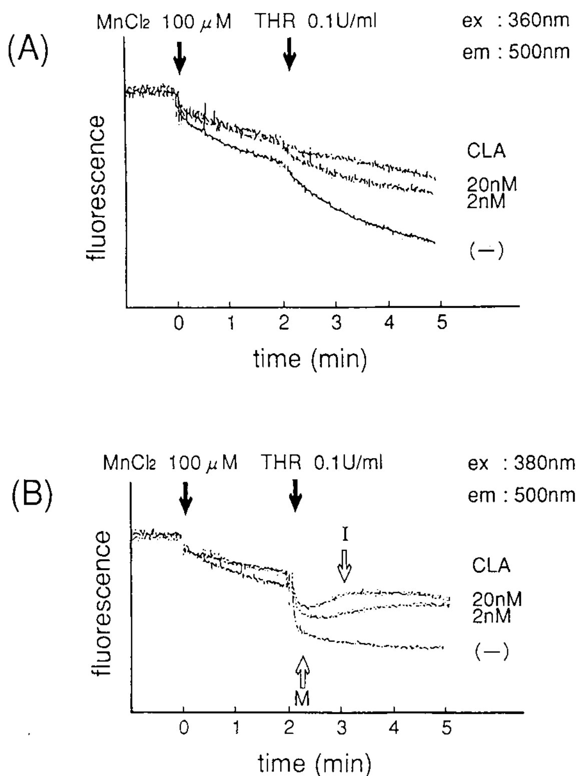

Fig. 2. Effects of calyculin A on Mn^{2+} influx in thrombin-stimulated platelets. Fura-2 AM loaded human platelets (1.0×10^8/ml) were suspended in Ca^{2+} free HEPES-glucose buffer and treated with calyculin A (CLA) (2 nM, 20 nM) or 0.5% ethanol (vehicle) at 37°C for 3 min prior to the addition of 100 μM $MnCl_2$. Following 2 min incubation, platelets were stimulated by thrombin (0.1 U/ml). The quench of fura-2 fluorescence by Mn^{2+} influx was determined at an excitation wavelength (ex) of 360 nm **(A)** and the quench by both Ca^{2+} mobilization and Mn^{2+} influx was determined at 380 nm **(B)** as described in Materials and Methods. An emission wavelength (em) was 500 nm. I, influx; M, mobilization.

ined in thrombin-stimulated platelets since Mn^{2+} is considered to share the same ion channel with Ca^{2+} [8–10]. Preincubation with calyculin A for 5 min did not alter the resting level of fluorescence intensity. However, when Mn^{2+} (100 μM) was added, a small but significant quench in fluorescence was observed at 360 nm or 380 nm excitation wavelength (Fig. 2), which is due to a basal leak of Mn^{2+} into the cells [8]. The addition of thrombin (0.1 U/ml) induced a rapid and strong quench of florescence at 360 nm (i.e., the influx of external Mn^{2+} into the cytoplasm) (Fig. 2A). The pretreatment of calyculin A suppressed thrombin-induced Mn^{2+} quench of fluorescence in a dose-related manner, indicating that Mn^{2+} influx is inhibited by calyculin A. When fura-2 fluorescence was monitored at 380 nm excitation (Fig. 2B), the rapid quench of fura-2 fluorescence after thrombin stimulation (shown by arrow M in Fig. 2B), which is considered to result from intracellular Ca^{2+} mobilization [9], was not significantly inhibited by calyculin A. However, the subsequent, sustained quench of the fluorescence (shown by arrow I in Fig. 2B) caused by Mn^{2+} influx [8] was suppressed in a dose-related manner. Furthermore, when calyculin A was added in the presence of 1 mM $CaCl_2$ 3 min after thrombin stimulation, at which Ca^{2+} channel is considered to be open [11,12], the fluorescence at 340 nm excitation immediately decreased, indicating that the ongoing influx of Ca^{2+} is inhibited by calyculin A (Fig. 3). Similar results on Mn^{2+} (or Ca^{2+}) influx and intracellular Ca^{2+} mobilization were obtained with tautomycin (40–400 nm) and okadaic acid (0.2–2 μM).

