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Silver Ion Induces a Cyclosporine A-Insensitive Permeability Transition in Rat Liver Mitochondria and Release of Apoptogenic Cytochrome *c*

Mohamad Radwan Almofti[*,1,2], Tomokazu Ichikawa[1], Kikuji Yamashita[3], Hiroshi Terada[5] and Yasuo Shinohara[1,2,4]

[1]Faculty of Pharmaceutical Sciences, The University of Tokushima, Shomachi 1, Tokushima 770-8505; [2]Single-Molecule Bioanalysis Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Hayashi-cho, Takamatsu 761-0395; [3]School of Dentistry, The University of Tokushima [4]Institute for Genome Research, The University of Tokushima, Kuramotocho 3-18, Tokushima 770-8503; [5]Faculty of Pharmaceutical Sciences, Tokyo University of Science, Shinjuku-ku, Tokyo 162-0826

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Various reagents are known to open the mitochondrial permeability pore (PTP) and induce a permeability transition (PT), releasing apoptogenic proteins from the intermembrane space and triggering apoptosis. In this study, we examined the effect of Ag^+, a known cytotoxic sulfhydryl-reactive heavy metal, on isolated rat liver mitochondria. The following results were obtained: (1) Upon addition, Ag^+ instantly induced mitochondrial swelling and acceleration of respiration. (2) Cyclosporine A, a specific inhibitor of classical PT, was ineffective against the effect of Ag^+, indicating that silver ions induced non-classic PT. (3) Sulfhydryl reagents such as reduced glutathione completely inhibited the effects of Ag^+ on the mitochondria. (4) Experimental results using polyethylene glycol indicated that Ag^+ induced opening of a pore in the inner mitochondrial membrane, which could be PTP of another open state or a distinct pore. (5) Electron microscopic analysis of mitochondria treated with Ag^+ showed a novel mitochondrial configuration that was apparently different from that of normal mitochondria or Ca^{2+}-treated mitochondria. (6) Ag^+ also induced the release of apoptogenic cytochrome *c* in a CsA-insensitive but GSH-sensitive manner. These results suggest that Ag^+ promotes a nonclassical permeability increase in the mitochondrial inner membrane that is clearly distinguishable from the classical PT and releases apoptogenic cytochrome *c* in a classical PT-independent manner.

Abbreviations: AIF, apoptosis inducing factor; BHT, butylated hydroxytoluene; BKA, bongkrekic acid; CsA, cyclosporine A; DTT, dithiothreitol; GSH, reduced glutathione; PEG, polyethylene glycol; PT, permeability transition; PTP, PT pore; RLM, rat liver mitochondria; TEM, transmission electron microscopy.

The mitochondrial inner membrane is highly impermeable even to tiny solutes and ions, since the electrochemical potential difference of H^+ across the inner membrane formed by oxidation of respiratory substrate is needed as a driving force for ATP synthesis. However, under certain conditions such as in the presence of Ca^{2+} and inorganic phosphate (P_i), the inner mitochondrial membrane becomes permeable to solutes and ions up to 1,500 Da. This phenomenon is referred to as the mitochondrial permeability transition (PT), and this classical PT is believed to reflect the opening of a proteinaceous pore (PTP) in the inner mitochondrial membrane (*1*, *2*). The PT is well established to be involved in the early step of most cases of programmed cell death, *i.e.*, apoptosis, since it results in the release of apoptogenic proteins from the mitochondrial intermembrane space, such as cytochrome c and apoptosis inducing factor (AIF), into the cytoplasm, triggering the apoptotic cascade (*3*, *4*). The hallmarks of this PT include the specific requirement for matrix calcium ions and inhibition by the immunosuppressant cyclosporin A (CsA).

Because CsA appeared to be an effective and potent inhibitor of mitochondrial PT regardless of the trigger used, PT has been discussed historically as the sole mechanism responsible for generalized increases in mitochondrial permeability (*1*, *5*). However, a number of laboratories have reported increases in mitochondrial permeability, induced by agents such as signal peptides (*6*), butylated hydroxytoluene (BHT) (*7*), thyroxine (*8*), and palmitic acid (*9*), that are CsA-insensitive and clearly distinguishable from the increase caused by the classical PT. Moreover, sizing experiments have demonstrated the existence of pores of multiple sizes (*10–11*)

Several sulfhydryl group-reactive heavy metals, such as Fe^{2+}, Cu^{2+}, Pb^{2+}, and Cd^{2+}, have been reported to induce the mitochondrial PT (*1*, *12*, *13*). In addition, the Ag^+ is a well-known sulfhydryl group-reactive cytotoxin (*14–17*). Ag^+ has been suggested to injure cells by oxidizing the sulfhydryl groups of important cell membrane proteins, which results in a change in membrane permeability as well as in disruption of mitochondrial functions (*14–17*). However, the actions of Ag^+ on isolated mitochondria have never been deeply investigated.

