Silver Ion Triggers Ca^{2+} Release from Intracellular Store Sites in Saponin-Treated HL-60 Cells (43247)

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Abstract. **We examined the effect of silver ion on Ca^{2+} mobilization from intracellular stores in permeabilized HL-60 cells using a filtration method and $^{45}Ca^{2+}$. In HL-60 cells preloaded with Ca^{2+} in the presence of ATP, micromolar concentrations of $AgNO_3$ elicited marked Ca^{2+} release within 1 min. The $AgNO_3$-induced Ca^{2+} release was not affected by the free Ca^{2+} concentration in the medium. Equivalent concentrations of $AgNO_3$ inhibited energy-dependent Ca^{2+} uptake as well as oxalate-supported Ca^{2+} uptake. In passive Ca^{2+} release experiments when ATP was completely depleted in the solution, $AgNO_3$ also triggered Ca^{2+} release. Sulfhydryl protecting agents such as 2-mercaptoethanol, dithiothreitol, and glutathione (reduced form) blocked the $AgNO_3$-induced Ca^{2+} release. From these results, we conclude that the apparent Ca^{2+} release induced by $AgNO_3$ is mainly due to inhibition of the Ca^{2+} pump with increased permeability for Ca^{2+} and partly due to a direct effect on the Ca^{2+} release channel, probably by modification of sulfhydryl groups on these proteins.** [P.S.E.B.M. 1991, Vol 197]

It is well known that cytosolic Ca^{2+} plays important roles in a variety of cellular functions, including secretion, metabolism, contraction, and cell growth in response to external stimuli (1, 2). Recently, the role of Ca^{2+} has been extended to cellular differentiation (3–5) and cellular death (namely, apoptosis) (6–8). Of equal importance are the mechanisms underlying the regulation of cytosolic Ca^{2+} concentrations. Ca^{2+} release from intracellular store sites has been shown to be the main source during the early phase of elevated free Ca^{2+} concentrations (9). Also inositol 1,4,5-trisphosphate (IP_3) is now widely accepted as a physiologic Ca^{2+} releasing agent in a wide variety of nonmuscle cells (10), and a guanine nucleotide-mediated Ca^{2+} translocation mechanism is proposed (11, 12). Furthermore, a Ca^{2+} channel sensitive to IP_3 has been purified (13). In skeletal muscle, much work has been done on the mechanisms of Ca^{2+} mobilization from the sarcoplasmic reticulum and excitation-contraction coupling (see Ref. 14 for review). Also, several reports indicated that heavy metals, especially silver ion, triggered Ca^{2+} release either from purified sarcoplasmic reticulum (15–17) or reconstituted (Ca^{2+}-Mg^{2+})ATPase vesicles (18). By analogy with muscle fibers, we examined the effect of silver ion on the mobilization of Ca^{2+} from the intracellular store sites in HL-60 cells. HL-60 is a well-characterized human promyelocytic cell line and is a good model for studying the mechanism of cellular proliferation and differentiation (see Ref. 19 for review). In this cell line, the IP_3-mediated process in response to external stimuli in differentiated cells has been studied successfully (20), and there has been growing interest in the role of intracellular Ca^{2+} ion on the differentiation process (3, 21, 22). In addition, the presence of IP_3-sensitive Ca^{2+} store sites in noninduced cells has also been shown (23), although the nature and physiologic relevance of Ca^{2+} store sites in noninduced cells have not been fully understood.

Materials and Methods

Materials. HL-60 cells were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in Iscove's modified Dulbecco's medium (from GIBCO Laboratories) supplemented with 10% fetal calf serum (from Boehringer Mannheim Biochemicals), in a humidified incubator with a 5% CO_2 atmosphere. $^{45}CaCl_2$ was obtained from Du Pont-

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New England Nuclear; saponin was from ICN Nutritional Biochemicals; IP_3 was prepared from erythrocyte ghosts according to the method of Downes *et al.* (24); hexokinase (Type III), ruthenium red, heparin (M_r 4000–6000), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), and glutathione (GSH) were from Sigma. Other reagents were commercial preparations of reagent grades.

