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Inhibition of Rabbit Platelet Aggregation by Nucleoside 5′-Alkylphosphates

CORRELATION WITH INHIBITION OF AGONIST-INDUCED CALCIUM INFLUX

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ABSTRACT. We investigated the effects of uridine 5′-alkylphosphates on agonist-induced aggregation, increased intracellular calcium concentration $[Ca^{2+}]_i$, and Ca^{2+} (Mn^{2+}) influx in washed rabbit platelets. Uridine 5′-hexadecylphosphate (UMPC16) and uridine 5′-eicosylphosphate (UMPC20) at a concentration of 1×10^{-5} M inhibited platelet aggregation induced by platelet-activating factor (PAF), thrombin, arachidonic acid, and ADP. UMPC16 did not cause significant interference in the binding of [^{3}H-*acetyl*]PAF to platelets. The inhibition of PAF-induced platelet aggregation by UMPC16 was dependent upon the addition time; UMPC16 was ineffective at 60 sec when the extracellular calcium uptake reached the maximum level in PAF-stimulated platelets. Furthermore, UMPC16 inhibited guanosine 5′-*O*-(3-thiotriphosphate)-induced platelet aggregation but did not affect ionophore A23187- and calcium-independent agonist phorbol 12-myristate 13-acetate-induced platelet aggregation. UMPC16 markedly inhibited the Ca^{2+} (Mn^{2+}) influx induced by PAF and ADP, and partly inhibited the $[Ca^{2+}]_i$ increase induced by the receptor-mediated stimulation. On the other hand, UMPC16 did not affect the $[Ca^{2+}]_i$ increase and Ca^{2+} (Mn^{2+}) influx induced by ionomycin. These experiments suggest that inhibition of calcium influx associated with receptor-mediated platelet activation may be involved in the action of UMPC16. BIOCHEM PHARMACOL **60**;2:197–205, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. agonist-induced Ca^{2+} influx; intracellular calcium; fura 2; Mn^{2+} uptake; nucleoside 5′-alkylphosphates; platelet aggregation

Platelet activation, induced by various physiological or pathological stimuli, consists of several processes, operating in parallel, which culminate with a biological end point characterized by aggregation of the cells. Early events include turnover of inositol phospholipids, production of inositol polyphosphates, calcium influx and mobilization of intracellular calcium, production of diacylglycerol and free arachidonic acid, and the phosphorylation of various proteins [1–7]. Recently, it has been reported that thiazolo[2,3-*a*]pyrimidine derivatives possess potent calcium antagonistic activity [8], and that 1,3-bis(4-*sec*-butylphenyl)tetrahydropyrimidin-2-ylidenamine is a potent blocker of the neuronal Ca^{2+} channel [9]. We have reported that nucleoside 5′-alkylphosphates inhibit the sexual agglutination between **a** and α haploid cells of *Saccharomyces cerevisiae*, whereas they do not affect yeast cell growth [10]. However, the mechanism by which nucleoside 5′-alkylphosphates inhibit sexual agglutination remains unclear. It has been shown that divalent metal ions are required for both sucrose- and dextran-induced agglutination of *Streptococcus* mutant cells [11], and that ristocetin-induced platelet agglutination stimulates GPIb/IIIa-dependent calcium influx [12]. We were interested in determining whether uridine 5′-alkylphosphates possess calcium antagonistic activity. Increased free $[Ca^{2+}]_i$§ due to mobilization of intracellular pools of calcium and extracellular calcium inflow is a critical early event in platelet activation by agonists binding to membrane receptors [3, 4]. Therefore, if uridine 5′-alkylphosphates are potent calcium inflow blockers, it is possible that uridine 5′-alkylphosphates inhibit receptor-mediated platelet activation, such as platelet aggregation. Hence, the present experiments were undertaken to study the effect of uridine 5′-alkylphosphates on platelet activation (aggregation) induced by PAF, thrombin, ADP, arachidonic acid, GTPγS, PMA, and ionophore A23187. Furthermore, as part of the same experimental approach,

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§ *Abbreviations:* AMPC16, adenosine 5′-hexadecylphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; CMC, critical micellar concentration; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; UMPC8, uridine 5′-octylphosphate; UMPC12, uridine 5′-dodecylphosphate; UMPC16, uridine 5′-hexadecylphosphate; UMPC20, uridine 5′-eicosylphosphate; and UMPC24, uridine 5′-tetracosylphosphate.

the binding pattern of agonist (PAF) to platelets, as well as changes in cytosolic $[Ca^{2+}]_i$ and Ca^{2+} (Mn^{2+}) influx by the agonists, was investigated.

MATERIALS AND METHODS

Materials

UMPC8, UMPC12, UMPC16, and AMPC16 were synthesized and purified by silica gel column chromatography as described previously [10]. UMPC20 and UMPC24 were newly synthesized by precisely following the above procedure, except that eicosyl or tetracosyl alcohol was used as an alkyl donor of the synthetic reaction. 1-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) was purchased from Bachem Feinchemikalien AG. The calcium ionophore A23187, thrombin from bovine plasma, arachidonic acid, 1-hexadecanol, ADP, GTPγS, ionomycin calcium salt, and BSA (fraction V, essentially fatty acid-free) were purchased from the Sigma Chemical Co. The nucleoside 5′-alkylphosphates and lipids yielded a single spot on TLC using a silica gel 60H plate with a solvent system of chloroform–methanol–water (65:35:6, by vol.) or a single peak on HPLC using an ODS column with a solvent system of methanol–water–acetonitrile (14:40:45, by vol.). 1-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (222 Gbq/mmol, [^{3}H]*acetyl*]PAF) was purchased from New England Nuclear Japan. Fura 2-AM and fura 2 were obtained from Dojin. PMA, Ficoll-Paque, and pinacyanol chloride were obtained from Research Biochemicals International, Amersham Pharmacia Biotech, and Nacalai Tesque, respectively. Solvents were all of reagent grade.