In this study, we examined whether Ag^+ could induce mitochondrial PT and the subsequent release of apoptogenic proteins. For this purpose, we studied the effect of

[*]To whom correspondence should be addressed. Fax: +81-87-869-4113, E-mail: radwanjp@yahoo.com

Ag^+ on the respiration, swelling, and morphology of isolated rat liver mitochondria (RLM). To investigate the mechanism of its action, we examined the effects of specific inhibitors of classic PT and polyethylene glycols (PEGs) of different molecular weights. Release of the apoptogenic protein, cytochrome *c*, was also addressed.

Our study showed that Ag^+ induced instant acceleration of mitochondrial respiration and swelling, changes in mitochondrial morphology, and the release of the apoptogenic protein cytochrome *c*. Ag^+ effects were not inhibited by CsA but were blocked by GSH and DTT, suggesting that Ag^+ induces a nonclassical PT. The PEG sizing study suggests that Ag^+ opens a pore in the inner mitochondrial membrane rather than just perturbing the membrane. This pore could be either just another open state of the PTP or a distinct pore, which causes the nonclassical PT.

MATERIALS AND METHODS

Materials—Cyclosporin A (CsA) and bongkrekic acid were generous gifts from Novartis Pharma Inc. and Prof. Duine (Delft University of Technology), respectively.

Preparation of Mitochondria—Mitochondria were isolated from the liver of normal male Wistar rats according to the procedure described previously (*18*). Protein concentrations of mitochondrial preparations were determined by the Biuret method, with bovine serum albumin as a standard.

Measurement of Mitochondrial Respiration and Swelling—For measurements of oxygen consumption and turbidity of mitochondria, mitochondria were suspended in the $+P_i$ medium (250 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) to a final protein concentration of 0.7 mg/ml. They were then energized by addition of 5 mM succinate (plus 0.5 μg/mg protein rotenone) as a respiratory substrate. Time-dependent oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs, YSI 5331) at 25°C. Mitochondrial swelling was monitored at 25°C by measuring the turbidity of the reaction mixture at 540 nm with a Shimadzu dual wavelength spectrophotometer, model UV-3000.

Polyethylene Glycol Effects and Pore status—The effects of polyethylene glycols (PEGs) of different molecular weights (MW) were determined exactly as described by Pfeiffer *et al*. (*10*). Swelling was triggered in media consisting of 80% assay reagent and 20% 300 milliosmolar (mOsM) PEG.

For assessment of the pore status (opened or closed) of mitochondria that had already undergone swelling, 1.1 ml of a 300 mOsM solution of PEG600 or PEG6000 was added.

Transmission Electron Microscopic Analysis of Mitochondrial Configuration—TEM analysis of mitochondria untreated or treated with 100 μM calcium ions or 50 μM silver ions for 3 min at 25°C was carried out essentially as described previously (*18*) with a Hitachi H-800MT electron microscope.

Measurement of Mitochondrial Cytochrome c Release—To measure the release of cytochrome c from mitochondria, we placed an aliquot (100 μl) of mitochondrial suspension treated with silver ions in a microtube, promptly centrifuged it, and separated the supernatant from the precipitant. The precipitated mitochondria were resuspended in 100 μl of the incubation medium, and 5-μl aliquots of this suspension (P) and the supernatant (S) were individually subjected to SDS-PAGE. Cytochrome c retained in mitochondria or released into the incubation medium was detected with a specific antibody against cytochrome c, which was prepared by using a synthetic peptide with the amino acid sequence of HTVEKGGKHKTGPNLHGLFC as an antigen.

