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Study on the Cupric Phenanthroline-Induced Serotonin Release in Digitonin-Permeabilized Platelets

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Digitonin-permeabilized rabbit platelets released serotonin dose-dependently in response to cupric phenanthroline, which is a mild oxidant which catalyzes the formation of disulfide bridges. The serotonin release induced by cupric phenanthroline was inhibited by ethylene glycol bis(β-aminoethylether)*N*,*N*′-tetraacetic acid (EGTA), but EGTA did not restore the decrease in protein-bound free sulfhydryls of permeable platelets. An alteration in calcium movements was suggested to be implicated in the serotonin release from cupric phenanthroline-treated permeable platelets.

The agent suppressed the calcium uptake to calcium-storage sites, probably the dense tubular system, and enhanced the calcium efflux. Moreover, it tended to inhibit Ca^{2+}-adenosine triphosphatase of permeable platelets. On the other hand, a sulfhydryl modifier, chloromercuriphenylsulfonic acid, also decreased the amount of free sulfhydryls in protein and released serotonin in permeable platelets, but EGTA did not inhibit either reaction. Neither cupric phenanthroline nor chloromercuriphenylsulfonic acid elicited serotonin release from intact platelets. It is suggested that both agents act on intra-platelet target molecules to exert their effects, but the mechanisms involved are different.

Keywords——platelet activation; serotonin secretion; cupric phenanthroline; permeabilization; sulfhydryl modification; calcium movement

Introduction

Permeable platelets have been proved to have similar reactivity to intact platelets to physiological stimuli,[1] and may be useful to examine intra-platelet interactions.

Recently, inositol 1,4,5-trisphosphate was shown to activate permeable platelets,[1,2] and this action is mediated by the increase in intracellular calcium mobilization.[3] It was suggested that the formation of inositol 1,4,5-trisphosphate by physiological stimuli at an early stage of activation[4-7] leads to platelet secretion and aggregation. However, Lapetina *et al.* reported that thrombin induced serotonin secretion and aggregation independently of inositol phospholipid hydrolysis and protein phosphorylation in human platelets permeabilized with saponin,[8] and the activation of human platelets by adenosine diphosphate (ADP) and collagen may not be mediated by the formation of inositol 1,4,5-trisphosphate.[9-11] These conflicting results suggest that the platelet activation process is complicated.

The aggregation of intact platelets is inhibited when membrane disulfide bonds are reduced.[12,13] Cupric *o*-phenanthroline (CuPh), an oxidizing agent,[14] is known to cross-link proteins by disulfide bond formation,[15-17] and our previous study demonstrated that CuPh and Ca^{2+} each released histamine from digitonin-permeabilized rabbit platelets.[18] However, the significance of the change between free sulfhydryls and disulfide bonds for platelet response is not clearly understood yet. In this study, experimental effort was focused on the mechanism of the CuPh-induced serotonin release, and a relation between the inhibition of Ca^{2+}-dependent adenosine triphosphatase (ATPase) and the serotonin release was suggested

to exist. Moreover, another mechanism which does not reqire calcium mobilization was thought to operate when permeable platelets were treated with a sulfhydryl modifying agent, *p*-chloromercuriphenylsulfonic acid (CMPS).

Materials and Methods

Materials——Reduced nicotinamido adenine dinucleotide (NADH) (grade III, from yeast), phosphoenol pyruvate trisodium salt hydrate and oligomycin were obtained from Sigma (St. Louis, Mo., U.S.A.); $^{45}CaCl_2$ (24.6 mCi/mg calcium) was from Du Pont/New England Nuclear (NEZ-013, Boston, U.S.A.), antimycin A was from Boehringer Mannheim GmbH (Mannheim, F.R.G), *o*-phenanthroline, digitonin, trifluoperazine (TFP) dimaleate, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 1,4-dithiothreitol and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from Wako Pure Chemical Industries, Ltd. (Osaka), ATP was from Fluka AG (Buches, Switzerland), pyruvate kinase (type III, 213 units/mg) and lactate dehydrogenase (LDH, type II, 2930 units/ml) were from Toyobo Co., Ltd. (Osaka), *p*-chloromercuriphenylsulfonic acid (CMPS) monosodium salt was from Nakarai Chemicals, Ltd. (Kyoto), and membrane filters (type TM-2, cellulose nitrate, 0.45 μm) were from Toyo Roshi Co., Ltd. (Tokyo). All other reagents were of the highest grade commercially available.