Preparations of Saponin-Treated HL-60 Cells. Permeabilization of HL-60 cells with saponin was carried out using essentially the same method as described previously (25, 26). Briefly, cells (1×10^8) were incubated in a solution (5 ml) containing 130 m*M* KCl, 5 m*M* $MgCl_2$, 20 m*M* Tris-maleate (pH 6.80), 5 m*M* EGTA, 2 m*M* diisopropylfluorophosphate, 0.2% bovine serum albumin, and 100 μg/ml saponin for 5 min at 37°C. After centrifugation (200*g*, 5 min, 4°C), cells were washed twice in a solution of 100 m*M* KCl, 20 m*M* Tris-maleate (pH 6.80), and 0.2% bovine serum albumin, resuspended in the same solution and kept on ice.

Assay of Ca^{2+} Uptake and Release. Ca^{2+} uptake and release were assayed using the filtration method, using $^{45}Ca^{2+}$ as described previously (26, 27). The reaction mixture contained 100 m*M* KCl, 20 m*M* Tris-maleate (pH 6.80), 3 m*M* $MgCl_2$, 10 m*M* NaN_3, 0.12 m*M* $^{45}CaCl_2$ (5000–6000 cpm/nmol), specified concentration of EGTA to obtain the desired free Ca^{2+} concentration, and 1×10^6 cells/ml. After 10 min of preincubation at 37°C, Ca^{2+} uptake was initiated by the addition of 3 m*M* ATP. At appropriate time points, aliquots of the mixture were passed through glass fiber filters (Whatman GF/C, pore size 1.2 μm). The filters were washed twice with 2 ml of the above solution without $^{45}Ca^{2+}$, dried, and the radioactivity was counted. In the assay for Ca^{2+} release, agents in 1/100 volume of the reaction mixture were added at the specified time. Ca^{2+} remaining in the cells was determined as described above. The apparent binding constant of EGTA for Ca^{2+} was assumed to be 1.0×10^6 M^{-1} at pH 6.80 (28).

Assay of Passive Ca^{2+} Release. Assay of passive Ca^{2+} release was carried out according to the method reported previously (29). Saponin-treated HL-60 cells accumulated Ca^{2+} in 500 μl of the reaction mixture as described above. After 10 min, 100 μl of the mixture was passed through a filter for estimation of Ca^{2+} uptake. At the same time, 400 μl of the above mixture was diluted into 4 ml of a dilution solution containing 0.1 *M* KCl, 20 m*M* Tris-maleate (pH 6.80), 5 m*M* EGTA, 10 m*M* NaN_3, and the specified concentration of $AgNO_3$. At the specified time points, 1 ml of the mixture was passed through a filter and thus the amount of Ca^{2+} remaining in cells could be determined. In these experiments, 10 m*M* glucose and 4.4 units/ml of hexokinase were added 1 min before dilution in order to deplete remaining ATP. The depletion of ATP was confirmed using the method of Greengard (30).

Results

$AgNO_3$-induced Ca^{2+} Release from Saponin-Treated HL-60 Cells. Saponin-treated HL-60 cells accumulated Ca^{2+} in the presence of 3 m*M* ATP. The uptake reached steady-state level within 10 min and remained constant in the course of the experiment. Application of 10 μ*M* $AgNO_3$ induced rapid Ca^{2+} release, and within 1 min almost all of the accumulated Ca^{2+} was released (Fig. 1). The time course of this Ca^{2+} release is much the same as that reported in skeletal muscle sarcoplasmic reticulum (15). In Figure 2, the concentration dependency of Ca^{2+} release induced by $AgNO_3$ is shown. The half-maximal and maximal effects of $AgNO_3$ were observed at 2.5 and 10 μ*M*, respectively. These values are almost the same as those reported in skeletal muscle (15, 16) and platelets (31). To elucidate the mechanism of Ca^{2+} release, the following experiments were performed.

Effect of Free Ca^{2+} Concentrations on the Ca^{2+} Release Induced by $AgNO_3$. Saponin-treated HL-60 cells accumulated Ca^{2+} in the presence of ATP at free Ca^{2+} concentrations ranging from 3.7×10^{-8} to 2.4×10^{-5} *M* adjusted with Ca^{2+}-EGTA buffer (Fig. 3). The maximal amount of accumulated Ca^{2+} was 0.9 nmol/10^6 cells, which was 2-fold that reported in human peripheral lymphocytes (26) and almost the same as that reported in guinea pig peritoneal macrophages (25). The apparent affinity constant for the Ca^{2+} uptake was