Buffers

(1) Basic Tyrode's solution: NaCl, 8.01 g/L; KCl, 0.195 g/L; $MgCl_2 \cdot 6H_2O$, 0.215 g/L; $NaHCO_3$, 1.02 g/L; glucose, 1.01 g/L; pH 7.2. (2) Tyrode/gelatin without Ca^{2+} with EDTA: same as Buffer 1 but containing gelatin (2.5 g/L) and Na_2 EDTA, 0.373 g/L; pH 6.5. (3) Tyrode/gelatin with Ca^{2+}: same as Buffer 1 but containing gelatin (2.5 g/L) and $CaCl_2 \cdot 2H_2O$ (0.143 g/L); pH 7.2. (4) HEPES-buffered saline with EDTA: HEPES, 1.00 g/L; NaCl, 8.01 g/L; KCl, 0.195 g/L; dextrose, 1.01 g/L; Na_2 EDTA, 0.373 g/L; pH 7.4.

CMC of Uridine 5′-Alkylphosphates

CMC was determined by measuring the visible absorption (595 nm) of a dye, pinacyanol chloride, as a function of uridine 5′-alkylphosphates [13]. The visible absorbance of the dye solution increases remarkably at CMC because of the increased solubility of the dye in the uridine 5′-alkylphosphate. CMC is defined by the intersection of the straight line fits of the absorbance data for concentrations below and above CMC.

Preparation of Rabbit Platelets

Plastic containers or siliconized glassware were used for all platelet preparation and stimulation procedures. Washed rabbit platelets were obtained by a modification of the procedure described previously [14]. Each 45-mL portion of rabbit blood was withdrawn into 5 mL of 41 mM citric acid–85 mM trisodium citrate–2% glucose solution and centrifuged at 170 *g* for 10 min. The supernatant platelet-rich plasma was underlayered with 10 mL of Ficoll-Paque and then was centrifuged at 750 *g* for 20 min. The platelet layer was mixed gently with 40 mL of HEPES-buffered saline containing 0.1 mM EDTA (pH 7.2) underlayered with 0.2 mL of Ficoll-Paque and centrifuged at 750 *g* for 10 min. Again the platelet layer was suspended in 40 mL of HEPES-buffered saline containing 0.1 mM EDTA (pH 7.2) and centrifuged at 750 *g* for 10 min. The pellet was resuspended in Tyrode/gelatin buffer containing 0.1 mM EDTA (pH 6.5) at a concentration of 1.0×10^9 cells/mL.

Platelet Aggregation Assay

Platelets (1×10^8 cells, 0.4 mL) were stimulated in Tyrode/gelatin buffer (pH 7.2) containing 1 mM Ca^{2+}. Aggregation activity was measured as the change in light transmission using an aggregometer (Nikko Hematracer, PAT-2A). Uridine 5′-alkylphosphates in 0.05% ethanol/0.05% DMSO/saline were added to the system 1 min before addition of the PAF solution unless otherwise stated. For the GTPγS-induced platelet aggregation assay, platelet membranes were permeabilized by a 1-min incubation with saponin (3.3 μg/mL) to allow for the delivery of non-permeable GTP to the platelet cytosol. The percent inhibition of platelet aggregation was calculated as [(control response − inhibited response)/control response] × 100. An IC_{50} was generated from the regression analysis of the concentration–response curve.

Binding Studies with [^{3}H-Acetyl]PAF

Platelets in Tyrode/gelatin buffer (pH 7.2) containing 0.1% BSA and 1 mM Ca^{2+} (2.5×10^8 cells/mL) were incubated with UMPC16 (1×10^{-5} M) for 1 min and then were exposed to [^{3}H-*acetyl*]PAF (1×10^{-9} M) at 37°. The binding assay was done in polypropylene tubes. After a 3-min incubation, 400 μL of the platelets was withdrawn, layered on 50 μL of dibutyl phthalate/dioctyl phthalate (3:1, v/v), and separated from the suspending medium by centrifugation (10,000 *g*, 60 sec), followed by washing with 400 μL of 0.1% BSA/saline solution. The platelet pellet was resuspended in 300 μL of 1% (w/v) Triton X-100, and the platelet-bound radioactivity was measured by liquid scintillation counting. Specific binding was calculated as the difference between [^{3}H-*acetyl*]PAF binding in the absence or presence of a 1000-fold molar excess of unlabeled PAF.

Measurement of Cytosolic Free Calcium Levels

Rabbit platelets (1×10^9 cells/mL) suspended in HEPES-buffered saline containing 0.1 mM EDTA (pH 7.4) were incubated with 1×10^{-6} M fura 2-acetoxymethyl ester for 45 min at 37°, washed once to remove extracellular dye, and resuspended at a concentration of 1×10^9 cells/mL of Tyrode/gelatin buffer containing 0.1 mM EDTA (pH 6.5). Aliquots (0.48 mL) of cells were dispensed into round-bottom test tubes, and the external Ca^{2+} ($[Ca^{2+}]_0$) concentration was adjusted to 1 mM with $CaCl_2$. These samples were then transferred to a purpose-built cuvette holder and thermostatically maintained at 37° in a fluorescence spectrophotometer CAF-100 constructed by Japan Spectroscopic. The cell suspension was stirred continuously with a magnetic stirrer. Following equilibration at 37° for at least 5 min, the cells were pretreated with 0.01 mL of uridine 5′-alkylphosphates at the indicated concentrations for 1 min followed by the addition of 0.01 mL of agonist at the indicated concentrations. Fura-2 fluorescence was monitored continuously at excitation wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}) (each with ±5 nm). The corresponding emission signals (500 ± 10 nm) as well as the ratio signal (F_{340}/F_{380}), referred to as $R_{340/380}$, a measure of $[Ca^{2+}]_i$, were recorded. Absolute $[Ca^{2+}]_i$ was calculated by the ratio method [15] from the following equation:

$$[Ca^{2+}]_i = K_d \cdot B \cdot [(R - R_{min})/(R_{max} - R)]$$

where K_d is the dissociation constant of fura-2 for Ca^{2+}, assumed *in vivo* to be 224 nM [14]; B is the ratio of F_{380} in Ca^{2+}-free solution to that in Ca^{2+}-containing solution; R is the fluorescence ratio at F_{340}/F_{380}; R_{max} was obtained by the addition of 0.01 mL of 20% Triton X-100, and R_{min} was obtained by the addition of 0.01 mL of 150 mM Tris/450 mM EGTA after determination of R_{max}.