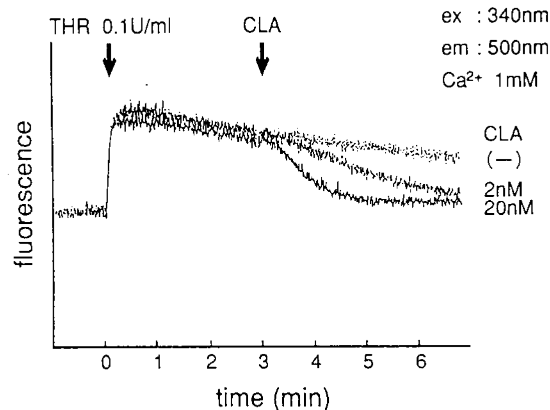

Fig. 3. Effects of calyculin A on Ca^{2+} influx in the late phase of thrombin stimulation. Fura-2 AM loaded platelets (1.0×10^8/ml) were incubated with 1 mM $CaCl_2$ at 37°C for 5 min and activated by thrombin (0.1 U/ml). Calyculin A (CLA) (2 nM, 20 nM) or 0.5% ethanol (vehicle) was added 3 min after thrombin stimulation. Fura-2 fluorescence was determined at an excitation wavelength (ex) of 340 nm and at an emission wavelength (em) of 500 nm as described in Materials and Methods.

DISCUSSION

Hori et al. reported that calyculin A, tautomycin, and okadaic acid inhibit protein phosphatase 1 with the different potency: calyculin A (IC_{50} = 5 nM) > tautomycin (IC_{50} = 32 nM) > okadaic acid (IC_{50} = 224 nM) [6]. In the present study, we demonstrated that these cell-permeable inhibitors suppressed thrombin- (0.1 U/ml) induced platelet reactions in the presence of Ca^{2+} but not in the absence of Ca^{2+} with the same potency sequence as for protein phosphatase 1 (Table I). Although the difference in cell permeability of these inhibitors is not well elucidated yet, these results suggest that protein

phosphatase 1 is involved in thrombin-induced Ca^{2+} influx.

Receptor operated Ca^{2+} channel (ROC) is considered to play a major role in Ca^{2+} influx in thrombin-stimulated platelets [13–16]. Therefore, the downregulation of Ca^{2+} influx by protein phosphatase inhibitors may result from the inhibition of ROC. On the other hand, it is also possible that these protein phosphatase inhibitors prevent the activation of thrombin receptor or phospholipase C pathway. However, we demonstrated that these inhibitors do not suppress thrombin- (0.1 U/ml) induced phosphorylation of P47 (preckstrin) and P20 (myosin light chain), major substrates of protein kinase C and Ca^{2+}/calmodulin dependent MLCK [1,3]. Furthermore, calyculin A did not inhibit the formation of inositol-1,4,5-trisphosphate (IP3) in thrombin-stimulated platelets (unpublished data). Thus, protein phosphatase 1 is considered to be involved in the regulation of ROC rather than in the regulation of thrombin receptor or phospholipase C pathway.

The biochemical properties of Ca^{2+} channel in platelets remain unknown. However, glycoprotein (Gp) IIb/IIIa complex is demonstrated to regulate agonist-induced Ca^{2+} influx in platelets [17–20]. In fact, calyculin A inhibited the formation of GpIIb/IIIa complex in thrombin-stimulated platelets, which can be detected by flow cytometry using PAC1 [21], a monoclonal antibody against the activated form of GpIIb/IIIa complex (unpublished data). Thrombin is also known to induce the phosphorylation of threonine residues located in the cytoplasmic domain of Gp IIIa, probably by activating protein kinase C [22]. However, it remains to be elucidated whether the phosphorylation of Gp IIIa truly regulates the opening of Ca^{2+} channel in platelets.

In conclusion, our data suggest that protein phosphatase 1 may upregulate Ca^{2+} influx by dephosphorylating unknown protein(s) involved in platelet ROC.

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