Preparation of Rabbit Platelets and Digitonin Treatment——Blood samples were collected from mature male Japanese-White rabbits (2.5—4.0 kg) through ear arterial vessels. Blood containing sodium citrate (0.38%) was centrifuged (250 × *g* for 15 min) at 23 °C to remove erythrocytes and leukocytes. Platelets were recovered from the resultant supernatant by centrifugation (1500 × *g* for 7 min), washed twice with modified Tyrode's buffer (10 mM HEPES, pH 7.3/137 mM NaCl/2.7 mM KCl/ 0.42 mM NaH_2PO_4/2mM $MgCl_2$/2 mM glucose/1 mM ethylenediaminetetraacetic acid (EDTA)/0.1% bovine serum albumin), and resuspended in buffer A (30 mM HEPES, pH 7.0/100 mM KCl/20 mM NaCl). The number of platelets was counted with a platelet counter (Toa Medical Electr. Co., Ltd., Kobe), and the number of platelets in the washed platelet preparation was adjusted to 1.0×10^9/ml with the same buffer. In a typical experiment, the platelet suspension was warmed to 37 °C and then 1/10 volume of digitonin (240 μM) was added.

The mixture was incubated for 10 min, then platelets were sedimented by centrifugation (1500 × *g* for 5 min), and resuspended in buffer A at the same platelet count. These permeabilized platelets were reacted with various substances followed by centrifugation (12000 × *g* for 1 min). The serotonin content of the supernatant was determined.

Serotonin, LDH and ATP Measurements——The permeable platelet suspension was incubated for the indicated time, then centrifuged and the resulting supernatant was used for the determination of LDH, ATP and serotonin. Serotonin was assayed directly as total 5-hydroxy-indoles by measuring the fluorescence emitted at 535 nm upon excitation at 295 nm in 3 M HCl.[19-21] A sample volume of 0.25 ml was homogenized in 1.25 ml of cold 0.1 M HCl with a vortex mixer and the protein was precipitated by the addition of 0.5 ml of 10% $ZnSO_4$ followed by 0.25 ml of 1 M NaOH. After centrifugation, the supernatant solution (1.8 ml) was collected and acidified with 0.67 ml of concentrated HCl. Fluorescence was measured with a Shimadzu difference spectrofluorophotometer (RF-503A, Shimadzu Corporation, Kyoto) and serotonin content was estimated by comparison with standard concentrations of serotonin creatinine sulfate treated in the same manner as the samples. LDH (EC 1.1.1.27), which is a cytoplasmic marker enzyme,[22] was quantified by the method of Kornberg.[23] ATP was determined by the luciferin–luciferase method described by Lunden *et al.*[24] Total ATP, LDH and serotonin were determined by solubilizing platelets with 0.1% Triton X-100, and released substances were expressed as percent of the total amounts.

Ca^{2+} Movements——Ca^{2+} uptake was measured by using ^{45}Ca and the Millipore filtration technique.[25] To 40 μl of permeabilized platelet suspension (1×10^9/ml) were added 10 μl each of 10 mM Mg/ATP and 500 μM $CaCl_2$ containing $^{45}Ca^{2+}$ (0.05 μCi/μl). The platelet suspension was incubated at 25 °C, diluted with 1 ml of buffer A at a specified time and immediately passed through a membrane filter (0.45 μm). The filter was washed with the same medium without $^{45}Ca^{2+}$, dried and counted for radioactivity with a liquid scintillation counter (LSC-900, Aloka).

Ca^{2+} efflux was determined by essentially the method described by Chiesi.[26] Passive efflux of Ca^{2+} was measured, after $^{45}Ca^{2+}$ had been loaded into the platelets (0.8×10^9/ml, 25°C, 3 h) in buffer A containing 2 mM Mg/ATP, 100 μM $CaCl_2$ and 0.03 μCi/μl of ^{45}Ca. The efflux experiment was started by diluting Ca^{2+}-loaded platelets 20 times with buffer A containing 0.25 mM ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA). The platelets were incubated for various times, then the mixture was filtered (within 10 s). The filter was washed, dried and counted for radioactivity.