The uptake of Mn^{2+} was monitored by the quenching of fura-2 fluorescence excited at 360 nm (F_{360}), a wavelength not sensitive to change in $[Ca^{2+}]_i$ [15–17]. Within each experiment, three to four determinations of each inhibitor addition were carried out. To minimize time-dependent effects on platelet responsiveness, leakage of the dye, or redistribution of fluorescence label, experiments were designed to be completed within 1 hr.

Data Presentation

Results shown in the tables and figures are representative of experiments performed at least twice using separate platelet preparations and are presented as an average of duplicates (±range of the mean) unless otherwise stated. The statistical significance of differences was analyzed using an unpaired *t*-test. Analysis was conducted using Statview software (Abacus Concepts). Differences were considered significant at $P < 0.05$.

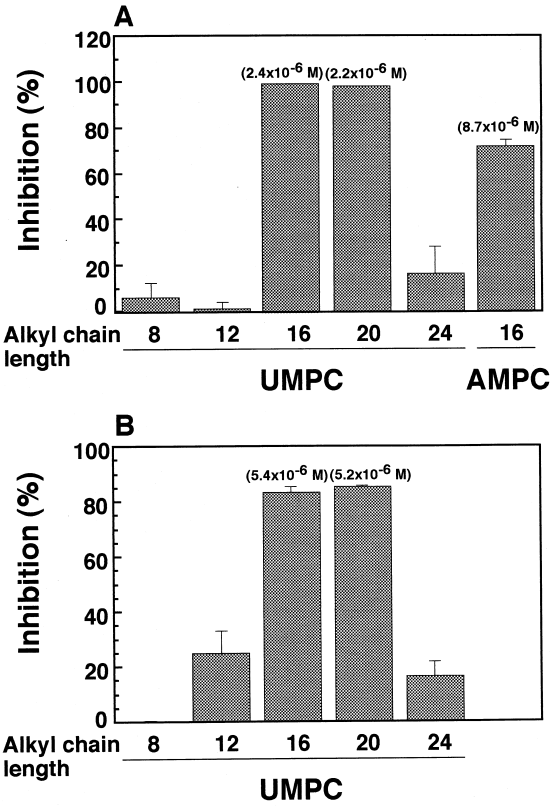

FIG. 1. Effect of uridine 5′-alkylphosphates on PAF (A)- and thrombin (B)-induced platelet aggregation. Washed rabbit platelets (1×10^8 cells, 0.38 mL) in Tyrode/gelatin buffer (pH 7.2) containing 1 mM $CaCl_2$ were preincubated with UMPC8, UMPC12, UMPC16, UMPC20, UMPC24, and AMPC16 in 2.0% ethanol/2.0% DMSO/saline (0.01 mL) at the indicated concentrations and then exposed to PAF in 0.1% BSA (final concentration, 8×10^{-11} M) (A) and thrombin in saline (final concentration, 0.05 U/mL) (B) for 2 and 4 min, respectively, at which time the maximum responses were obtained in the presence of vehicle. Percent inhibition of platelet aggregation was calculated as described in Materials and Methods, and the results are presented as averages of duplicates (±range of the mean). The numbers in parentheses are the IC_{50} values.

RESULTS

Effect of Nucleoside 5′-Alkylphosphates on PAF- and Thrombin-Induced Platelet Aggregation

The behavior of UMPC8, UMPC12, UMPC16, UMPC20, UMPC24, and AMPC16 on agonist-induced platelet aggregation was assayed. These nucleoside 5′-alkylphosphates prevented the PAF- and thrombin-induced platelet aggregation to varying degrees, as shown in Fig. 1 A and B. The uridine 5′-alkylphosphates containing a C16 or C20 span methylene chain length of the polar head group (UMPC16 and UMPC20) inhibited the PAF- and thrombin-induced platelet aggregation more strongly than UMPC8, UMPC12, and UMPC24, indicating that the alkyl chain moiety of uridine 5′-alkylphosphate plays an important role in expressing biological activity. The IC_{50} value (2.4×10^{-6} M) of UMPC16 toward 8×10^{-11} M PAF-induced aggregation was almost the same as that of UMPC20 (2.2 ×

