The K_v channel blocker 4-aminopyridine enhances Ag^+ uptake: A scanning electrochemical microscopy study of single living cells

Dongping Zhan, Fu-Ren F. Fan, and Allen. J. Bard*

Department of Chemistry and Biochemistry, University of Texas, 1 University Station A5300, Austin, TX 78712-0165

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We report that silver ion (Ag^+) uptake is enhanced by 4-aminopyridine (4-AP), a well known voltage-sensitive potassium ion channel (K_v) blocker. Both bacterial (*Escherichia coli*) and mammalian (3T3 fibroblast) cells were used as model systems. Ag^+ uptake was monitored with a scanning electrochemical microscope with an amperometric Ag^+ ion-selective electrode (Ag^+ -ISE) and the respiration rates of *E. coli* cells were measured by oxygen reduction at an ultramicroelectrode. The results showed that not only the amount but also the rate of silver uptake by the cells increased significantly when 4-AP was added to the solution. For fibroblasts, the Ag^+ uptake rate was 4.8×10^7 ions per cell per sec without 4-AP compared with 1.0×10^8 ions per cell per sec with 0.2 mM 4-AP. For *E. coli* cells, the uptake rate was 1.5×10^4 ions per cell per sec without 4-AP vs. 3.5×10^4 ions per cell per sec with 0.5 mM 4-AP and 5.9×10^4 ions per cell per sec with 1 mM 4-AP. Thus, 4-AP might be useful where silver is used as antimicrobial agent to speed its uptake.

E. coli | scanning electrochemical microscope | silver ion

Ag^+ has been used as an antimicrobial drug for centuries (1, 2). The antimicrobial activity of Ag^+ involves the complex reaction with membranes, enzymes, nucleic acids, and other cellular components. As a soft acid, Ag^+ interacts with electron donor groups containing nitrogen, oxygen, and sulfur that are present in microbial cells as amines, hydroxide groups, phosphates, and thiols. Thiol group-containing enzymes, such as lactate dehydrogenase, are proposed to be inhibited after binding of Ag^+ (3, 4). Ag^+ has also been shown to affect the respiratory chain (5–7). Other potential sites of action for Ag^+ are associated with energy-yielding reactions of the respiratory chain, collapse of proton motive force, and interference with phosphate uptake (8, 9). Ag^+ may also compete for cellular entry in an essential copper transport system (10, 11). Ag^+ accumulation may occur in two stages: a rapid, reversible, and metabolically independent surface binding followed by metabolically dependent, irreversible intercellular accumulation (12, 13). Silver toxicity has also been studied in mammalian cells such as fibroblasts and keratinocyte cells (14, 15).

The current antimicrobial use of silver has promoted concerns over silver resistance. Silver-resistant bacterial strains have been isolated from both clinical and environmental sources (16, 17). In Gram-negative bacteria, at least two general mechanisms effectively reduce Ag^+ access: the outer membrane porins and active efflux systems (18, 19). Most toxic heavy metal resistances result not from chemical detoxification, but from energy-dependent ion efflux from the cell via channels that function either as ATPases or as chemiosmotic cation/proton antiporters. The molecular genetics of a silver efflux pump has been described; the gene cluster for silver resistance contains a total of nine genes, seven of which have been named and two less-recognized ORFs that are still called ORFs: *silP ORF105 silA silB ORF96 silC silS silR silE* (20). The steady state between the uptake, absorption, and efflux processes determines the Ag^+ efficacy.

Although Ag^+ toxicity and resistance have been investigated extensively, to our knowledge, the mechanism of silver uptake remains uncertain. Here, we suggest a relationship between Ag^+ uptake and K_v channels through a study of the effect of 4-aminopyridine (4-AP), a well known broad-spectrum blocker of K_v channels (21, 22). The action of 4-AP on K_v channels is complicated and depends on many factors as has been discussed in detail (21–24). 4-AP can penetrate the cell membrane, act on the cytoplasmic side of K_v channels, block the K_v channels from assuming their “on” (K^+ passing) state, and become trapped in the channel once it is closed or inactivated. In general, the K_v channels with greater 4-AP sensitivity have a slower “off” rate. We show that 4-AP increases the rate of Ag^+ uptake and this suggests involvement of the K_v channels.