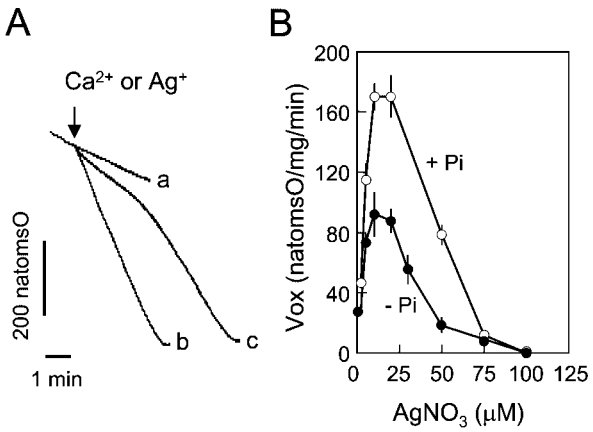

Fig. 1. **Effects of Ag^+ on mitochondrial respiration.** Mitochondria were suspended in $+P_i$ or $-P_i$ medium to final protein concentrations of 0.7 mg/ml, energized with 5 mM succinate (plus 0.5 μg/ml rotenone) at 25°C, and treated with various reagents. (A) Typical time courses of mitochondrial respiration with no addition (trace a) or with addition of 10 μM Ag^+ (trace b) and 100 μM Ca^{2+} (trace c). (B) Rates of respiration induced by Ag^+ at different final concentrations. Open and closed circles represent the results observed with $+P_i$ and $-P_i$ medium, respectively. Error bars represent standard deviations of at least 3 different experiments.

RESULTS

Ag^+-Induced Acceleration of Mitochondrial Respiration—Since acceleration of respiration is one of the most typical characteristics of mitochondrial PT, the effect of Ag^+ treatment on the rate of respiration was examined. Upon addition of Ag^+ to energized mitochondria in the presence of inorganic phosphate (P_i), immediate acceleration of respiration was observed, and this differed from Ca^{2+}-induced acceleration of respiration, in which a lag time was observed (Fig. 1A). Acceleration of mitochondrial respiration, in the presence or absence of Pi, was proportional to the dose of Ag^+ up to 10 μM, at which concentration almost maximal responses were observed (Fig. 1B). However, in the absence of P_i, the Ag^+ effects were approx. one-half of those in the presence of P_i (Fig. 1B). At Ag^+ concentrations higher than 10 μM, the rate of respiration was reduced and was completely inhibited at 100 μM. The latter effect may be attributed to the inhibitory effect of Ag^+ on the respiratory chain, since acceleration was not observed even after the addition of a protonophoric uncoupler, SF6847 (data not shown).

Stimulatory Effects of Ag^+ on Mitochondrial Swelling—Mitochondrial swelling is also considered to reflect the PT of the inner membranes. Therefore, effects of Ag^+ on mitochondrial swelling were examined. Immediate swelling was observed upon addition of Ag^+ to energized mito-

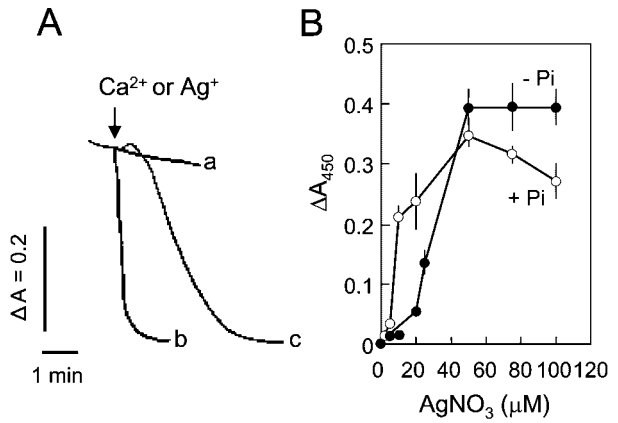

Fig. 2. **Effects of Ag^+ on mitochondrial swelling.** Experimental conditions were the same as in Fig. 1. (A) Typical time course of turbidity of mitochondrial suspension with no addition (trace a) or with addition of 50 μM Ag^+ (trace b) and 100 μM Ca^{2+} (trace c). (B) Swelling triggered by adding Ag^+ at different final concentrations. Open and closed circles represent the results observed with $+P_i$ and $-P_i$ medium, respectively. Error bars represent standard deviations of at least 3 different experiments.

