Study on the Cupric Phenanthroline-Induced β-Glucuronidase Release in Saponin-Permeabilized Polymorphonuclear Leukocytes

Isao ADACHI,* Akio MURASE, Masaharu UENO and Isamu HORIKOSHI

Department of Hospital Pharmacy, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan. *Received February 1, 1991*

Saponin-permeabilized polymorphonuclear leukocytes (PMNs) released β-glucuronidase, a lysosomal enzyme, dose-dependently in response to cupric phenanthroline (CuPh), a mild oxidant, which catalyzes the formation of disulfide bridges. The β-glucuronidase release induced by CuPh was inhibited by ethylene glycol bis(β-aminoethylether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid (EGTA). Both dithiothreitol (DTT) and *N*-(6-aminohexyl)-5-chloro-naphthalene sulfonamide (W-7) also inhibited the β-glucuronidase release induced by CuPh. CuPh elicited a decrease in protein-bound free sulfhydryls simultaneously, and this decrease was not restored by EGTA treatment. CuPh inhibited Ca^{2+} uptake into Ca^{2+} store sites, and promoted a Ca^{2+} efflux from Ca^{2+} store sites. It also inhibited Ca^{2+}-adenosine triphosphatase (ATPase) activity in permeable PMNs. DTT, a sulfhydryl reducing agent, suppressed both the β-glucuronidase release and the Ca^{2+} uptake in CuPh-treated permeable PMNs. On the other hand, chloromercuriphenylsulfonic acid (CMPS), a sulfhydryl modifier, decreased the amount of free sulfhydryls in protein and released β-glucuronidase in permeable PMNs dose-dependently, but EGTA did not inhibit either reaction. Neither CuPh nor CMPS released β-glucuronidase from intact PMNs.

These results indicate that both CuPh and CMPS act on intra-PMN target molecules to exert their influence, but the involved mechanisms are different in nature. Alteration in calcium movement is responsible for the β-glucuronidase release in the CuPh-treated permeable PMNs.

Keywords polymorphonuclear leukocyte; permeabilization; cupric phenanthroline; calcium mobilization; degranulation; beta-glucuronidase release; sulfhydryl reagent; disulfhyde linkage; saponin

Introduction

Intracellular sulfhydryl groups are essential to biological responses in various cellular systems. Oxidation of non-glutathione sulfhydryls was reported to be one of the biochemical changes which contributes to C5a-induced aggregation in polymorphonuclear leukocytes (PMNs).[1] It was suggested that changes in sulfhydryl status by reactive aldehydes modulate the activity of the plasma membrane reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase responsible for superoxide anion production in stimulated PMNs and pulmonary alveolar macrophages.[2] Rice and Barnea reported that copper-mediated oxidation of thiols regulated the release of luteinizing hormone releasing hormone from isolated hypothalamic granules in adult male rats. They proposed that copper, bound to an intracellular chelator (protein, peptide, or amino acid), oxidizes thiols of the granule, leading to a change in granule-membrane permeability and hence to the hormone release.[3]

Intracellular free Ca^{2+} has been implicated as a second messenger in stimulus-response coupling in various cells. Using permeable cells, extracellularly added Ca^{2+} stimulated mast cells,[4] adrenal medullary chromaffin cells,[5] GH_3 pituitary cells,[6] PC12 cells,[7] isolated gastric glands,[8] platelets[9] and neutrophiles.[10] Moreover, in permeable platelets, sulfhydryl-reacting reagents decreased the protein-bound free sulfhydryls, changed Ca^{2+} movement, and elicited a release reaction. The report suggested that protein-bound free sulfhydryls play an important role in intracellular Ca^{2+} metabolism and hence various biochemical and biological functions of platelets.[11] However, the significance and role of the exchange between free sulfhydryls and disulfide bonds for lysosomal enzyme release in PMNs, especially in relation to intracellular Ca^{2+} movement, are not yet clearly understood.