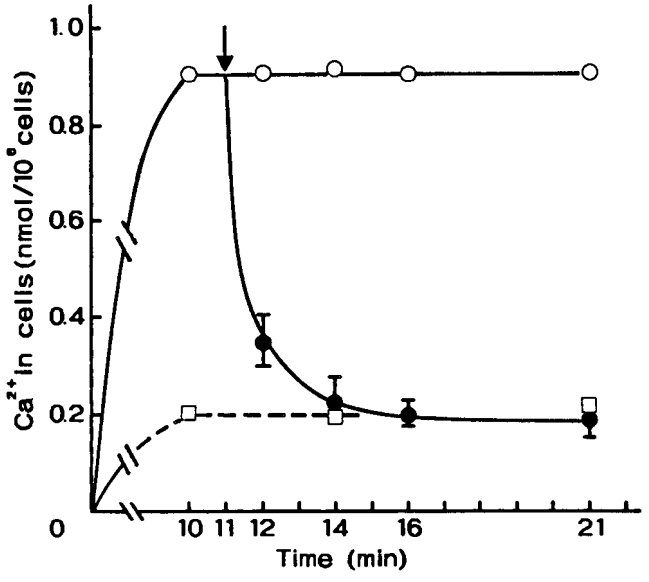

Figure 1. Time course of Ca^{2+} release induced by $AgNO_3$. The assay was initiated by the addition of 3 m*M* ATP. At the time indicated by the arrow, $AgNO_3$ was added to give a final concentration of 10 μ*M*. ○, Control; ●, 10 μ*M* $AgNO_3$; □, ATP free. Free Ca^{2+} concentration in the reaction mixture was 2.4×10^{-5} *M*. Each point, mean ± SE of three experiments, each done in duplicate.

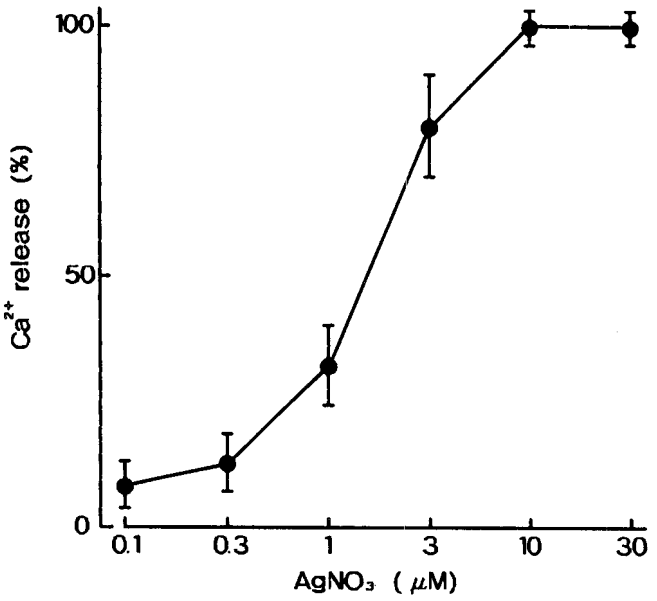

Figure 2. Dose dependence of Ca^{2+} release induced by $AgNO_3$. The Ca^{2+} release is expressed as percentage of the amount of released Ca^{2+}, at 10 min after the addition of various concentrations of $AgNO_3$, relative to the energy-dependent Ca^{2+} uptake. Each point, mean ± SE of three experiments, each done in duplicate.