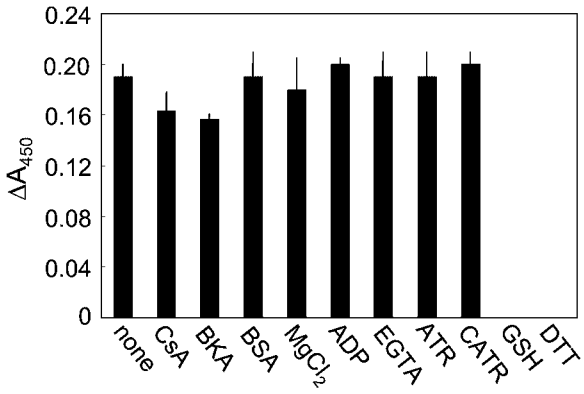

Fig. 3. **Effects of various inhibitors of classical PT on Ag^+-induced mitochondrial swelling.** Effects of CsA (1 μM), BKA (10 μM), EGTA (10 mM), CATR (5 μM), ATR (0.1 mM), $MgCl_2$ (5 mM), BSA (100 μM), ADP (0.5 mM), DTT (1 mM) or GSH (1 mM) on the swelling induced by 50 μM Ag^+. Error bars represent standard deviations of at least 3 different experiments.

chondria, which was again different from the delayed effect of calcium ions (Fig. 2A). Ag^+ induced mitochondrial swelling, in the presence and absence of P_i, in a dose- dependent manner (Fig. 2B). In the presence or absence of P_i, maximum swelling at about 50 μM, and a slight decrease in swelling at higher concentrations were observed. It is noteworthy that mitochondrial swelling caused by Ag^+ at low doses of up to 50 μM was increased significantly by the addition of P_i, but it was slightly inhibited at higher doses (Fig. 2B).

As Ag^+ induced acceleration of respiration and swelling of mitochondria, we concluded that Ag^+ induces mitochondrial PT. However, this PT seems to be different from the classical PT usually induced by Ca^{2+}, as it was immediate and only partially P_i-dependent, whereas the Ca^{2+} effect occurs after a lag time and is completely P_i-dependent.

It should be mentioned that Ag^+ was added to mitochondria as $AgNo_3$. Therefore, to check the effect of NO_3^- on mitochondria, we examined the effect of $NaNo_3$ on mitochondrial respiration or swelling. We found that $NaNo_3$ has no effect on mitochondrial respiration or swelling (data not shown), indicating that the effects of $AgNo_3$ on mitochondria are due to Ag^+ only.

Effects of Inhibitors of Classical PT on Ag^+ Effects on Mitochondria—To determine the molecular features of Ag^+ effects on the structure and functions of mitochondria, we next examined the effects of various inhibitors of classical PT on the respiration and swelling of mitochondria induced by Ag^+. As shown in Fig. 3, cyclosporin A (CsA), ADP, MgCl2 and bongkrekic acid (BKA), which are known inhibitors of classical PT (*19–22*), were not effective against Ag^+-induced mitochondrial swelling. In addition, carboxyatractyloside (CATR) and atractylside (ATR), which are known to stabilize the ADP/ATP carrier in the c state (*23*), and bovine serum albumin (BSA), which binds free fatty acids (*9*), had no effect on Ag^+-induced increase in membrane permeability. Ethylene glycol bis(b-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), a selective chelating reagent of Ca^{2+}, also had no inhibitory effect, indicating that Ag^+-induced mitochondrial permeability does not require matrix Ca^{2+}. Similar results were obtained for Ag^+-induced acceleration of mitochondrial respiration (data not shown).

Because Ag^+ reacts with SH groups to form hemi-silver sulfides, in preference to amino, imidazole, carboxyl, and phosphoryl moieties (*24*), reduced glutathione (GSH) and dithiothreitol (DTT), which maintain protein SH groups in their reduced form under oxidant stress (*25*), were used to probe the involvement of membrane protein SH groups in the mitochondrial response to Ag^+. Addition of DTT or GSH to mitochondria had no effect. However, their addition before Ag^+ resulted in complete inhibition of Ag^+-induced mitochondrial swelling and acceleration of respiration (Fig. 3). Exactly the same results were obtained for Ag^+-induced mitochondrial acceleration of respiration (data not shown). Since the inhibitory effects of DTT and GSH could represent an effect of these reagents to reduce the effective concentration of Ag^+, thereby preventing binding of the ion with the mitochondria, the effects of both reagents were measured after Ag^+ addition. When DTT or GSH was given after Ag^+, they stopped Ag^+-induced acceleration of respiration (data not shown). It should be noted that the effects of addition of GSH or DTT after Ag^+ on mitochondrial swelling could not be observed as Ag^+-induced swelling was instantaneous and finished within one second (see Fig. 2A). Together, these results suggest that Ag^+ increases mitochondrial permeability through its interaction with sulfhydryl groups of mitochondrial membrane proteins, and this effect is clearly distinguishable from the classical PT.