In this report, PMNs were permeabilized with a cholesterol-complexing agent—saponin—and thus, permeabilized PMNs responded to *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), a physiological stimulator for PMNs. These experiments revealed that permeabilized PMNs are similar to intact PMNs in regard to their response to physiological stimulators. These permeable PMNs secreted a lysosomal enzyme, β-glucuronidase, in response to cupric phenanthroline (CuPh) or chloromercuriphenylsulfonic acid (CMPS). Using these permeable PMNs, the effect of CuPh, a sulfhydryl oxidizer,[12] and CMPS, a sulfhydryl-modifying agent, on protein-bound sulfhydryl content and Ca^{2+} movement was examined to evaluate the relation among intracellular sulfhydryl modification, Ca^{2+} movement, and β-glucuronidase release.

Materials and Methods

Materials NADH (grade III, from yeast), W-7, fMLP, phospho(enol)-pyruvate trisodium salt hydrate, NAD 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.); Adenosine triphosphate (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, Japan); *p*-chloromercuriphenylsulfonic acid monosodium salt, *p*-nitrophenyl-β-D-glucuronide and ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Nakarai Chemical Ltd. (Kyoto, Japan); 1,10-*ortho*-phenanthroline, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 1,4-dithiothreitol (DTT) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and membrane filters (type TM-2, cellulose nitrate, 0.45 μm) were from Toyo Roshi Co. Ltd. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Preparation and Permeabilization of PMNs Male guinea pigs of the Hartley strain were intraperitoneally injected with 26 ml of 2.9% thioglycollate 15 h before the harvest of peritoneal exudate cells. The cells were harvested from the peritoneal cavity using modified Hanks' balanced salt solution (20 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 0.5 U/ml heparin). The collected cells were centrifuged (110 × *g*, at 4 °C, for 10 min) and washed with the same buffer. Afterwards leukocyte cell density was adjusted to

2.0×10^7/ml in a reaction buffer (30 mM HEPES, pH 7.0, 100 mM KCl, 20 mM NaCl), and saponin (Wako Pure Chemical) was added to the culture to make a 105 μg/ml concentration. The cell suspension was incubated for 10 min at 37 °C, washed with the reaction buffer by centrifugation ($150 \times g$, at 4 °C, for 5 min), and resuspended in the reaction buffer at appropriate cell density. Thus permeabilized PMNs were incubated with various reagents such as CuPh. In the case of CuPh, a 3/1 molar ratio of *o*-phenanthroline/$CuSO_4$ was used. Hereafter, CuPh concentration denotes the *o*-phenanthroline concentration.

β-Glucuronidase and LDH Measurement β-Glucuronidase activity was assayed with *p*-nitrophenyl-β-D-glucuronide (Nakarai Chemical Ltd.) as a substrate. The assay mixture contained: 50 mM sodium acetate buffer (pH 5.0), 0.1% (w/v) Triton X-100, 1 mM *p*-nitrophenyl-β-D-glucuronide and culture supernatant. Permeabilized PMNs (0.8×10^7/ml: 400 μl) were placed in conical centrifugation tubes and incubated for 15 min at 37 °C in the presence of various reagents, and the reaction was terminated by centrifugation ($6000 \times g$, at room temperature, for 1 min). Aliquots of supernatants (50 μl) were obtained and submitted to the measurement of β-glucuronidase and LDH activities. β-Glucuronidase activity was determined by measuring the *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucuronide.[13] LDH [EC 1.1.1.27], which is a cytoplasmic marker enzyme,[14] was quantified by the method of Kornberg.[15] Total β-glucuronidase and LDH were determined by solubilizing PMNs with 0.5% Triton X-100, and released substances were expressed as a percentage of the total amounts.