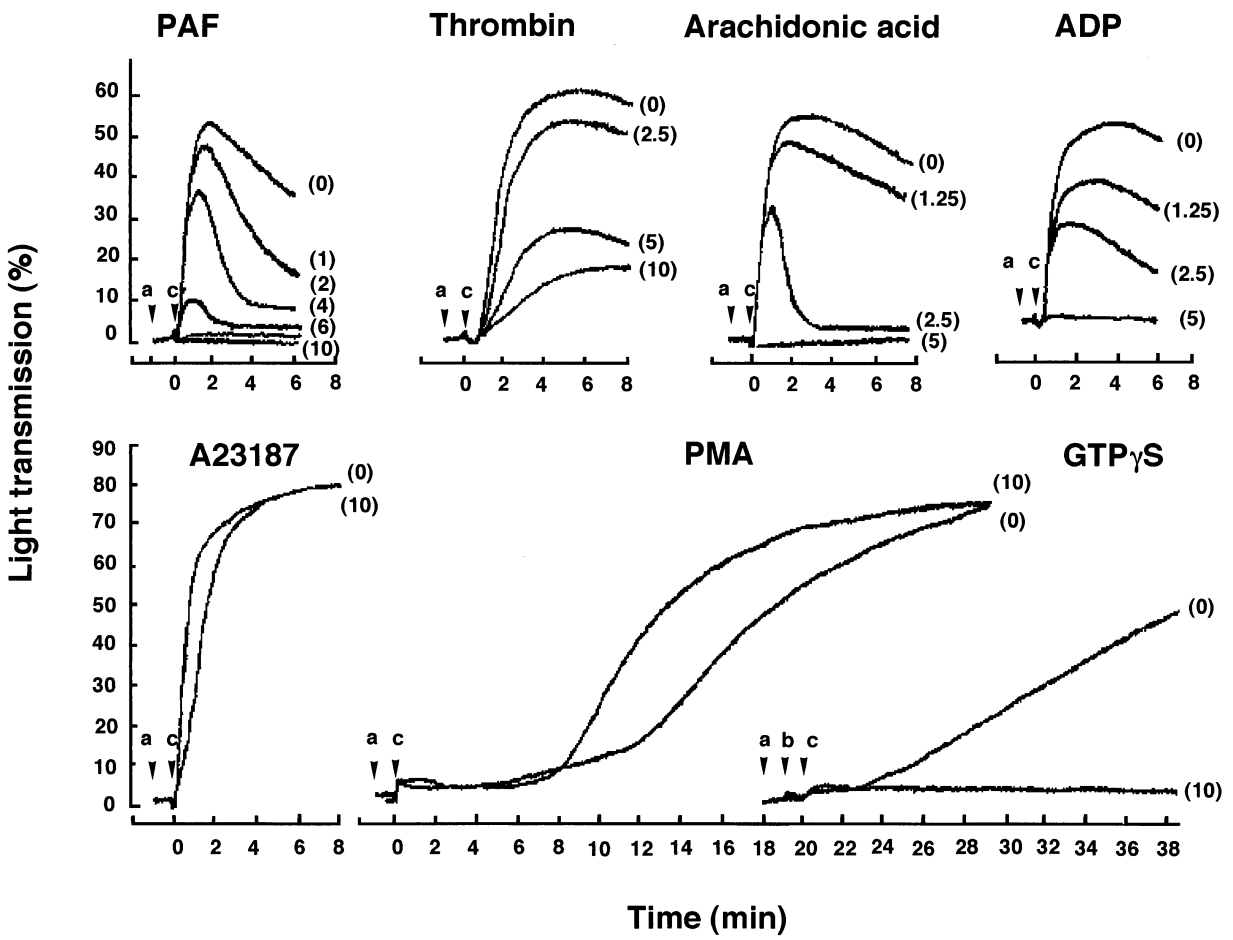

FIG. 2. Effect of UMPC16 on agonist-induced platelet aggregation. Platelets (1×10^8 cells, 0.38 mL) were preincubated with UMPC16 at various concentrations for 1 min (a), as described in the legend for Fig. 1, and then exposed to agonists (0.01 mL) except GTPγS (c), PAF in 0.1% BSA/saline (final concentration, 8×10^{-11} M), thrombin in saline (final concentration, 0.05 U/mL), arachidonic acid in 4% ethanol/saline (final concentration, 2×10^{-5} M), ADP in saline (final concentration, 2.5×10^{-5} M), A23187 in 4% DMSO/saline (final concentration, 2×10^{-7} M), and PMA in 4% ethanol/saline (final concentration, 2×10^{-8} M). GTPγS (final concentration, 2×10^{-5} M) (c) was added to platelets preincubated with UMPC16 (final concentration, 1×10^{-5} M) (a) for 1 min and then permeabilized with saponin (3.3 μg/mL) (b) for another 1 min. The numbers in parentheses are the concentrations of UMPC16 (μM).

10^{-6} M) (Fig. 1). The concentrations were markedly lower than the CMC values of UMPC16 and UMPC20 (1.88×10^{-4} and 1.32×10^{-4} M, respectively). In addition, AMPC16 showed the ability to inhibit the PAF-induced platelet aggregation; the extent of inhibition of 8×10^{-11} M PAF-induced aggregation by AMPC16 (IC_{50} value, 8.7×10^{-6} M) was lower than that by UMPC16. On the other hand, 1-hexadecanol at concentrations of 1–10 μM, which contains an alkyl moiety comparable to that of UMPC16, did not suppress PAF-induced platelet aggregation (data not shown).

Effect of UMPC16 on Platelet Aggregation Induced by Various Agonists

To investigate whether the inhibition of platelet aggregation by nucleoside 5′-alkylphosphates was only of receptor-mediated stimulation, the effect of UMPC16 on platelet aggregation induced by various agonists was assayed. As shown in Fig. 2, PAF-, thrombin-, arachidonic acid-, ADP-, and GTPγS-induced platelet aggregation was reduced markedly by UMPC16. The concentration-dependent effects of UMPC16 and agonist on platelet aggregation are recorded in Figs. 2 and 3. The magnitude of inhibition was dependent on the concentration of UMPC16 and agonist. The IC_{50} values of PAF (8×10^{-11} M)-, thrombin (0.05 U/mL)-, arachidonic acid (2×10^{-5} M)-, and ADP (2.5×10^{-5} M)-induced aggregation were similar (2.4×10^{-6}, 5.4×10^{-6}, 2.1×10^{-6}, and 2.2×10^{-6} M, respectively). In contrast, platelet aggregation induced by PMA at concentrations of 2×10^{-9} to 2×10^{-7} and A23187 at concentrations of 2×10^{-8} to 2×10^{-7} M was not prevented by 1×10^{-5} M UMPC16. These results indicate that UMPC16 inhibited receptor-mediated stimulation,

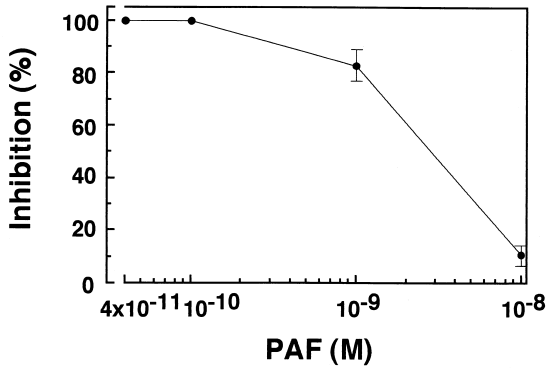

FIG. 3. Inhibitory action of UMPC16 on platelet aggregation by PAF at various concentrations. Platelets were preincubated with UMPC16 (final concentration, 1×10^{-5} M) for 1 min and then exposed to PAF for 2 min, as described in the legend for Fig. 1. Percent inhibition of platelet aggregation was calculated as described in Materials and Methods, and the results are presented as averages of duplicates (±range of the mean).

whereas the protein kinase C pathway activated by PMA and Ca^{2+} influx induced by A23187 were not influenced.