Ag^+ Opens a CsA-Insensitive Pore in the Mitochondrial Inner Membrane—Several thiol reagents were reported to induce PT and open the classical PTP (*1*, *26*, *27*). However, the above results indicate that Ag^+ seems not to open PTP in its classical form when it is opened by Ca^{2+} and P_i, since inhibitors of the opening classical PTP were not effective against the Ag^+-induced increment in permeability of the mitochondrial inner membrane. Therefore, we next examined whether the interaction of Ag^+ with sulfhydryl groups in mitochondrial inner membrane proteins results in just a perturbation of the membrane

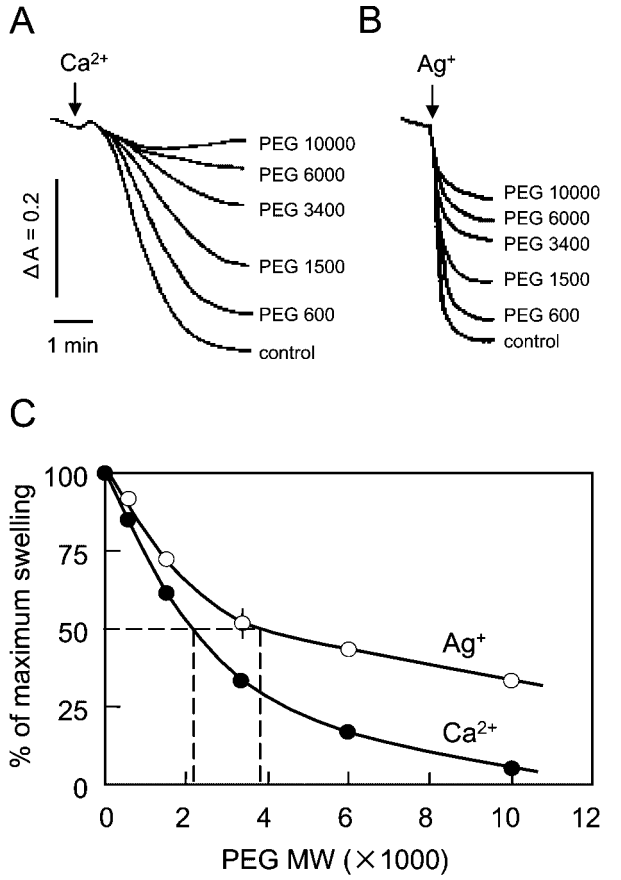

Fig. 4. **Ag⁺ opens a pore rather than disrupting mitochondrial membrane structure.** Time courses of mitochondrial swelling induced by 100 μM calcium ions (A) or by 50 μM Ag⁺ (B) in the absence or presence of 300 mOsM PEGs with various MWs. (C) Sizes of the pores induced by 50 μM Ag⁺ or by 100 μM Ca^{2+} estimated by curve fitting. Fifty percent inhibition of Ag⁺-induced swelling was estimated to be caused by PEG3800. The classical PTP induced by Ca^{2+} was 50% inhibited by PEG2200. Error bars represent standard deviations of at least 3 different experiments.