Ca^{2+} Movements Ca^{2+} uptake was measured by using $^{45}Ca^{2+}$ and the Millipore filtration technique. Permeabilized PMNs (0.62×10^7/ml: 260 μl) were suspended in the reaction buffer containing 1.5 mM Mg/ATP, 77 μM $CaCl_2$ and 1.0 μCi $^{45}Ca^{2+}$, and incubated at 25 °C for 30 min. The cell suspension was passed through a membrane filter (0.45 μm). The filter was washed twice with the same medium without $^{45}Ca^{2+}$ of 1.0 ml, dried and counted for radioactivity with a liquid scintillation counter (LSC-900, Aloka). The ratio of Ca^{2+} uptake was calculated dividing the value by that of the control experiment, which contained saline in place of CuPh.

Ca^{2+} efflux was determined by the method described by Chiesi.[16] After $^{45}Ca^{2+}$ had loaded into the permeable PMNs (1.0×10^8, 25 °C, 3 h) in a buffer containing 1.7 mM Mg/ATP, 87 μM $CaCl_2$ and 10 μCi/ml of $^{45}Ca^{2+}$ (25 mCi/mg), a passive efflux experiment of Ca^{2+} was started by diluting Ca^{2+}-loaded PMNs 27 times with the reaction buffer containing 0.25 mM EGTA and various reagents. The PMNs were incubated for 10 min, then the mixture was filtered (within 10 s). The filter was washed and dried, and its radioactive count was determined. The Ca^{2+} efflux was expressed by the ratio of remaining $^{45}Ca^{2+}$ count in permeable PMNs of a test sample-treated to that in the control culture.

Quantification of Sulfhydryl Groups in Proteins The quantification of PMN sulfhydryl groups in proteins was carried out by the method of Yamada *et al.*[17] The precipitate of PMNs was suspended in 200 μl of distilled water after being treated with various reagents (CuPh, EGTA *etc.*). The suspension was mixed with a precipitating solution containing 1.67% *m*-phosphoric acid, 0.02% ethylenediaminetetraacetic acid disodium salt, and 30% NaCl. It was then allowed to stand for about 2 h at room temperature, followed by centrifugation at $8500 \times g$ for 1 min. The precipitate was used for the quantification of protein-bound sulfhydryl groups, and the denatured protein sediment was neutralized by the addition of 0.1 ml of 0.3 M Na_2HPO_4 and solubilized by adding 1 ml of 1% sodium dodecyl sulfate (SDS). A 0.2 ml solution was saved for protein quantification. The rest of the solution was mixed with a 10 mM DTNB solution of 81.8 μl. After incubation for 30 min at room temperature, 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. Protein content was determined according to Lowry *et al.*[18] using bovine serum albumin as a standard. The amount of free sulfhydryls in proteins was expressed as pmoles per μg protein.

Ca^{2+}-Dependent ATPase Activity ATPase activity was essentially measured as described by Neet and Green,[19] using NADH, pyruvate kinase and LDH. Typically, permeable PMN suspension (1.0 ml, 0.8×10^7/ml) was incubated with CuPh at 37 °C for 10 min. CuPh was removed by centrifugation ($150 \times g$ for 5 min at 25 °C). PMN pellet was resuspended to the same cell density in the reaction buffer. An aliquot (350 μl) of cell suspension was diluted to 1.5 ml with a solution which contained 0.55 mM phospho(enol)pyruvate, 0.2 mM NADH, 6.5 mM $MgCl_2$, 1.3 mM ATP, 1.3 units/ml LDH, 1.3 units/ml pyruvate kinase, and 1.3 mM EGTA and 2.6 mM $CaCl_2$. Changes of absorbance at 340 nm (NADH) were recorded using a double-beam spectrophotometer (model 200-20, Hitachi Ltd., Tokyo) at room temperature. Basal ATPase activity was measured following the addition of 1.3 mM ATP. Thereafter, 2.6 mM $CaCl_2$ was added, and the Ca^{2+}-requiring ATPase activity was detected by a decrease in light absorption at 340 nm. Ca^{2+}-dependent ATPase activity was calculated from the difference between the initial velocities of the Ca^{2+}-requiring ATPase and basal ATPase, and it is expressed as nmols of ATP converted per min per 10^7 PMNs.