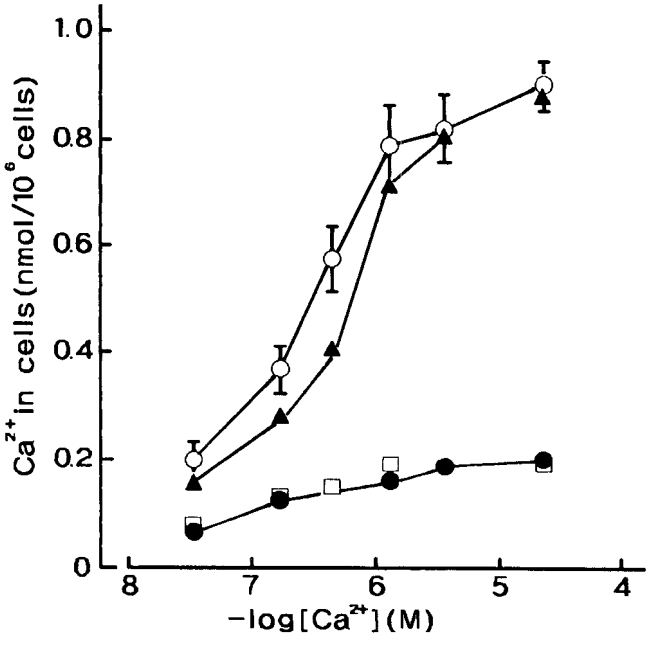

Figure 3. Effect of free Ca^{2+} concentrations on Ca^{2+} uptake and Ca^{2+} release induced by $AgNO_3$ or IP_3. Experimental procedures were as outlined in the legend to Figure 1. The accumulated Ca^{2+} at 10 min after the addition of 3 m*M* ATP and the retained Ca^{2+}, at 10 min after the addition of the indicated agents under various free Ca^{2+} concentrations, are plotted on the same scale. ○, Accumulated Ca^{2+}; ▲, 5 μM IP_3; ●, 10 μM $AgNO_3$; □, ATP free. Each point, mean ± SE of three experiments, each done in duplicate.

approximately $1.5 \times 10^6\ M^{-1}$, indicating the presence of high-affinity binding sites for Ca^{2+}. The demonstrated Ca^{2+} uptake is presumably by nonmitochondrial stores, as has been reported in other tissues (26), because Ca^{2+} uptake by mitochondria was blocked by the continuous presence of azide in the medium (25). In Figure 3, the remaining Ca^{2+} 10 min after the addition of 10 μM $AgNO_3$ or the physiologic Ca^{2+} releasing agent, IP_3 (5 μM), is shown using the same scale. The time course of Ca^{2+} release induced by IP_3 and its effective concentrations are almost the same as those reported in other tissues (26, 29, 32). The maximal effect was obtained at 5 μM concentration of IP_3. In this condition, about 60% of the accumulated Ca^{2+} was retained in the stores, suggesting the presence of an IP_3-insensitive Ca^{2+} pool (12). Ca^{2+} release induced by $AgNO_3$ was not affected by the free Ca^{2+} concentrations in the medium, in contrast to that by IP_3, which was inhibited over 1×10^{-6} *M* free Ca^{2+} concentration, as was shown in other tissues (26, 29, 32). It is notable that the free silver ion concentrations in each medium may vary due to the presence of various concentrations of EGTA. In addition, inclusion of heparin (20 μg/ml), which has been shown to be a specific antagonist of the physiologic receptor for IP_3 (33), to the uptake medium elicited no effect on the Ca^{2+} release induced by $AgNO_3$ (data not shown). In our preliminary experiments, heparin did show the same effect, i.e., "competitive, reversible and potent antagonism of IP_3-activated Ca^{2+} release" as shown in permeabilized DDT_1 MF-2 smooth muscle cell line (33). These observations suggest that silver ion may be acting at different binding sites from that of IP_3. We also examined the effect of ruthenium red, a dye that was shown to inhibit Ca^{2+} release induced by $AgNO_3$ in skeletal muscle terminal cisternae (16, 17) and human platelet membranes (31). The dye (up to 20 μM), however, did not show any blocking effect (data not shown). This observation may also be interpreted as in the case of IP_3, although the possibility remains that the Ca^{2+} store sites in HL-60 cells lack the specific binding sites for the dye.

Effect of $AgNO_3$ on ATP-Dependent Ca^{2+} Uptake by Saponin-Treated HL-60 Cells. Inclusion of 10 μM $AgNO_3$ in the Ca^{2+} uptake medium completely abolished ATP-dependent Ca^{2+} uptake (data not shown). Because Ca^{2+} efflux can be enhanced by either inhibition of Ca^{2+} uptake or stimulation of Ca^{2+} release, we examined the first component independently from the latter using potassium oxalate. Oxalate can be used to examine the unidirectional Ca^{2+} influxes in vesicular structures because it keeps the free Ca^{2+} concentration at a low level in the vesicles by forming Ca-oxalate precipitates and makes the Ca^{2+} efflux components negligible (27). As shown in Figure 4A, in the presence of 10 m*M* oxalate, Ca^{2+} uptake by HL-60 cells was markedly stimulated (0.6 n*M*/min/1×10^6 cells). Fig-