The scanning electrochemical microscope (SECM) has become a useful tool for exploring bioprocesses occurring on single living cells, because SECM can provide a spatial image of electrochemical (EC) reactivity as well as cell topography (25–27). Our group reported investigations on the action of multidrug resistance (MDR) pumps on menadione in human Hep G2 and yeast cells (28, 29). In this article, we used an amperometric Ag^+ ion-selective electrode (Ag^+ -ISE) to monitor the Ag^+ uptake by *Escherichia coli* and fibroblasts in real time (30). The cell viability for different Ag^+ treatments was characterized by studying cell respiration by SECM. The results showed that Ag^+ uptake by *E. coli* and fibroblasts are enhanced significantly when 4-AP is present, suggesting involvement of the K_v channel. Cell proliferation was inhibited and the toxic effect of Ag^+ was promoted, causing loss of cell viability. This suggests possible applications in Ag^+ -based therapeutics and conceivably applications in bioremediation for environmental purposes (31).

Results

Electrochemical Detection with the Micropipet Supported Ag^+ -ISE.

The electrochemistry of the interface between two immiscible electrolyte solutions (ITIES) provides a sensitive voltammetric method to detect the ion concentration in aqueous solution (32, 33). Recently, we developed an Ag^+ -ISE tip supported by a micrometer-sized micropipet as an SECM tip to determine the Ag^+ concentration in the vicinity of single living cells (30). The internal solution was 5 mM silver ionophore IV and 5 mM bis(triphenylphosphoranylidene)ammonium tetrakis(4-chlorophenyl)borate (BTTPATPBCl) dissolved in 1,2-dichloroethane (DCE). The solution was injected into a micropipet, which was then inserted into a sulfate medium. Because DCE is immiscible with the aqueous sulfate medium, an ITIES was formed at the tip of the micropipet. A silver tetrakis(4-chlorophenyl)borate

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*To whom correspondence should be addressed. E-mail: ajbard@mail.utexas.edu.

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Fig. 2. Ag^+ concentration changes with time when fibroblasts exposed to (i) 0.1 mM Ag_2SO_4 (magenta circle) and (ii) 0.1 mM Ag_2SO_4 and 0.2 mM 4-AP (green triangle). (Inset) Schematic diagram for detection of Ag^+ concentration in the bulk drop.

for a group of experiments was calibrated with a standard aqueous solution of 100 μM Ag_2SO_4 . In this article, all of the Ag^+ concentrations were calculated from the results from DPV because the peak current response of DPV could be determined more conveniently and precisely, especially with low silver concentrations.

Fig. 2 *Inset* shows a schematic diagram of the set-up used for monitoring Ag^+ uptake by a monolayer of fibroblast cells. The Ag^+ -ISE was positioned ≈ 1 mm above the cells, which is far enough to show the concentration change in the bulk of the drop. After the sulfate medium drop was replaced by a 100- μl drop of sulfate medium containing 100 μM Ag_2SO_4 , the change of Ag^+ concentration with time was recorded immediately, and then as a function of time, by DPV. The Ag^+ concentrations in the drop at different times after the Ag^+ -containing sulfate medium was applied to the fibroblasts in the presence or absence of 0.2 mM 4-AP is shown in Fig. 2. Note that the amount of Ag^+ taken up by the fibroblasts in the presence of 4-AP was significantly larger than the case in which 4-AP was absent. The rate of Ag^+ uptake with 4-AP was also faster because the time when steady state was achieved was shortened.

Fig. 3. Ag^+ concentration changes with time when *E. coli* implanted collagen gel exposed to: (i) 0.1 mM Ag_2SO_4 , collagen gel without *E. coli* (black squares); with *E. coli*: (ii) 0.1 mM Ag_2SO_4 (red circles), (iii) 0.1 mM Ag_2SO_4 and 0.5 mM 4-AP (green triangles), and (iv) 0.1 mM Ag_2SO_4 and 1 mM 4-AP (blue inverted triangles). (Inset) Schematic diagram for detection of Ag^+ concentration in the bulk drop.

Effect of 4-AP on Silver Uptake by *E. coli*. We also examined Ag^+ uptake in *E. coli*, a model used frequently for the evaluation of antimicrobial drugs. Because *E. coli* cells do not attach spontaneously to the bottom of the Petri dish, collagen type I was used to immobilize *E. coli* following the procedure reported elsewhere (37). Fig. 3 *Inset* shows the schematic diagram for monitoring the Ag^+ uptake by *E. coli* cells immobilized in a 3D collagen type I gel. Same as in the fibroblast studies, the Ag^+ concentration change in the bulk solution was monitored with time by DPV. Fig. 3 shows typical Ag^+ uptake by *E. coli* for different cases. Because the collagen gel is a hydrogel, silver ions can readily diffuse into it, as shown in curve 1 in the absence of *E. coli*, where a steady state is attained after ≈ 2 min with only a slight decrease in the Ag^+ concentration. Curve 2 shows the case of *E. coli*-collagen exposure to $100 \mu\text{M}$ Ag^+ , whereas curves 3 and 4 show Ag^+ with two different concentrations of 4-AP. When the concentration of 4-AP was 0.5 mM , the amount of Ag^+ taken up by *E. coli* was significantly promoted and the time taken to reach steady state decreased. These results are similar to the case of mammalian fibroblasts.