Results

Permeabilization of Guinea Pig PMNs Guinea pig PMNs were treated with various concentrations of saponin as described in Materials and Methods, and leakages of LDH and β-glucuronidase were assessed simultaneously in order to establish optimum conditions for permeabilization. The leakage of β-glucuronidase was less than that of LDH (a cytoplasmic marker enzyme) in every saponin concentration examined. The percent releases of LDH and β-glucuronidase were 50 and 5, respectively, when PMNs were treated with 105 μg/ml of saponin for 10 min. This result indicates that the plasma membrane became permeable, while the lysosomal granule membrane remained intact under these experimental conditions. The permeabilized PMNs released β-glucuronidase in response to fMLP, as in the case of intact PMNs. This indicates that there is minimal disturbance to the topography of the surface membrane receptor for fMLP and of its function for granule secretion.

Effect of CuPh on β-Glucuronidase Release from Permeable PMNs CuPh (an -S–S- cross-linker) released β-glucuronidase from saponin-permeabilized PMNs dose dependently up to 600 μM (Fig. 1). Under these experimental conditions, constant leakage of LDH (about 50%) was observed, and it was dependent on the saponin concentration but not on the concentration of CuPh (up to 1 mM). No release of β-glucuronidase occurred from intact PMNs (not treated with saponin) by CuPh up to the concentration of 600 μM. EGTA inhibited the release of β-glucuronidase induced by CuPh by about 70% with a 45 μM EGTA concentration, and the inhibition by EGTA was dose-dependent (Fig. 2A). Over the concentration of 90 μM EGTA, β-glucuronidase release was decreased almost to the basal (control) level. These results indicate that the increase in free calcium concentration is requisite for the CuPh-induced β-glucuronidase release. Figure 2B demonstrates that CuPh formed disulfide linkages and decreased the amount of free sulfhydyls in proteins. However, EGTA did not block the CuPh-mediated disulfide formation as shown

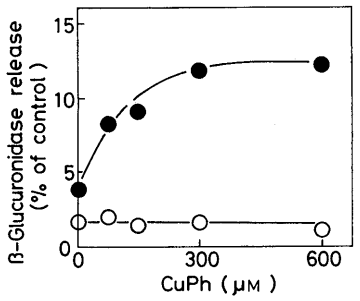

Fig. 1. Effect of CuPh on β-Glucuronidase Release in Permeable PMNs

Permeabilized PMNs (●) or intact PMNs (○) were incubated for 15 min at 37 °C with CuPh at the indicated concentrations. After the incubation, the PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as a percent release with respect to the total amount of β-glucuronidase in the PMN suspension. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and representative of three others.

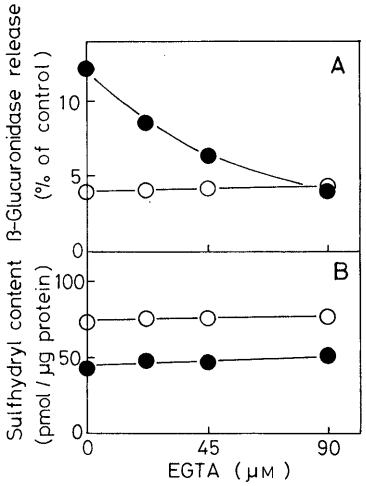

Fig. 2. Effects of EGTA on β-Glucuronidase Release (A) and Protein-Bound Free Sulfhydryls (B) in Permeable PMNs When Treated with CuPh

Permeabilized PMNs were incubated for 15 min with EGTA at the indicated concentrations in the absence (○) or presence (●) of 300 μM CuPh.

A: After the incubation, the PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as percent release with respect to the total amount of β-glucuronidase in the permeable PMN suspension.

B: The precipitated PMNs 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.