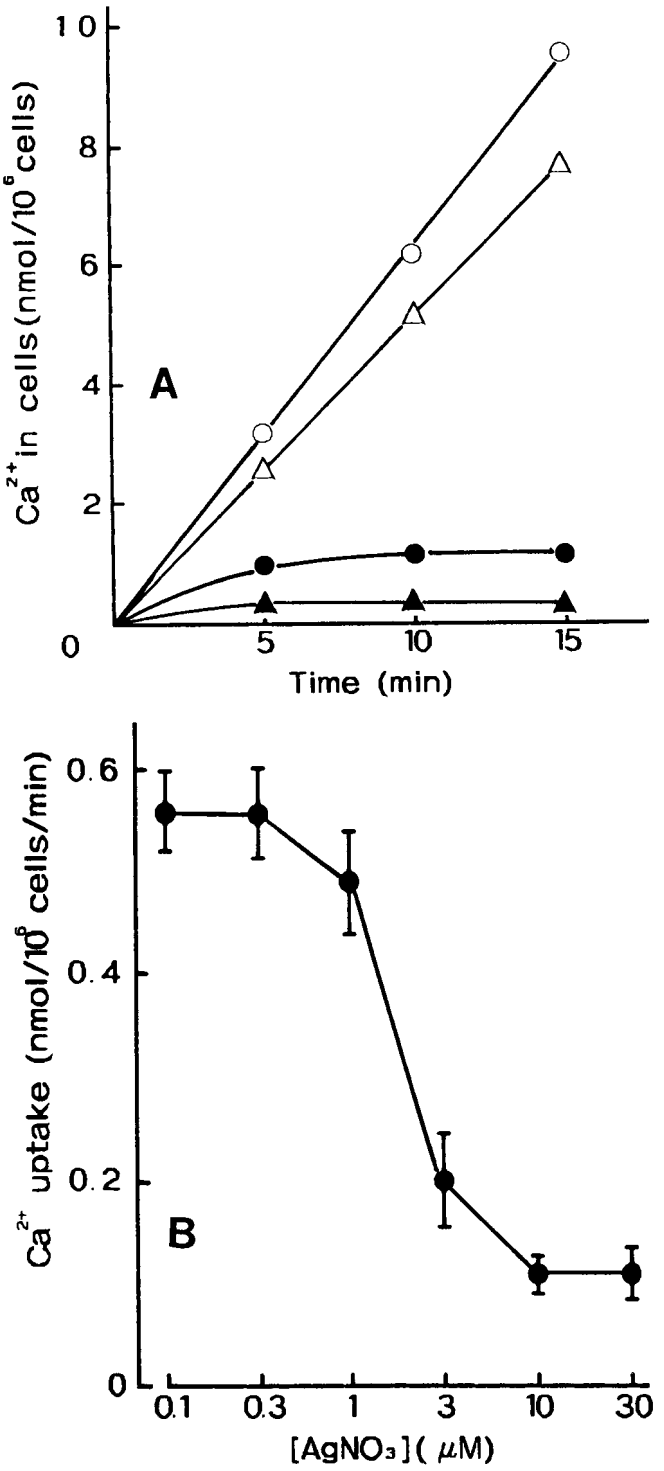

Figure 4. Effect of $AgNO_3$ on the unidirectional Ca^{2+} uptake supported by oxalate. The reaction mixture contained 10 m*M* potassium oxalate in addition. The assay was initiated by the addition of 3 m*M* ATP. Free Ca^{2+} concentration in the mixture was 2.4×10^{-5} *M*. (A) Time course of Ca^{2+} uptake in the presence of 10 m*M* potassium oxalate and indicated concentrations of $AgNO_3$ are shown. ○, Control; △, 1 μM $AgNO_3$; ▲, 10 μM $AgNO_3$; ●, oxalate free. Each point, mean of three experiments, each done in duplicate. (B) Dose-dependent blocking effects of $AgNO_3$ on Ca^{2+} uptake supported by oxalate. Note that the Ca^{2+} uptake is expressed as nmol/10^6 cells/min. Each point, mean ± SE of three experiments, each done in duplicate.