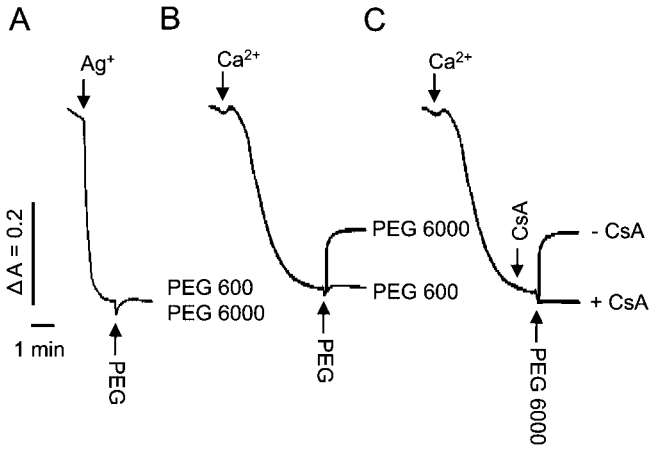

Fig. 5. **The pore opened by Ag⁺ closes spontaneously.** Effects of PEGs on the mitochondria preswollen by 50 μM Ag⁺ (A) and 100 μM Ca^{2+} (B and C). In (A) and (B), 1.1 ml of 300 mOsM PEG600 or PEG6000 was added to the preswollen mitochondria. In (C), 1 μM CsA was added before the addition of PEG6000.

structure or in opening of a pore. For this purpose, we examined the effects of polyethylene glycols (PEGs) with different molecular weights (MW), *i.e.*, different Stokes radii, on Ag⁺-induced swelling of mitochondria according to the protocol of Pfeiffer *et al*. (*10*).

Similar to the inhibitory effects of PEGs on the swelling induced by Ca^{2+} (Fig. 4A), which is known to open the classical PTP, a decreased degree of swelling induced by Ag⁺ was observed with PEGs with higher MW (Fig. 4B). The degrees of swelling obtained with PEGs with various MW were expressed as percentages of the swelling observed in the absence of PEG; the PEG MW required to inhibit swelling by 50% was estimated graphically (Fig. 4C). As depicted in Fig. 4C, Ag⁺ opened a significantly larger pore than the classical PTP opened by Ca^{2+} with Pi, and their sizes were equivalent to the sizes of PEG3800 and PEG2200, respectively. Thus, we concluded that Ag⁺ also opens a pore in the inner mitochondrial membrane.

The Pore Opened by Ag⁺ Closes Spontaneously—The pore status of swollen mitochondria, *i.e.*, whether the pore is open or closed, can be also assessed by adding PEG. If the pore is open in swollen mitochondria, addition of PEG6000 will result in a significant water efflux (shrinkage) of these mitochondria. But if the pore is closed, the effective internal and external osmotic strengths will be approximately equal (~300 mOsM), and added PEG will have no effect on volume, regardless of PEG size. However, addition of PEG600 will alter neither the effective external osmotic strength nor the mitochondrial volume.

Addition of PEG600 or PEG6000 to mitochondria swollen by Ag⁺ resulted in no appreciable change in absorbance; that is no shrinkage occurred (Fig. 5A). In contrast, although PEG600 produced no shrinkage in mitochondria swollen by Ca^{2+}, PEG6000 elicited a dramatic increase in absorbance (Fig. 5B). These results suggest that, following mitochondrial swelling by Ca^{2+}, the classical PTP remained open, whereas the Ag⁺-induced pore was closed. This proposition is supported by the following experiment: Following PTP induction and mitochondrial swelling by Ca^{2+}, CsA was added to close the PTP. The subsequent addition of PEG6000 resulted in no shrinkage (Fig. 5C), analogous to the traces seen with Ag⁺ (see Fig. 5A). These results strongly suggested that while the classical PTP induced by Ca^{2+} remained open after the completion of swelling, the pore opened by Ag⁺ closed spontaneously. It appears, however, that this pore does not close completely. State 4 respiration continues unabated after the completion of swelling induced by Ag⁺, indicating that this pore is still permeable, at least to protons.

Mitochondria Treated with Ag⁺ Differed in Appearance from Those Treated with Ca^{2+}—We further examined the membrane structure of mitochondria treated with Ag⁺ by using transmission electron microscopy (TEM). As shown in Fig. 6, when the classical PT was induced by Ca^{2+}, significant mitochondrial swelling and increase in mitochondrial volume, compared to the control mitochondria, were observed. In addition, the structure of the inner mitochondrial membrane disappeared as reported previously (*19*, *28*, *29*). However, mitochondria treated with