Characterization of Inhibitory Action of UMPC16 on Receptor-Mediated Platelet Aggregation

Since the inhibition of Ca^{2+} influx by the capacitative calcium influx blocker econazole has been reported to be reversed immediately upon addition of 0.3% (w/v) BSA [18], we further investigated the effect of BSA on the inhibitory action of UMPC16 to PAF-induced platelet aggregation. After rabbit platelets (2.5×10^8 cells/mL) in Tyrode/gelatin buffer, pH 7.2, containing 1 mM $CaCl_2$ were preincubated with UMPC16 (1×10^{-5} M) for 1 min and then incubated with PAF (1×10^{-9} M) for 2 min, BSA dissolved in saline [final concentration, 0.3% (w/v)] or saline was added to the platelets. Three minutes later, the extent of platelet aggregation was 58.3 ± 6.5 or 12.5 ± 6.5% in the absence and presence of UMPC16, respectively. This result indicates that the inhibition of PAF (1×10^{-9} M)-induced platelet aggregation by UMPC16 (1×10^{-5} M) was not reversed upon the addition of 0.3% (w/v) BSA. Next, we investigated whether supplemental calcium reversed the inhibition of platelet aggregation by UMPC16 using PAF as an agonist. Increasing the concentration of calcium ranging from 1 to 10 mM in platelet suspension did not reverse UMPC16 inhibition of PAF-induced aggregation, indicating that UMPC16 is not a calcium chelator. Furthermore, to determine whether UMPC16 was effective even after the addition of agonist (PAF), the influence of addition time of UMPC16 on platelet aggregation was studied. A 1-min incubation with 1×10^{-5} M UMPC16 before the addition of 8×10^{-11} M PAF caused 100% inhibition of PAF-induced aggregation (Fig. 4A). However, when platelets (2.5×10^8 cells/mL) preincubated with 1×10^{-5} M UMPC16 for 10 sec, 30 sec, 1 min, and 2 min were immediately diluted four times, 1×10^{-9} M PAF-induced

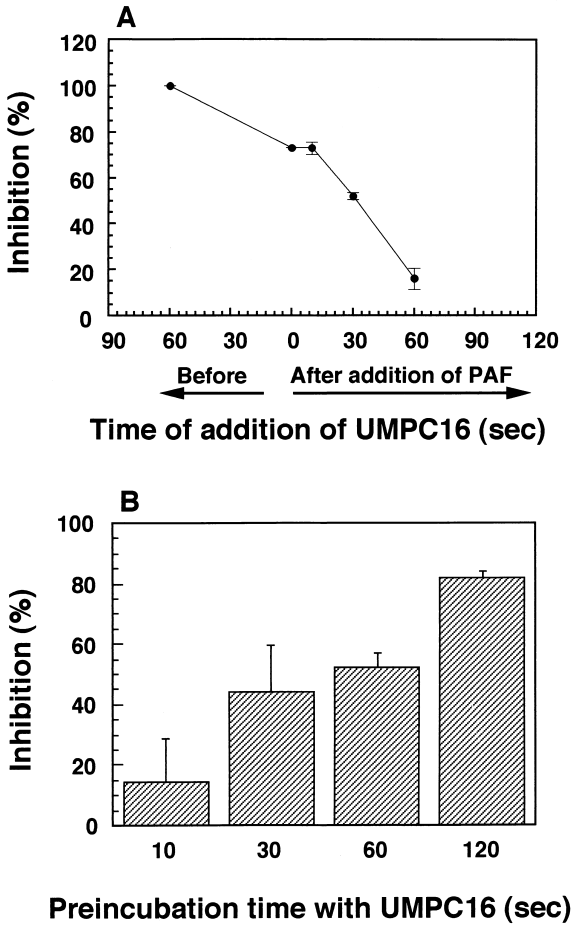

FIG. 4. Effect of addition of UMPC16 at various times (A) and preincubation time with UMPC16 (B) on PAF-induced platelet aggregation. (A) UMPC16 (final concentration, 1×10^{-5} M) or vehicle was added to platelets at the indicated times before and after addition of PAF (final concentration, 8×10^{-11} M). (B) Platelets were preincubated with UMPC16 (final concentration, 1×10^{-5} M) for the indicated periods, immediately diluted four times, and then exposed to PAF (final concentration, 1×10^{-9} M). Percent inhibition of platelet aggregation was calculated as described in Materials and Methods, and the results are presented as averages of duplicates (±range of the mean).

platelet aggregation was suppressed to 85.7 ± 14.3, 56.0 ± 15.5, 47.6 ± 4.7, and 17.9 ± 2.1% of vehicle-treated control, respectively, in spite of the fact that UMPC16 at the indicated concentration of 2.5×10^{-6} M slightly inhibited aggregation (Fig. 4B). Furthermore, when UMPC16 was added to platelets 10 sec, 30 sec, and 1 min after the addition of PAF, platelet aggregation occurred to the extent of 25, 48, and 84% of the control experiments, respectively. These observations indicated that UMPC16 was not an effective inhibitor if added after the agonist, and that the expression of inhibitory action by UMPC16 required reaction time with platelets. To investigate the possibility that UMPC16 inhibits the binding of agonist to its receptor, the influence of UMPC16 on [^{3}H-*acetyl*]PAF binding to platelets was examined. PAF has been shown to