ure 4A also shows that 10 μM $AgNO_3$ completely abolished the supporting effect of oxalate on the Ca^{2+} uptake. Figure 4B shows the relationship between $AgNO_3$ concentration in the medium and the inhibition of Ca^{2+} uptake. The maximal inhibitory effect of $AgNO_3$ was observed at 10 μM, which was the same as that used in the Ca^{2+} release experiment. From these results, it is proposed that Ca^{2+} release induced by $AgNO_3$ may be due to inhibition of the Ca^{2+} pump in the intracellular store sites of saponin-treated HL-60 cells. However, the rapid Ca^{2+} release shown in Figure 1 cannot be simply explained by this mechanism, because simple inhibition of the pump by depleting ATP using hexokinase and glucose exhibited almost no release during the first minute and subsequently exhibited very slow release (data not shown). Therefore, the results obtained here should not be attributed merely to the inhibition of the pump, because similar results would be obtained if the permeability of the stores for Ca^{2+} were to be dramatically increased by silver ion.

Passive Ca^{2+} Release from Saponin-Treated HL-60 Cells. We also examined the effect of $AgNO_3$ on the passive release of Ca^{2+} from saponin-treated HL-60 cells (Fig. 5). The energy-dependent Ca^{2+} uptake component was completely abolished because ATP was depleted by the addition of hexokinase (4.4 units/ml) and glucose (5 m*M*), and the dilution solution contained 5 m*M* EGTA, which extremely reduced the free Ca^{2+} concentration in the medium. $AgNO_3$ markedly induced Ca^{2+} release, and 5 min after the start of Ca^{2+} release, almost all the stored Ca^{2+} was released. In contrast, 40% of

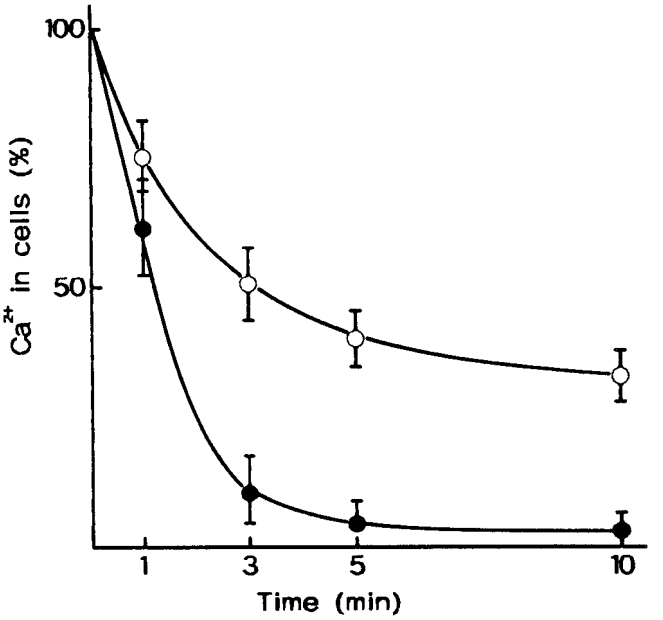

Figure 5. Effect of $AgNO_3$ on the passive release of Ca^{2+}. The Ca^{2+} in cells was relative to that at 10 min after the addition of 3 m*M* ATP. The dilution was made at time 0. ○, Control; ●, 10 μM $AgNO_3$. Each point, mean ± SE of three experiments, each done in duplicate.

accumulated Ca^{2+} was retained within the cells in the absence of $AgNO_3$. The maximal effect of $AgNO_3$ was obtained at 10 μM (data not shown), which was the same value obtained in the experiments of Ca^{2+} uptake. From these passive efflux experiments, it is proposed that there exists the unidirectional efflux pathway for Ca^{2+}, which is opened by silver ion. This efflux pathway would consist of either the putative Ca^{2+} release channels sensitive to silver ion, or simply the (Ca^{2+}-Mg^{2+})-ATPase (Ca^{2+} pump) proteins that had been changed conformationally to acquire the high permeability for Ca^{2+} (18). We could not distinguish these two mechanisms in this series of experiments, but the result that the effective $AgNO_3$ concentration required for the inhibition of Ca^{2+} pump and the stimulation of Ca^{2+} release in passive efflux experiments is the same, suggesting the latter, that the Ca^{2+} pump itself is acting as Ca^{2+} releasing site in our systems.