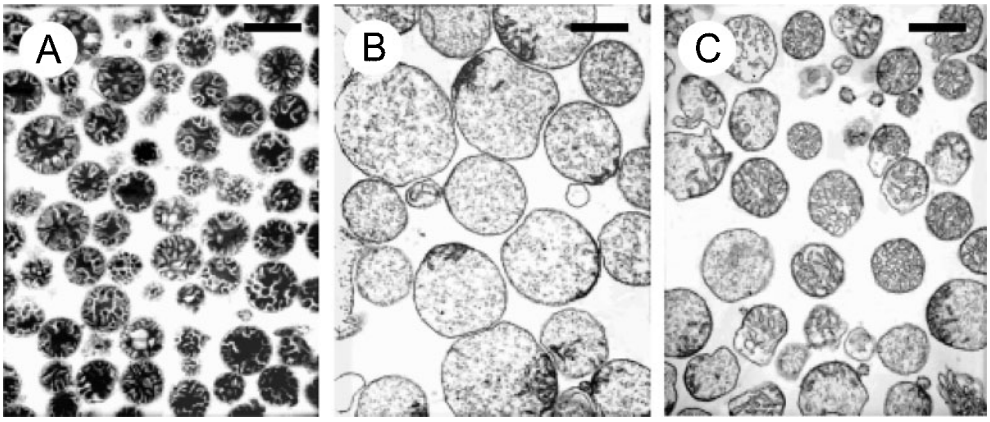

Fig. 6. **Transmission electron microscopic observations of configurations of mitochondria treated with Ag^+.** Experimental conditions were the same as in Fig. 1. (A) Mitochondria without further treatment. (B) Mitochondria treated with 100 μM Ca^{2+}. (C) Mitochondria treated with 50 μM Ag^+. Treatments were performed for 3 min at 25°C in $+P_i$ medium. Bars indicate 1.0 μm. The panels shown are typical of multiple evaluations.

Ag^+ had novel configurations, in which there was less increase in mitochondrial volume, and the structure of inner mitochondrial membrane did not completely disappear (Fig. 6C). Presumably, these differences reflect the differences between the molecular features of pores opened in the inner mitochondrial membrane by Ca^{2+} and Ag^+, of which the former remain open after induction, while the latter close spontaneously.

Treatment of Mitochondria with Ag^+ Caused Significant Release of Cytochrome c—When the classical PT is induced, remarkable changes occur in mitochondrial functions and morphologies, and mitochondrial cytochrome *c* is released. This release is known to trigger subsequent steps of apoptosis (*3*, *4*). Thus, we next examined whether cytochrome *c* is released from mitochondria after treatment with Ag^+.

As shown in Fig. 7, when mitochondria were treated with 50 μM Ag^+, a significant amount of cytochrome c was released from mitochondria. However, unlike the Ca^{2+}-induced release, cytochrome c release induced by Ag^+ was not prevented by CsA but was completely inhibited by GSH. This result is consistent with the results of Ag^+-induced acceleration of respiration and swelling.

DISCUSSION

Mitochondria have been believed to be the major site of energy conversion (*i.e.*, oxidative phosphorylation) in living cells. However, recent studies have also revealed the importance of mitochondria in the regulation of cell fates. In particular, dysfunction of mitochondria, by induction of PT accompanied with the release of cytochrome *c*, was found to be a key process in regulation of the apoptotic process (*3*, *4*). However, the mechanisms by which cytochrome *c* is released from mitochondria accompanied with dysfunction of mitochondria are still not yet fully understood. To understand these mechanisms, experiments using isolated mitochondria seem to be very important, as well as those using whole cells, and investigations of mitochondrial status under various conditions are very important.

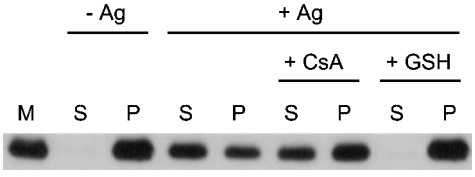

Fig. 7. **Release of apoptogenic cytochrome *c* from mitochondria induced by Ag^+.** During the measurement of respiration (3 min after addition of Ag^+), aliquots of mitochondrial suspension were placed in a microtube, and the mitochondria were separated by prompt centrifugation. Supernatants containing cytochrome c released from 3.5 μg of mitochondrial proteins were subjected to Western analysis using the specific antibody against cytochrome *c*. A typical result obtained with 3 independent mitochondrial preparations is shown. Lane "M" represents total mitochondrial proteins (3.5 μg). Where indicated, 1 μM CsA or 1 mM GSH was added before addition of Ag^+.