Sulfhydryl Groups of Platelets——The quantification of platelet sulfhydryl groups in protein was carried out by a modification of the method of Yamada *et al.*[27] Permeable platelet suspension was reacted with various substances, then centrifuged at 12000 × *g* for 1 min to give a platelet pellet. The pellet was suspended in 0.2 ml of doubly-distilled water, mixed with 1 ml of precipitating solution consisting of 1.67% *m*-phosphoric acid, 0.02% EDTA/2Na, and 30% NaCl, allowed to stand for about 2 h at 25°C, and centrifuged at 10000 × *g* for 30 s. The denatured protein sediment was neutralized by the addition of 0.1 ml of 0.3 M Na_2HPO_4 followed by solubilization using 1 ml of 1% sodium

dodecyl sulfate, and the precipitate was used for the assay of protein-bound sulfhydryl groups. An aliquot (0.1 ml) of 0.01 M DTNB solution was added to the mixture. The whole was incubated for 30 min at room temperature, then the optical density was measured at 412 nm. The content of sulfhydryl groups was calculated on the basis of a molar extinction coefficient of 1.16×10^4.

Ca^{2+}-Dependent ATPase Activity——ATPase was measured at 37°C essentially as described by Neet and Green.[28] Permeable platelet suspension (1.0 ml) (1.0×10^9/ml) was incubated in buffer A containing 2 units of pyruvate kinase/ml, 2 units of LDH/ml, 5 mM phosphoenolpyruvate and 200 μM NADH. Basal ATPase activity was measured after the addition of 0.5 mM ATP in a double-beam spectrophotometer (model 200-20, Hitachi Ltd., Tokyo) in terms of the decrease in light absorption at 550 nm. Thereafter, 1 mM $CaCl_2$ was added followed by 1 μM A23187, and the ATPase activity was measured. Ca^{2+}-dependent ATPase activity was calculated from the initial velocity of decrease in light absorption and is expressed as nmol of ATP converted per min per 10^9 platelets.

Results

Washed platelets were treated with various concentrations of digitonin as described in Materials and Methods, and leakages of LDH, ATP and serotonin were assessed simultaneously in order to establish optimum conditions for permeabilization. LDH is a cytoplasmic marker enzyme and both serotonin and ATP are constituents of granule contents. Digitonin treatment released LDH from platelets dose-dependently (up to 100 μM) and the leakages of serotonin and ATP were less than that of LDH at every digitonin concentration examined. A digitonin concentration of 20 μM was chosen, which elicited about 16% LDH leakage but only about 3% serotonin and 2% ATP leakages during 10 min incubation, and this was added to the platelet suspension routinely for preparing the permeable platelets unless otherwise indicated. Digitonin-permeabilized platelets showed the same responses (aggregation and release of serotonin) to thrombin as obtained with intact platelets, indicating that there is minimal disturbance to the topography of surface membrane receptors for the agonist.

Figure 1A illustrates the serotonin release from permeable platelets by CuPh. A molar ratio of *o*-phenanthroline/$CuSO_4$ of 3/1 was used. Hereafter, CuPh concentration denotes the *o*-phenanthroline concentration. It released serotonin dose-dependently up to 300 μM. Because CuPh interfered with serotonin quantification at higher concentration (data not shown), the apparent serotonin content was adjusted on the basis of calibration curves. EGTA inhibited the serotonin release induced by CuPh by about 60% at 50 μM EGTA concentration (Fig. 1A), and the inhibition by EGTA was dose-dependent (data not shown). These results are in good agreement with our previous study[18] using histamine as a marker of dense granule content, and suggest that the increase in free calcium concentration is requisite for the CuPh-induced serotonin release. Figure 1B demonstrates that CuPh formed disulfide linkages and decreased the amount of free sulfhydryls in proteins. Protein-bound sulfhydryl groups decreased by 25% during a 15 min incubation at 150 μM CuPh, and the extent of decrease roughly correlates with that of release of serotonin. EGTA did not affect the CuPh-induced decrease in free sulfhydryl groups. This indicates that EGTA did not inhibit the effect of CuPh directly.

Figure 2 demonstrates the calcium uptake in digitonin-permeabilized platelets. Although permeable platelets accumulated calcium in a time-dependent manner in the absence of exogenous ATP, the degree of uptake was greatly enhanced by the addition of ATP. The ATP-dependent calcium uptake was initially time-dependent, and became slight after about 20 min of incubation (Fig. 2A). Platelets showed negligible calcium uptake with or without ATP when they were not treated with digitonin (data not shown). Accumulation of calcium was dependent on ATP concentration; it increased linearly up to about 0.6 mM ATP and reached the maximum level at 1 mM ATP concentration (Fig. 2B), but decreased slightly over 2 mM ATP concentration (data not shown). These experiments indicate that the plasma membrane of digitonin-treated platelets is permeable to calcium ion and the active transport system to calcium storage sites of permeable platelets was revealed to be intact. Figure 2A also