Effects of Sulfhydryl Protecting Agents on the $AgNO_3$-Induced Ca^{2+} Release. The actions of heavy metals have been explained by binding to sulfhydryl groups on a protein in sarcoplasmic reticulum (15–17). To elucidate the mechanism of action of silver ion, we applied sulfhydryl protecting agents such as DTT, 2-ME, and GSH. When these agents were added to the assay medium, all of these agents elicited marked blocking effect on the $AgNO_3$-induced Ca^{2+} release within the concentration range that had no effect on Ca^{2+} uptake. The concentration-dependent blocking effects of these agents are shown in Figure 6. The half maximal blocking effects of DTT, 2-ME, and GSH were observed at 3, 10, and 100 μM, respectively, and the maximal blocking effects were observed at 10 μM, 100 μM, and 1 mM, respectively. DTT was most effective, probably because DTT has two sulfhydryls on its molecule. Interestingly, in the course of these experiments, we found that GSH, the endogenous constituent in living cells, inhibits Ca^{2+} accumulation at high concentrations over 2 mM. This effect was not observed in any other tissues. High concentrations of GSH may induce lysis of the saponin-treated cells in our assay system. DTT and 2-ME had no effect on Ca^{2+} uptake up to 10 mM. Similar blocking effects were observed when these agents were added concomitantly with $AgNO_3$. On the other hand, when these agents were added to the medium following $AgNO_3$ treatment, we could not observe the reuptake of Ca^{2+} to the saponin-treated cells (data not shown). However, it is notable that our results obtained here do not merely imply that silver ion is occluded after binding but also imply that the Ca^{2+} store sites might possess extremely high-affinity binding sites for silver ion. Only 10 μM $AgNO_3$ was applied to the solution. From these results, it is suggested that silver ion acts by modifying the sulfhydryls on proteins of the intracellular Ca^{2+} stores sites and, once bound to the sulfhydryls, reversal of the effect is difficult even in the presence of excess amounts of powerful reducing agents.

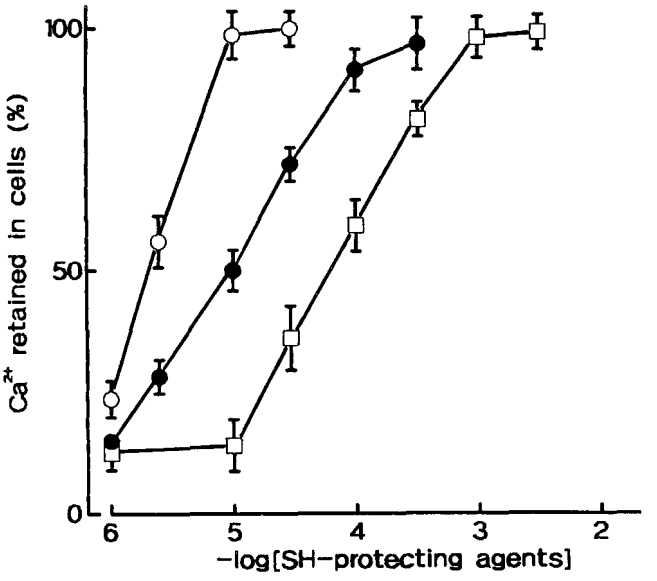

Figure 6. Dose-dependent blocking effects of sulfhydryl (SH)-protecting agents on the Ca^{2+} release induced by $AgNO_3$. Experimental procedures were as described in the legend of Figure 1, except that various concentrations of SH-protecting agents were included in the uptake medium. Ca^{2+} uptake was measured 10 min after the addition of ATP, and subsequently 10 μM $AgNO_3$ was added to the medium. The Ca^{2+} retained in cells was measured 10 min after the addition of $AgNO_3$, and expressed relative to the accumulated Ca^{2+}. The accumulated Ca^{2+} was not affected by the presence of these agents, except at high concentrations (>2 mM) of GSH. ○, DTT; ●, 2-ME; □, GSH. Each point, mean ± SE of three experiments, each done in duplicate.