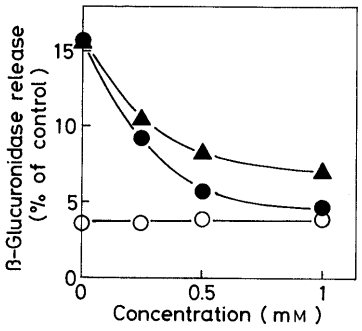

Fig. 3. Inhibitory Effect of DTT or W-7 on the CuPh-Induced β-Glucuronidase Release

Permeabilized PMNs were incubated in the absence (○) or presence (●, ▲) of 300 μM CuPh. The PMN suspensions contained DTT (○, ●) or W-7 (▲) at the concentrations indicated at abscissa. After incubation for 15 min at 37 °C, β-glucuronidase release was assessed. Results are presented from one experiment performed in duplicate, which is representative of two others.

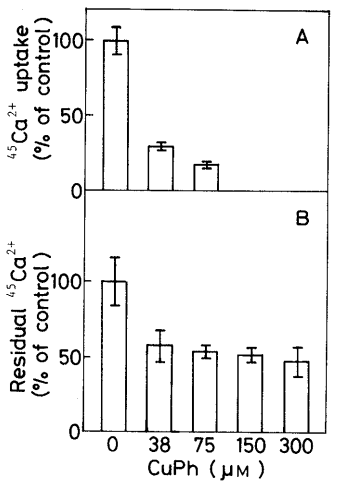

Fig. 4. Effects of CuPh on Active Uptake (A), and Passive Efflux (B) of Calcium

A: Permeabilized PMNs were incubated in the presence of 1.5 mM Mg/ATP for 30 min at 25 °C. The PMN suspensions contained CuPh at the concentrations indicated at abscissa. Calcium uptake was measured as described in the text, and expressed as the percent of control culture.

B: Permeabilized PMNs were incubated for 3.0 h at 25 °C in the presence of 1.7 mM Mg/ATP, then diluted 27 times with the reaction buffer containing 0.25 mM EGTA. After being incubated for 10 min, PMNs were collected on a membrane filter and washed with the same buffer to eliminate unincorporated Ca^{2+}. Experimental details were as described in Materials and Methods. Residual amounts of ^{45}Ca in permeable PMNs were determined. Data are means ± S.D. ($n=3$).

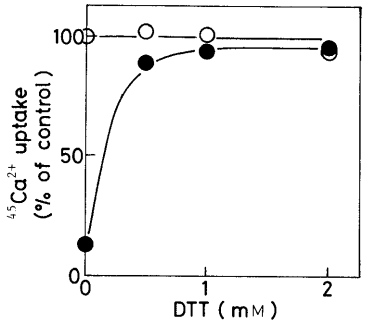

Fig. 5. Effect of DTT on the CuPh-Induced Inhibition of Calcium Uptake

DTT was added to permeabilized PMNs to give the indicated concentrations at abscissa with 75 μM CuPh (●) or without CuPh (○). After 30 min incubation at 25°C, active calcium uptake was assessed as in Fig. 4A. Results are presented from one experiment performed in duplicate, which is representative of two others.

in Fig. 2B. Protein-bound free sulfhydryl groups decreased by 34% with 300 μM CuPh concentration, and the extent of the decrease roughly correlated with that of the release of β-glucuronidase (data not shown). Thus, EGTA did not directly inhibit the oxidative reaction of CuPh.

Effects of DTT and W-7 on the β-Glucuronidase Release Induced by CuPh DTT, a sulfhydryl reducer, inhibited the β-glucuronidase release induced by CuPh dose-dependently as shown in Fig. 3. Half maximum inhibition of the CuPh-induced β-glucuronidase release was achieved by about 0.25 mM DTT. A DTT concentration of 1 mM completely inhibited the β-glucuronidase release from permeabilized PMNs, and the sulfhydryl quantity in proteins was recovered with DTT treatment (data not shown). W-7, an inhibitor for calmodulin, diminished the β-glucuronidase release from permeabilized PMNs elicited by CuPh. Half maximum inhibition was observed at the 300 μM concentration of W-7. Neither CuPh, W-7 nor DTT influenced the β-glucuronidase assay itself.