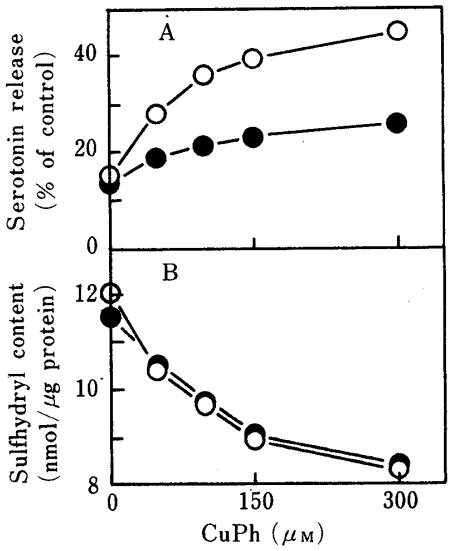

Fig. 1. Effects of CuPh on Serotonin Release (A) and Protein-Bound Free Sulfhydryls (B) in Permeable Platelets

Permeabilized platelets were incubated for 15 min with CuPh at the indicated concentrations in the absence (○) or presence (●) of 50 μM EGTA. (A) After the incubation, the platelets were centrifuged, and the supernatant was obtained. Released serotonin in the supernatant was quantified and expressed as percent release with respect to the to total amount of serotonin in the permeable platelet suspension. (B) The precipitated platelets were used for the quantification of free sulfhydryls in protein. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of three others.

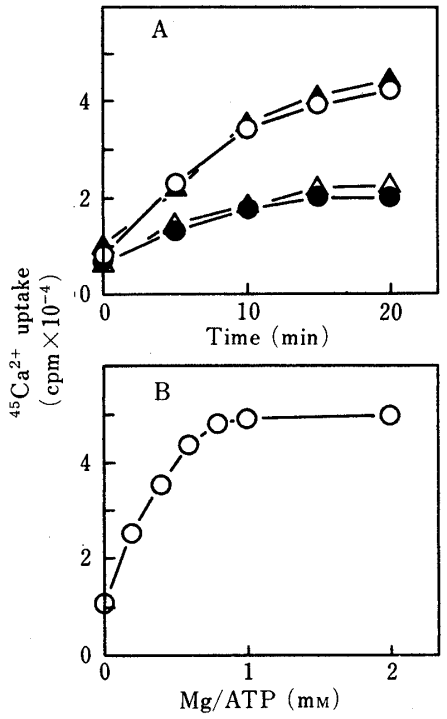

Fig. 2. Calcium Uptake by Permeable Platelets (A), and Its Dependency on ATP (B)

(A) Permeabilized platelets were incubated in the absence (●) or presence (○, △, ▲) of 2 mM Mg/ATP. The platelet suspension contained 50 μM of antimycin A with 25 μM oligomycin (▲), 100 μM TFP (△), or the vehicle (○). After incubation for the indicated time, at abscissa, calcium uptake was terminated by collecting the platelets on a membrane filter. (B) Permeabilized platelets were incubated for 1 h in the presence of various concentrations of Mg/ATP. Calcium uptake was measured as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of two others.

demonstrates that the active calcium uptake in permeable platelets was mediated not by mitochondria but by the calmodulin-dependent sites. Antimycin A and oligomycin, inhibitors of mitochondria, did not prevent the active calcium uptake in permeable platelets. On the other hand, TFP, which is a calmodulin inhibitor and prevents calcium uptake by endoplasmic reticulum and the dense tubular system in neutrophils and platelets, respectively, completely abolished the ATP-dependent calcium accumulation at 100 μM concentration. These results indicate that the active calcium transport in permeable platelets was toward a calmodulin-dependent non-mitochondrial pool (probably the dense tubular system) under our experimental conditions.

The inhibition by EGTA of serotonin release suggests that the release reaction by CuPh was mediated by the increase in cytoplasmic calcium concentration, so the effect of CuPh on calcium movement was examined (Fig. 3). CuPh decreased the active calcium uptake in permeable platelets dose-dependently (Fig. 3A). Dithiothreitol, a disulfide reducing agent, inhibited the effect of CuPh at 300 μM concentration (data not shown). Calcium efflux induced by CuPh from ^{45}Ca-prelabeled permeable platelets is shown in Fig. 3B. CuPh released Ca^{2+} dose- and time-dependently from permeable platelets which had actively accumulated ^{45}Ca. There are many factors which influence calcium movement, so the mechanism of the altered