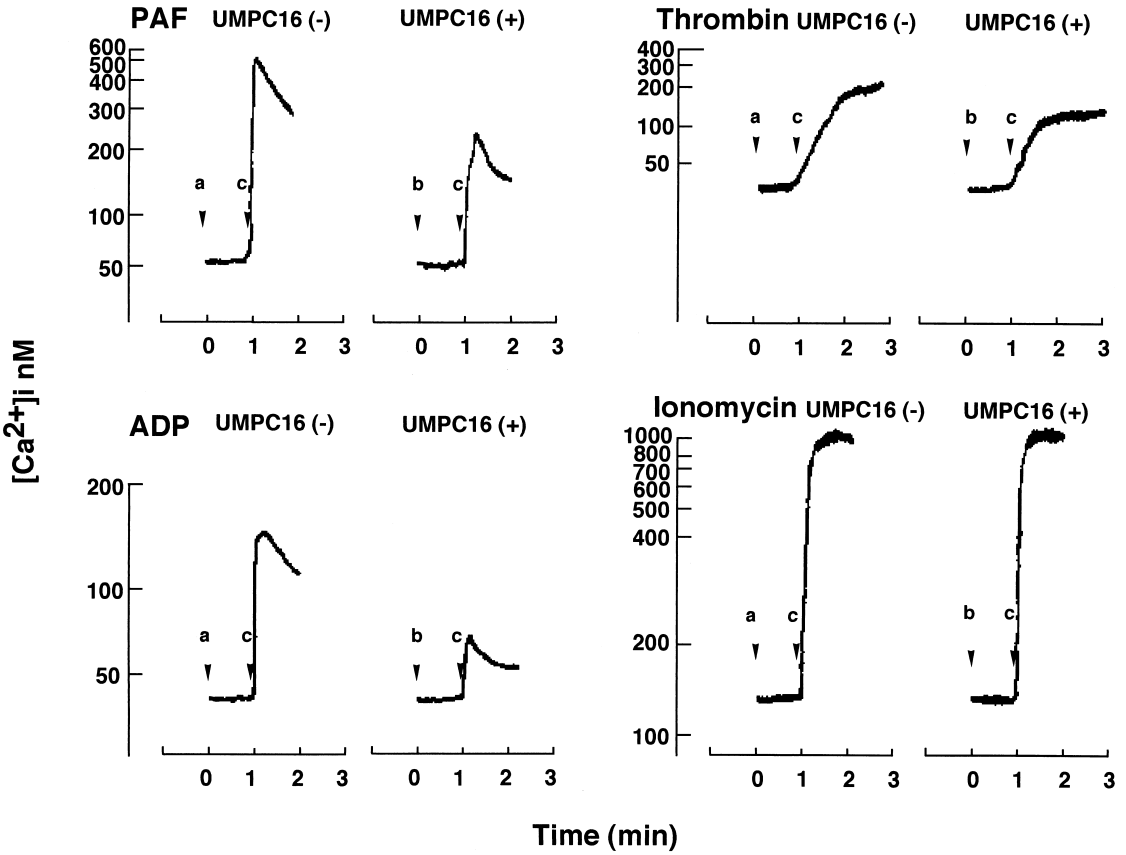

FIG. 5. Effect of UMPC16 on PAF-, thrombin-, ADP-, and ionomycin-induced intracellular platelet calcium elevation. UMPC16 or vehicle was preincubated with platelets directly in the 37° water-jacketed block of the instrument for 1 min. Maximum response was determined by stimulating with agonists in the presence of vehicle. Additions as indicated were vehicle (a), UMPC16 (b, 1×10^{-5} M) and agonists (c; PAF, 8×10^{-11} M; thrombin, 0.05 U/mL; ADP, 2.5×10^{-5} M; ionomycin, 1.6×10^{-7} M). Results from one of at least four different experiments are shown.

possess specific and non-specific binding sites on rabbit platelets [19]. The specific binding of PAF was not inhibited significantly by UMPC16; the specific binding of PAF at 1×10^{-9} M was 1.9 ± 0.9 and 1.5 ± 0.2 fmol/10^8 cells in the absence and presence of 1×10^{-5} M UMPC16, and the non-specific binding of PAF was 9.4 ± 1.4 and 8.3 ± 0.3 fmol/10^8 cells in the absence and presence of 1×10^{-5} M UMPC16 (N = 6).

Effect of UMPC16 on PAF-, Thrombin-, ADP-, and Ionomycin-Induced $[Ca^{2+}]_i$ Response

Figure 5 shows the agonist-induced $[Ca^{2+}]_i$ peak in the absence and presence of UMPC16, calculated from F_{340}/F_{380}. In the presence of 1 mM Ca^{2+}, the mean basal platelet $[Ca^{2+}]_i$ was 52.0 ± 18.4 nM (N = 15). Whereas 1×10^{-5} M UMPC16 did not alter basal platelet $[Ca^{2+}]_i$ and did not inhibit 4×10^{-8} and 1.6×10^{-7} M ionomycin-induced $[Ca^{2+}]_i$ response, receptor-mediated stimulation (8×10^{-11} M PAF, 0.05 U/mL of thrombin, and 2.5×10^{-5} M ADP)-induced $[Ca^{2+}]_i$ increase was inhibited by 1×10^{-5} M UMPC16; the extents of the inhibition toward PAF (8×10^{-11} M)-, thrombin (0.05 U/mL)-, and ADP (2×10^{-5} M)-induced $[Ca^{2+}]_i$ increase were 65, 34, and 78% compared with that of the vehicle-treated control group, respectively (Table 1). The $[Ca^{2+}]_i$ increase in platelets stimulated by these agonists was due to both release of Ca^{2+} from the intracellular stores and entry through the plasma membrane. The inhibition of the receptor-mediated $[Ca^{2+}]_i$ increase by UMPC16 was not 100% (Fig. 5 and Table 1), but the inhibition of the platelet aggregation was 100%, suggesting that the inhibition of $[Ca^{2+}]_i$ responses by UMPC16 might result from the inhibition of the receptor-mediated Ca^{2+} influx. Accordingly, PAF-, ADP-, and ionomycin-induced Ca^{2+} entries in fura-2-loaded platelets were examined using Mn^{2+} added to the external medium as a marker for Ca^{2+} entry, since the intracellular dye fluorescence is quenched by Mn^{2+} binding [20]. Figure 6 illustrates the effects of 1×10^{-5} M UMPC16 on the acceleration of Mn^{2+} uptake (quenching of F_{360}) induced by 8×10^{-11} M PAF, 2.5×10^{-5} M ADP, and 1.6×10^{-7} M ionomycin. The slow decline in fluorescence with excitation at 360 nm was probably due to a slow basal leak of Mn^{2+} into the cells. Both agonists evoked a fall in fluorescence with excitation at 360 nm. UMPC16 strongly