Discussion

In this study, we showed that micromolar concentrations of $AgNO_3$ induced rapid Ca^{2+} release from intracellular store sites in HL-60 cells. Silver ion was employed in this study because it has some distinct properties among heavy metals. First, silver ion is assumed to be absent in biologic systems, and, if present, there is no evidence that it participates in cellular enzymatic functions including redox oxidoreductive reactions yielding superoxide species believed to affect many biochemical functions (34). Second, silver ion has a high affinity for sulfhydryl groups, and its mode of action is presumably simply attributable to its binding to sulfhydryls on proteins in intracellular Ca^{2+} store sites as shown in other tissues (15–17) and in this study. Finally, it is a monovalent cation that has relatively low affinity for EGTA (35) and enabled us to examine Ca^{2+} movement in extremely low and therefore near physiologic Ca^{2+} concentrations in cytosol. In fact, according to the silver ion/EGTA dissociation constant of 1.12 mM at pH 6.80 (36), the free silver ion concentrations used in the experiments shown in Figures 3 and 5 were calculated to be ranging from 2.5 to 9.2 μM and 1.8 μM, respectively. Nevertheless, $AgNO_3$ elicited similar

dose-dependence of Ca^{2+} release irrespective of the EGTA concentrations in each medium. These facts are easily explained assuming that the intracellular Ca^{2+} store sites possess extremely high-affinity binding sites for silver ion as shown in Figure 6 and that the silver ion/EGTA binding is transient.

By using this assay system, we showed first that silver ion triggered Ca^{2+} release from intracellular store sites in HL-60 cells. As shown in Figure 3, silver ion elicited Ca^{2+} release independent of free Ca^{2+} concentrations in the medium, and 10 μM $AgNO_3$ released almost all of the Ca^{2+} stored in contrast with IP_3. This observation suggests that silver ion might act at different sites from that for IP_3. The inference is also supported by the observation that low molecular weight heparin, which was shown to be one of the specific antagonists for IP_3 receptor (33), had no effect on the $AgNO_3$-induced Ca^{2+} release. However, Adunyah and Dean (31) showed that IP_3-sensitive channel possesses essential sulfhydryls reactive with silver ion in purified platelet inner membranes. Thus, it remains to be known whether silver ion might react with IP_3-sensitive channels in subfractionation studies yielding IP_3-sensitive Ca^{2+} pool (12). Additionally, ruthenium red, a dye that was shown to inhibit Ca^{2+} release induced by silver ion in terminal cisternae of skeletal muscle (16, 17) and platelet membranes (31), had no effect in our whole cell system. Then we examined the effect of silver ion on unidirectional Ca^{2+} influx supported by oxalate, and showed that the Ca^{2+} influx was inhibited in similar concentration range as was observed in Ca^{2+} release. This observation is consistent with the proposal by Gould *et al.* (18), that silver ions could trigger Ca^{2+} release by interaction with (Ca^{2+}-Mg^{2+})-ATPase in reconstituted systems and that the ATPase itself could act as a pathway for rapid Ca^{2+} release. Our results that $AgNO_3$ could induce Ca^{2+} release in passive release experiments could also be explained by this mechanism that (Ca^{2+}-Mg^{2+})-ATPase itself would act as an ionophoric site, although the existence of putative Ca^{2+} release channels sensitive to silver ion could not be excluded. The observation that sulfhydryl protecting agents such as 2-ME, DTT, and GSH block the $AgNO_3$-induced Ca^{2+} release suggests that silver ions react specifically with sulfhydryls on proteins on presumably the endoplasmic reticulum, because soluble components were removed during saponin treatment. We could not observe reuptake of Ca^{2+} by these agents after $AgNO_3$-induced Ca^{2+} release, but Adunyah and Dean (31) showed minimal reuptake of Ca^{2+} released by 1 μM $AgNO_3$ with 2 mM DTT in human platelet membranes. Partial reversal of *p*-chloromercuribenzenesulfonate-induced Ca^{2+} release with DTT was also reported by the same investigators and by Bindoli *et al.* (37). Silver ions would have markedly high affinity for sulfhydryls (15) and once bound to them, dissociation would be difficult even in the presence of excess amounts of powerful reducing agents. In conclusion, our results clearly indicate that silver ion induces Ca^{2+} release from intracellular Ca^{2+} store sites by the modification of sulhydryl groups on Ca^{2+} pump, although the putative Ca^{2+} channel might be modified concomitantly by silver ion. Although the action of silver ion is apparently not physiologic, in a pathologic state when the physiologic sulfhydryl-protecting agent GSH is reduced or depleted, sulfhydryl modification might occur, leading to the perturbation of cellular Ca^{2+} homeostasis. Our observation may also explain the mechanism of cellular death related to the lasting rise of intracellular Ca^{2+} (6–8).

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