Effects of CuPh on Uptake and Efflux of Ca^{2+}, and Ca^{2+}-Dependent ATPase The inhibition of β-glucuronidase release by EGTA suggests that the release reaction by CuPh was mediated by an increase in cytoplasmic calcium concentration, so the effects of CuPh on calcium movements were examined (Fig. 4). CuPh inhibited Ca^{2+} uptake and promoted Ca^{2+} efflux from cytoplasmic Ca^{2+} store sites in permeable PMNs dose-dependently. CuPh resulted in 70% and 82% inhibition of Ca^{2+} uptake at 38 and 75 μM, respectively (Fig. 4A). The addition of 0.5 mM DTT, a disulfide reducing agent, recovered Ca^{2+} uptake almost to the control level even in the presence of 75 μM CuPh. The percent of Ca^{2+} uptake was not changed up to a concentration of 2 mM DTT alone (Fig. 5).

Ca^{2+} efflux was expressed as a percent of $^{45}Ca^{2+}$

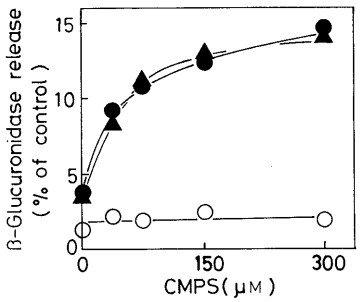

Fig. 6. Effect of CMPS on β-Glucuronidase Release in the Presence or Absence of EGTA in Permeable PMNs

Permeabilized PMNs were incubated for 15 min with CMPS (●, ▲) at the indicated concentrations in the absence (●) or presence (▲) of 0.25 mM EGTA. Intact PMNs were also treated with CMPS (○) as the control culture. After the incubation, PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as the percent release compared to the total amount of β-glucuronidase in the permeable PMN suspension. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of two others.

remaining in the intracellular Ca^{2+} store sites after incubation. CuPh promoted Ca^{2+} efflux from cytoplasmic Ca^{2+} store sites dose-dependently. The concentration of CuPh of 75 μM enhanced the $^{45}Ca^{2+}$ efflux and decreased the remaining $^{45}Ca^{2+}$ amount to about 50% of the control incubation (Fig. 4B).

CuPh-treated and washed permeable PMNs showed decreased Ca^{2+}-dependent ATPase activity. Ca^{2+}-ATPase activity was inhibited by approximately 82% and 90% with the addition of 75 and 150 μM CuPh, respectively. Specific activity of control cell suspension was 12.2 nmol/min/10^7 PMNs.

Effect of CMPS on Permeable PMNs CuPh catalyzes the air-oxidation of sulfhydryl groups to disulfides and is an efficient reagent to cross-link the sarcoplasmic reticulum Ca^{2+}-ATPase molecules. Thus, we examined whether disulfide cross-linking is essential for the β-glucuronidase release in permeable PMNs. Permeable PMNs were treated with CMPS, and the β-glucuronidase release was determined (Fig. 6). CMPS, which chemically modifies free sulfhydryl groups but does not form cross-links, was incorporated in the permeable PMNs which also released β-glucuronidase dose-dependently from permeabilized PMNs. However, this had no effect on intact PMNs. Both CMPS and CuPh released β-glucuronidase and decreased the amount of protein-bound free sulfhydryls (data not shown), but a difference in sensitivity to EGTA was found. EGTA did not inhibit the β-glucuronidase release induced by CMPS (Fig. 6). The mechanisms involved in the β-glucuronidase release elicited by these two sulfhydryl modifiers were thought to be different from each other.