TABLE 1. Effect of UMPC16 on PAF-, thrombin-, ADP-, and ionomycin-induced intraplatelet calcium elevation

Agonist	Elevated $[Ca^{2+}]_i$ (nM)		Inhibition (%)
	Saline	UMPC16 (1×10^{-5} M)	
PAF (8×10^{-11} M)	474.7 ± 30.3	167.4 ± 17.9*	65
Thrombin (0.05 U/mL)	182.1 ± 18.6	120.4 ± 13.6†	34
ADP (2.5×10^{-5} M)	118.1 ± 12.4	26.4 ± 3.2*	78
Ionomycin (4×10^{-8} M)	204.9 ± 14.4	230.1 ± 9.5	0
(1.6×10^{-7} M)	756.0 ± 94.4	789.5 ± 149.0	0

Platelets (10^9 cells/mL) were incubated and treated with vehicle or UMPC (final concentration, 1×10^{-5} M) for 1 min, as described in the legend of Fig. 5, and then exposed to PAF (final concentration, 8×10^{-11} M), thrombin (final concentration, 0.05 U/mL), ADP (final concentration, 2.5×10^{-5} M), or ionomycin (final concentrations, 4×10^{-8} M and 1.6×10^{-7} M). Maximum $[Ca^{2+}]_i$ levels were determined from fura-2 fluorescence as described in Materials and Methods. The results shown are means ± SD of 4–6 determinations.

*†Significantly different from vehicle-treated control group: *$P < 0.001$, and †$P < 0.01$.

prevented the acceleration of Mn^{2+} uptake induced by receptor-mediated stimulation (PAF and ADP), whereas the inhibition of ADP-induced uptake of Mn^{2+} by UMPC16 was not reversed upon addition of 0.3% (w/v) BSA. There was no significant difference between the uptake of Mn^{2+} in vehicle-treated control platelets and that in PAF- or ADP-stimulated platelets in the presence of UMPC16 (Fig. 6). On the other hand, UMPC16 did not affect the acceleration of Mn^{2+} uptake induced by ionomycin.

DISCUSSION

The data provided by the present study show that nucleoside 5′-alkylphosphates directly inhibited PAF-, thrombin-, arachidonic acid-, and ADP-induced platelet aggregation in a concentration-dependent manner (Figs. 1 and 2). Arachidonic acid is metabolized by cyclooxygenase and thromboxane A_2 synthetase to endoperoxides and thromboxane A_2, which are considered to induce activation of the phospholipase C pathway [20]. Stimulation by PAF, thrombin, thromboxane A_2, and ADP is effected through each receptor and induces phospholipase C activation, which is associated with the protein kinase C system on phosphorylation of the 40-kDa protein. Thus, UMPC16 probably inhibited these receptor-mediated signaling pathways, including Ca^{2+} influx, in platelet aggregation (Fig. 2).

Next, we examined the influence of UMPC16 on the binding of agonist to its receptor, using PAF among these agonists. The compound did not affect significantly the specific binding of PAF to platelets, indicating that the action of UMPC16 was not due to inhibition of the binding of agonists to their receptors. Interestingly, UMPC16 did not affect calcium ionophore A23187-induced platelet aggregation (Fig. 2). Furthermore, UMPC16 did not inhibit PMA-induced platelet aggregation (Fig. 2). PMA directly activates protein kinase C to phosphorylate 40-kDa protein in platelets, which does not require extracellular Ca^{2+} [21]. However, UMPC16 did inhibit GTPγS-induced platelet aggregation (Fig. 1). Guanine nucleotide-binding proteins (G proteins) are thought to couple receptor-associated events, including activation of membrane ion channels [22–27]. Not only receptor-mediated platelet activation but also G protein activation with GTPγS has been shown to result in entry of extracellular Ca^{2+} into endothelial cells from the human umbilical vein [28]. On the other hand, PAF, thrombin, arachidonic acid, and ADP have been reported to induce Ca^{2+} entry through the plasma membrane and Ca^{2+} release from intracellular stores [3, 16, 17]. Our results indicate that UMPC16 may be active in antagonizing Ca^{2+} channels coupled with G-protein receptor. Accordingly, we herein determined the effects of UMPC16 on Ca^{2+} movements in platelets, including Ca^{2+} release from intracellular stores and Ca^{2+} entry through plasma membrane. As expected, although UMPC16 did not affect ionomycin-induced $[Ca^{2+}]_i$ increase, the compound prevented PAF-, thrombin-, and ADP-induced $[Ca^{2+}]_i$ increase (Fig. 5 and Table 1). However, the inhibition of $[Ca^{2+}]_i$ increase by UMPC16 was not 100%. PAF- and ADP-induced uptake of Mn^{2+}, used as a Ca^{2+} surrogate for entry through the plasma membrane, was blocked almost completely by UMPC16 (Fig. 6). Accordingly, the current observations suggest that the mode of action of UMPC16 may be attributable to blockage of calcium influx mediated by agonist-specific binding to its receptor. The inhibitory action of UMPC16 on the uptake of Mn^{2+} was not reversed by BSA, suggesting that the mechanism of action by UMPC16 appears to be one pertaining to a specific event at the plasma membrane, unlike econazole, possibly involving alterations in plasma fluidity/structure [18]. On the other hand, increasing the concentration of calcium in the suspending medium did not reverse UMPC16 inhibition of PAF-induced aggregation (Fig. 4), which was not consistent with the mode of action of the calcium blocker verapamil [29].