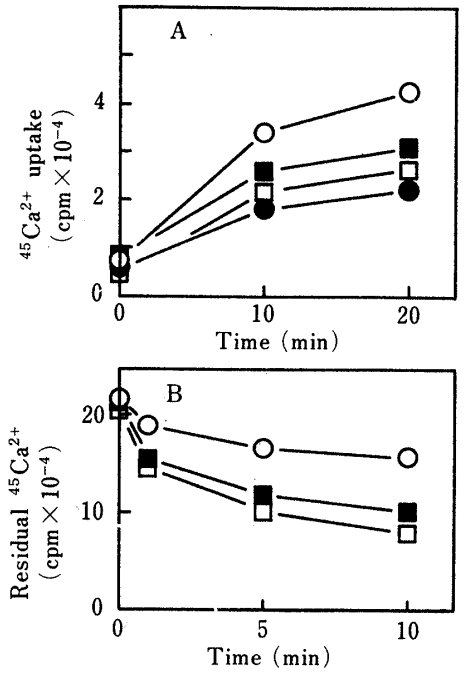

Fig. 3. Effects of CuPh on Active Uptake (A), and Passive Efflux (B) of Calcium

(A) Permeabilized platelets were incubated in the absence (●) or presence (○, □, ■) of 2 mM Mg/ATP for the indicated time. The mixture contained 150 μM CuPh (■), 300 μM of CuPh (□), or the vehicle (○). Calcium uptake was measured as described in the text. (B) Permeabilized platelets were incubated for 3 h in the presence of 2 mM Mg/ATP, then diluted 20 times with buffer A containing 0.25 mM EGTA. After being incubated for the indicated time, platelets were collected on a membrane filter and washed with the same buffer to eliminate unincorporated Ca^{2+}. The suspension contained 150 μM CuPh (■), 300 μM CuPh (□), or the vehicle (○). Residual amounts of ^{45}Ca in permeable platelets were determined. Data are presented from one experiment performed in duplicate, and are representative of two others.

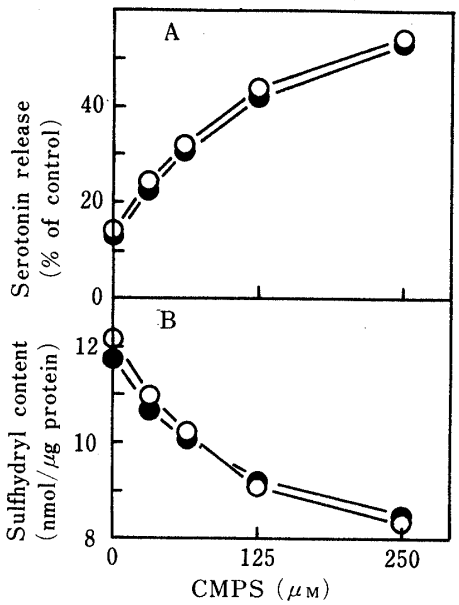

Fig. 4. Effects of CMPS on Serotonin Release (A) and Protein-Bound Free Sulfhydryls (B) in Permeable Platelets

Permeabilized platelets were incubated for 15 min with CMPS at the indicated concentrations in the absence (○) or presence (●) of 50 μM EGTA. (A) After the incubation, platelets were centrifuged, and the supernatant was obtained. Released serotonin in the supernatant was quantified and expressed as percent release compared to the total amount of serotonin in the permeable platelet suspension. (B) The pellet was used for the quantification of free sulfhydryls in protein. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of three others.

calcium movement induced by CuPh could not be determined only from these experimental results. Considering that CuPh facilitates passive efflux of calcium from skeletal muscle sarcoplasmic reticulum by polymerizing and inhibiting Ca^{2+}-ATPase,[26] it is probable that CuPh acts on Ca^{2+}-dependent ATPase in permeable platelets and releases calcium ion.

ATPase activity could not be determined in the presence of CuPh, because it interferes with the enzyme-coupled measurement of ATPase activity. Thus, permeable platelets were treated with CuPh and washed twice with buffer A. These platelet preparations revealed decreased values of Ca^{2+}-dependent ATPase.

The values were 1.3, 1.4, and 8.1 nmol/min per 10^9 platelets when platelets were treated with CuPh at 600, 150, and 38 μM concentration, respectively. The control value was 13.7 nmol/min per 10^9 platelets. These CuPh-treated and washed platelets showed a distinct decrease in Ca^{2+}-ATPase activity compared to control experiments.