Discussion

Artificially permeabilized PMNs are useful for both the examination of effects of ordinarily impermeable solutes and the investigation of signal transduction mechanisms. Saponin makes a complex (micelle) with a cholesterol on plasma membrane, and is able to selectively permeabilize the plasma membrane.[20] The saponin treatment of PMNs leaked approximately 50% of LDH and less than 10% of β-glucuronidase under our experimental conditions. β-Glucuronidase is a hydrolytic enzyme which is contained in a primary granule (azurophil),[21,22] and the release of β-glucuronidase represents the degranulation of PMNs. Smolen and Stoehr[23] reported that permeabilization of neutrophils was irreversible because the cells were still responsive to calcium ions and were trypan blue-positive even after washing out the saponin.[23] In permeabilized platelets, extracellularly added calcium elicited a release reaction.

PMNs play a major role in acute phase infections and immune responses and release various biologically-active substances in response to physiological stimulants. However, the function of intracellular sulfhydryl groups of proteins in the secretion reaction of PMNs is not yet well understood. Therefore, we wished to clarify whether or not sulfhydryl modifiers affect a lysozomal enzyme release in PMNs as in the case of platelets. The CuPh-induced β-glucuronidase release in permeable PMNs was inhibited by DTT. DTT reduces disulfide groups, so it is thought that DTT reduced disulfide bonds which were formed by CuPh-mediated cross-linking, and decreased the release of β-glucuronidase from permeable PMNs. We also demonstrated that CuPh, an air-oxidized-sulfhydryl agent, released β-glucuronidase from permeable PMNs but not from intact PMNs in the absence of exogeneous calcium ions, and the action was considerably inhibited by EGTA. However, the effect of CuPh on the cross-link formation of sulfhydryl groups was not inhibited by EGTA. Therefore, the action of CuPh was thought to be mediated by calcium ions indirectly. Target molecules of these sulfhydryl reagents must not be on the outer cell surface since intact PMNs did not respond at all to either CuPh or CMPS.

Since CuPh inhibits Ca^{2+}-ATPase of sarcoplasmic reticulumn[24] and H^+, K^+-ATPase of gastric vesicles[25] in cell-free systems, it is reasonable to assume that CuPh modified and inhibited Ca^{2+}-ATPase of permeabilized PMNs, blocked Ca^{2+} uptake, and accelerated Ca^{2+} leakage. An increase in cytoplasmic Ca^{2+} concentration is known to activate protein kinase C and calmodulin-dependent light chain kinase in platelets.[26-28] Actually, inhibition of Ca^{2+}-ATPase was observed in permeable PMNs treated with CuPh. CuPh also inhibited active calcium uptake, and enhanced calcium efflux from permeable PMNs. CuPh was suggested to work on the Ca^{2+}-dependent ATPase, causing leakage of Ca^{2+}, activating protein phosphorylation, and consequently releasing β-glucuronidase. Since W-7, a calmodulin inhibitor,[29] suppressed the CuPh-induced β-glucuronidase release from permeable PMNs, calmodulin might play a role in this release reaction. Based on these experimental results, the CuPh-induced β-glucuronidase release appeared to be closely related to intracellular calcium ion mobilization. In fact, β-glucuronidase was released by the addition of exogeneous calcium to the permeabilized PMNs (data not shown).

CMPS, a sulfhydryl modifying agent, reacts with free sulfhydryl groups without forming cross-links. CuPh but not CMPS influences the KCl conductance of gastric vesicles made from the parietal cells of hog gastric mucosa.[25] We wished to determine whether chemical cross-linking is a prerequisite for the elicitation of β-glucuronidase release in permeable PMNs. Since CMPS also released β-glucuronidase from permeable PMNs, cross-linking itself was not a prerequisite for the release reaction. Moreover, β-

glucuronidase release by CuPh and CMPS showed different sensitivities to inhibition by EGTA. EGTA blocked neither the decrease in free sulfhydryls induced by CuPh and CMPS nor the release of β-glucuronidase induced by CMPS. However, the β-glucuronidase release by CuPh was considerably inhibited by EGTA. These results suggest that the β-glucuronidase release by CMPS was not mediated by an elevation in calcium ion concentration. CMPS stimulated the incorporation of $^{32}PO_4$ from γ-^{32}P-ATP in total proteins and 70 kilodaltons band on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (unpublished data). It is possible that CMPS stimulated protein kinases directly and released β-glucuronidase even in the presence of EGTA.