In this study, we investigated the effects of Ag^+, a well-known sulfhydryl group-reactive cytotoxin (*14–17*), on isolated rat liver mitochondria. Ag^+ was found to cause immediate acceleration of mitochondrial respiration and swelling, and these effects were insensitive to inhibitors of classical PT such as CsA and BKA, and did not require the presence of Ca^{2+}. Thus, Ag^+ was concluded to increase mitochondrial permeability in a way that is clearly distinguishable from the classical PT and the opening of classical PTP usually induced by Ca^{2+} and P_i.

Ag^+ is known to react strongly with SH groups to form stable hemi-silver sulfides, but it could also react with other chemical groups (*24*). However, the fact that Ag^+-induced mitochondrial swelling and acceleration of respiration were totally prevented and immediately reversed by GSH and DTT (Fig. 3) suggests that they were the result of a reversible interaction of Ag^+ with SH-bearing ligands at the mitochondrial inner membrane.

Classical PT usually induced by Ca^{2+} is known to be completely P_i-dependent; however, Ag^+-induced PT was only partially P_i-dependent. Although the reason for this partial dependence on P_i is not clear at present, these results support the conclusion that the effects of Ag^+ on mitochondria are different from the Ca^{2+}-induced classical PT, which is completely P_i-dependent.

To examine whether Ag^+ simply perturbs the mitochondrial inner membrane or indeed opens a pore, the status of the mitochondrial inner membrane was further examined by measuring the permeability of PEGs of different molecular weights according to Ref. *10*. Comparison of the inhibition by PEG of the mitochondrial swelling induced by Ca^{2+}, which is known to open classical PTP, and by Ag^+ suggests that Ag^+ also induces the open-

ing of a pore in the inner mitochondrial membrane. Whether this pore is just another open state of the classical PTP, which is larger in diameter and insensitive to classical PTP opening inhibitors, or a distinct pore is not clear; these possibilities are currently being investigated. However, in either case, the pore opened by Ag^+ closes spontaneously, unlike the classical PTP, which remains open.

The status of mitochondrial membranes treated with Ag^+ was further investigated by the TEM appearance. The mitochondria treated with Ag^+ showed a novel configuration and their appearance was distinct from those of untreated mitochondria (control) and Ca^{2+}-treated mitochondria. Mitochondria swollen by Ag^+ appeared smaller than those swollen by Ca^{2+}, and the inner membrane was still partially visible, while it was completely absent in mitochondria swollen by Ca^{2+}. The reason why mitochondria treated with Ag^+ showed such a novel configuration is still uncertain. But the results taken together suggest that it is attributable to opening of a different pore by Ag^+. It is also possible, however, that Ca^{2+} causes the continuous opening of the pore, while Ag^+ induces only transient opening of the same pore.

When classical PT is induced, apoptogenic cytochrome *c* is known to be released from mitochondria (*3*, *4*). Thus, we also examined whether cytochrome *c* is released when mitochondria are treated with Ag^+. As a result, remarkable amount of cytochrome c was found to be released from mitochondria in a CsA-insensitive but GSH- and DTT-sensitive manner. It is difficult to discuss, at this stage, the mechanism causing the release of cytochrome *c*, but it is clear that only a transient increment in permeability of the mitochondrial membrane caused by Ag^+ was also associated with the release of cytochrome *c*. In addition, whether this cytochrome *c* release is able to induce apoptosis and/or necrosis in living cells needs further investigations in whole cells.

In summary, we characterized the effects of Ag^+ on isolated rat liver mitochondria. Ag^+ was found to induce transient non-classical PT in the mitochondrial inner membrane, which was characterized by the induction of mitochondrial swelling and acceleration of respiration, but was clearly different from classical PT as it was not inhibited by classical PT inhibitors such as CsA. This non-classical PT seemed to have resulted from the opening of a pore, which could be just another open state of classical PTP with larger diameter or a distinct pore, but in either case, the pore appeared to close spontaneously. Ag^+ seemed to induce its effects on mitochondria through interaction with sulfhydryl groups of the mitochondrial inner membrane. All of these effects of Ag^+ on mitochondria resulted in the release of apoptogenic cytochrome *c* in a CsA-insensitive manner. These results support previous reports results using different reagents by presenting further evidence that cytochrome *c* could be released through nonclassical PT.

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