The behavior of PAF at concentrations higher than 10^{-8} M was not suppressed by 1×10^{-5} M UMPC16 (Fig. 3), similar to the inhibitory action of PAF antagonists such as WEB2086 and CV3988 [30] and serine protease inhibitors (preventing receptor-mediated platelet activation) [2]. As shown in Fig. 4A, when added to platelets previously

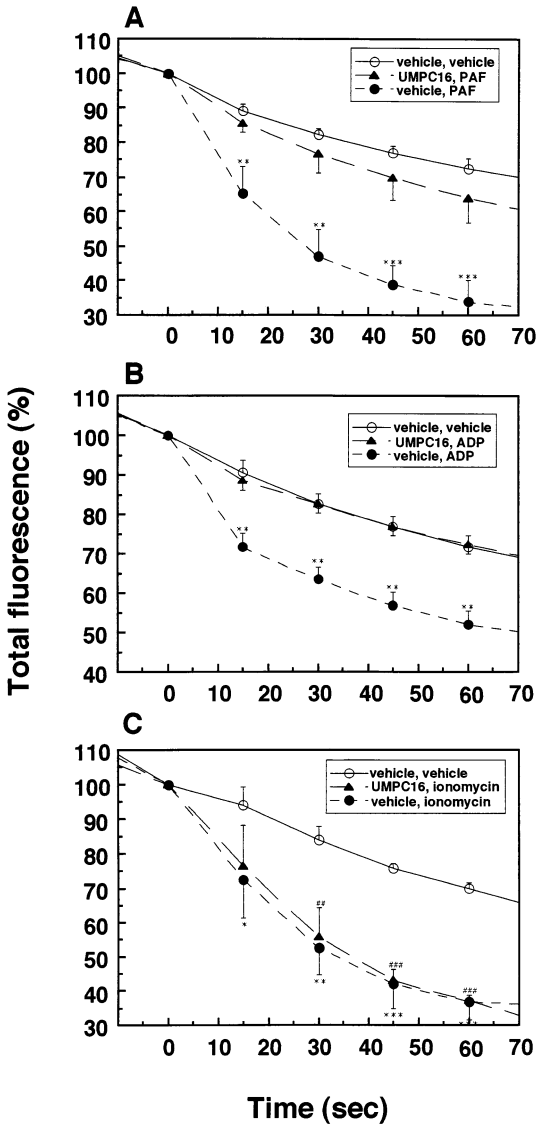

FIG. 6. Effect of PAF (A), ADP (B), and ionomycin (C) on fluorescence of fura-2-loaded platelets in the presence of $MnCl_2$. Platelets were suspended in Ca^{2+}-free medium, preincubated with UMPC16 (final concentration, 1×10^{-5} M) or vehicle for 1 min and with $MnCl_2$ (final concentration, 0.2 mM) for 1 min, and mixed with agonists (final concentration; PAF, 8×10^{-11} M; ADP, 2.5×10^{-5} M; ionomycin, 1.6×10^{-7} M) or vehicle at zero time. The results are presented as means ± SD of 3–4 experiments. Key: (*), (**), and (***) $P < 0.05$, 0.01, and 0.001 versus vehicle-treated control group; (##) and (###) $P <$ 0.01 and 0.001 versus vehicle-treated control group.

stimulated by PAF, the inhibitory action of UMPC16 disappeared time-dependently. Furthermore, when platelets were preincubated with UMPC16 and then immediately diluted in order to reduce the concentration of UMPC16, PAF-induced platelet aggregation was suppressed as the extent of the inhibition was increased in a manner dependent on the preincubation time (Fig. 4B). Accordingly, UMPC16 is likely to function by penetrating the plasma membrane of platelets or from the inside rather than the outside of platelets.

Palmitic acid (C16:0) and arachidic acid (C20:0) do not induce Ca^{2+} influx, but arachidonic acid, probably its metabolite thromboxane A_2, does accelerate the uptake of Ca^{2+} (Mn^{2+}) [31]. On the other hand, it has been reported that in rat thymocytes and in human neutrophils, in which the intracellular Ca^{2+} stores are depleted by treatment with the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin, unsaturated fatty acids, including arachidonic acid, inhibit capacitative Ca^{2+} entry, but saturated arachidic acid and arachidonic methyl ester do not [32]. The present results show that among nucleoside 5′-alkylphosphates, UMPC16 and UMPC20 were more effective inhibitors of platelet activation than AMPC16, UMPC8, UMPC12, and UMPC24 (Fig. 1). Therefore, not only the alkyl chain moiety of nucleoside 5′-alkylphosphates but also the polar head group may be involved in the inhibition of receptor-mediated Ca^{2+} entry. The inhibitory action of UMPC16 and UMPC20 was not associated with the CMC values. The current study agrees with the evidence that UMPC16 and UMPC20 more strongly inhibited sexual agglutination in *S. cerevisiae* than do AMPC16, UMPC8, UMPC12, and UMPC24 ([10] and Tanaka *et al.*, unpublished data). Although PAF production has been reported to be changed during the cell cycle of the yeast *S. cerevisiae*, probably as the regulator [33], inhibition by UMPC16 was not limited to PAF-induced platelet activation (Fig. 1). The present results suggest that the inhibition of intracellular calcium-dependent reactions, which are mediated through an unknown signaling system, may be involved in the inhibition of sexual agglutination by UMPC16. In conclusion, UMPC16 was an effective inhibitor of receptor-mediated Ca^{2+} influx in platelet activation through a specific mechanism of action.

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