References

1) E. H. Kraut and A. L. Sagone, Jr., *Blood*, **63**, 1056 (1984).
2) G. Witz, N. J. Lawrie, M. A. Amoruso and B. D. Goldstein, *Biochem. Pharmacol.*, **36**, 721 (1987).
3) G. E. Rice and A. Barnea, *J. Neurochem.*, **41**, 1672 (1983).
4) N. Chakravarty, *Life Sci.*, **39**, 1549 (1986).
5) S. P. Wilson and N. Kirshner, *J. Biol. Chem.*, **258**, 4994 (1983).
6) S. A. Ronning and T. F. J. Martin, *J. Biol. Chem.*, **261**, 7834 (1986).
7) S. C. Peppers and R. W. Holz, *J. Biol. Chem.*, **261**, 14665 (1986).
8) S. H. Norris and S. J. Hersey, *Am. J. Physiol.*, **249**, G408 (1985).
9) I. Adachi, C. Sumi, K. Furuhashi, H. Adachi, M. Ueno and I. Horikoshi, *J. Biochem.* (Tokyo), **100**, 1009 (1986).
10) J. E. Smolen, S. J. Stoehr and L. A. Boxer, *Biochim. Biophys. Acta*, **886**, 1 (1986).
11) I. Adachi, K. Furuhashi, M. Ueno and I. Horikoshi, *Chem. Pharm. Bull.*, **36**, 713 (1988).
12) K. Kobashi, *Biochim. Biophys. Acta*, **158**, 239 (1968).
13) R. H. Michell, M. J. Karnovsky and M. L. Karnovsky, *Biochem. J.*, **116**, 207 (1970).
14) D. S. Beattie, H. R. Sloan and R. E. Basford, *J. Cell Biol.*, **19**, 309 (1963).
15) A. Kornberg, "Methods Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Vol. 1, Academic Press, New York, 1955, p. 441.
16) M. Chiesi, *Biochemistry*, **23**, 3899 (1984).
17) K. Yamada, K. Kubo, K. Shuto and N. Nakamizo, *Thrombs. Res.*, **38**, 61 (1985).
18) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
19) K. E. Neet and N. M. Green, *Arch. Biochem. Biophys.*, **178**, 588 (1977).
20) G. J. Mick, T. Bonn, J. Steinberg and K. McCormick, *J. Biol. Chem.*, **263**, 10667 (1988).
21) C. C. Cox, Y. W. R. Dougher, R. B. Ganong, M. R. Bell, E. J. Niedel and R. Snyderman, *J. Immunol.*, **136**, 4611 (1986).
22) B. Dewald and M. Baggiolini, "Methods in Enzymology," ed. by G. D. Sabato and J. Everse, Vol. 132, Academic Press Inc., New York, 1986, p. 268.
23) J. E. Smolen and S. J. Stoehr, *J. Immunol.*, **134**, 1859 (1985).
24) D. E. Knight, T. J. Hallam and M. C. Scrutton, *Nature* (London), **296**, 256 (1982).
25) N. Takeguchi, R. Joshima, Y. Inoue, T. Kashiwagura and M. Morii, *J. Biol. Chem.*, **258**, 3094 (1983).
26) R. J. Haslam and J. A. Lynham, *Biochem. Biophys. Res. Commun.*, **77**, 714 (1977).
27) Y. Nishizuka, *Nature* (London), **308**, 693 (1984).
28) Y. Kawahara, Y. Takai, R. Minakuchi, K. Sano and Y. Nishizuka, *Biochem. Biophys. Res. Commun.*, **97**, 309 (1980).
29) H. Hidaka, T. Yamaki, M. Naka, T. Tanaka, H. Hayashi and R. Kobayashi, *Mol. Pharmacol.*, **17**, 66 (1980).