CuPh catalyzes the air-oxidation of sulfhydryl groups to disulfides[14] and is an efficient reagent to cross-link the sarcoplasmic reticulum Ca^{2+}-ATPase molecules.[29] Thus, we examined whether disulfide cross-linking is essential for the serotonin release in permeable platelets. Permeable platelets were treated with CMPS, and the serotonin release and the decrease in protein-bound free sulfhydryls were determined (Fig. 4). Incorporation of CMPS,

which reacts with free sulfhydryl groups but does not form cross-links, in the permeable platelets also released serotonin dose-dependently from permeabilized platelets, although it had no effect on intact platelets. CMPS, as well as CuPh released serotonin and decreased the amount of protein-bound free sulfhydryls, but a difference in sensitivity to EGTA was found. EGTA inhibited the serotonin release induced by CuPh, but did not inhibit that induced by CMPS. The mechanisms involved in the serotonin release elicited by these sulfhydryl modifiers were thought to be different from each other.

Discussion

Artificially permeabilized platelets are useful for both the examination of effects of ordinarily impermeable solutes and the investigation of signal transduction mechanisms. Exogenously added Ca^{2+} has been proven to elicit serotonin release in permeable platelets.[18,29] Recently, inositol 1,4,5-trisphosphate, one of the metabolic products which increase immediately in response to physiological stimuli such as thrombin,[4-7] has been reported to cause protein phosphorylation,[30] to release serotonin and to cause aggregation in permeable platelets.[1,2] These findings imply that permeable platelets have several advantages for the analysis of platelet activation.

Modification of thiol groups influences platelet aggregation, and the sensitivity of intact platelets to fibrinogen was suggested to be altered when they were treated with sulfhydryl-reducing agents.[12,13,31] However, the function of intracellular sulfhydryl groups in platelet activation is not yet understood. Our results demonstrated that CuPh, an air oxidized-sulfhydryl agent,[14] stimulates permeable platelets but not intact platelets to release serotonin in the absence of exogenous calcium, and the action was considerably inhibited by EGTA, a calcium ion chelator. CuPh inhibited active calcium uptake, and caused calcium efflux from calcium storage sites (probably the dense tubular system). There are two distinct sites of calcium storage in platelets. Mitochondria are known to accumulate calcium especially in high Ca^{2+} concentration media. The other site is the dense tubular system, a structure derived from megakaryocyte endoplasmic reticulum.[32] Active calcium uptake by permeable platelets under our experimental conditions was not affected by antimycin A with oligomycin but was decreased by treatment with TFP. This result indicates that the active calcium uptake depended on the dense tubular system of digitonin-permeabilized platelets. Since CuPh inhibits Ca^{2+}-ATPase of sarcoplasmic reticulum[26] and $(H^+ + K^+)$-ATPase of gastric vesicles[33] in cell-free systems, it is reasonable to assume that CuPh modified and inhibited Ca^{2+}-ATPase of the dense tubular system of permeable platelets, blocked Ca^{2+} uptake and accelerated Ca^{2+} leakage. Actually, inhibition of Ca^{2+}-dependent ATPase was observed on treatment with CuPh. Increase in cytoplasmic Ca^{2+} concentration is known to activate protein kinase C and calmodulin-dependent myosin light chain kinase in platelets.[34-37] CuPh was suggested to work on the Ca^{2+}-dependent ATPase, causing leakage of Ca^{2+}, activating protein phosphorylation, and consequently releasing serotonin.

CuPh is known to form protein disulfide bonds. On the other hand, CMPS, a sulfhydryl modifying agent, reacts with free sulfhydryl groups but does not form cross-links. CuPh but not CMPS influences the KCl conductance of gastric vesicles made from the parietal cells of hog gastric mucosa.[33] Thus, we wished to determine whether chemical cross-linking is prerequisite for the elicitation of serotonin release in permeable platelets. Figure 4 clearly demonstrates that CMPS, like CuPh, releases serotonin dose-dependently from permeable platelets. However serotonin releases induced by CuPh and CMPS showed different sensitivities to inhibition by EGTA. EGTA neither inhibited the decrease of free sulfhydryls induced by CuPh and CMPS nor the release of serotonin induced by CMPS. However, the serotonin release by CuPh was considerably inhibited by EGTA. These results suggest that

the serotonin release by CMPS was not mediated by the elevation of calcium ion concentration. Further work is necessary to differentiate the mechanisms of the serotonin release induced by CuPh and CMPS in order to cast light on the intracellular phenomena of platelet release reaction and activation, especially in relation to protein sulfhydryl groups.

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