To elucidate the validity of these results, experiments were also done with the *E. coli* suspensions. The *E. coli* cells were harvested through centrifugation and washed with sterile distilled water several times to remove the residual anions adhering to the outside surface of the cells. Then, *E. coli* cells were exposed to the Ag^+ -containing sulfate medium with different concentrations of 4-AP and DPVs were taken as a function of time. As shown in Fig. 4, the *E. coli* cells suspended in solutions took up Ag^+ much faster in the presence of 0.5 mM and 1 mM 4-AP. In the presence of 0.1 mM and 0.2 mM 4-AP, in the first 2 min, the relative amount of Ag^+ taken up was lower than that in the absence of 4-AP. Five minutes later, it reached nearly the same value. After 10 min, the amounts were slightly more than the latter. A dose that increases the uptake was estimated to be approximately ≥ 0.5 mM.

Another check on the silver uptake was the measurement of the respiration, and thus viability, of *E. coli* cells in the collagen gel. Because Ag^+ could be reduced to metallic silver, which might give a positive feedback for regular redox mediators such as ferrocenemethanol in our previous study, here we used a mercury hemisphere ultramicroelectrode as the SECM tip to

Fig. 4. Differential pulse voltammograms of Ag^+ concentration after *E. coli* suspended in different sulfate culture mediums for different time: (A) 2 min; (B) 5 min, and (C) 10 min. Black line, calibrate DPVs of 0.1 mM Ag_2SO_4 only; red line, *E. coli* exposure to 0.1 mM Ag_2SO_4 ; green line, *E. coli* exposure to 0.1 mM Ag_2SO_4 + 0.1 mM 4-AP; blue line, *E. coli* exposure to 0.1 mM Ag_2SO_4 + 0.2 mM 4-AP; cyan line, *E. coli* exposure to 0.1 mM Ag_2SO_4 + 0.5 mM 4-AP; and magenta line, *E. coli* exposure to 0.1 mM Ag_2SO_4 + 1 mM 4-AP.

monitor oxygen concentration above the living cells. The more active the cells were, the greater the oxygen consumed and, consequently, the lower the SECM tip current for oxygen reduction in the vicinity of the cells. In these experiments, 10 μl of *E. coli* implanted collagen was injected into a Teflon well with a diameter of 2 mm and a depth of 1 mm. Fig. 5 shows the lateral probe scanning results of cell viability in different cases. Fig. 5 *Inset* is a schematic diagram of a lateral probe scanning experimental arrangement. The tip was scanned across the interface between the Teflon and the collagen-containing well. In a

Fig. 5. SECM lateral probe scanning curves for oxygen reduction by using a mercury hemispheric electrode. The tip scanned from the Teflon substrate through the *E. coli* implanted collagen gel region after 10 min of exposure to sulfate medium: (i) red line, without Ag^+ ; (ii) green line, with 0.2 mM Ag^+ ; (iii) blue line, with 0.2 mM Ag^+ and 0.5 mM 4-AP; (iv) magenta line, with 0.2 mM Ag^+ and 1.0 mM 4-AP; (v) cyan line: no *E. coli* cells implanted. Because the cells consume oxygen, the tip currents above the cell region are lower than the Teflon region. The controlled one (cyan) shows the spatial effect of the slightly convex collagen gel.

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were mixed in a volume ratio of 8:1:1. Fifty microliters of *E. coli* implanted collagen was cast in a Teflon well (with a diameter of 4.2 mm and a depth of 0.6 mm.) and incubated in sterile hood at 37°C for 30 min to form a flat gel plane. After rinsing several times with the sulfate medium to remove the phosphate ions, the sample was fixed on the SECM stage for EC and SECM experiments. To elucidate the enhancement of Ag⁺ uptake by 4-AP, 200- μ l *E. coli* suspensions were mixed with 200 μ l of sulfate medium containing different concentrations of Ag⁺ and 4-AP for 2, 5, and 10 min, respectively, and then centrifuged. The clear solution was separated and the residual Ag⁺ was detected by the amperometric Ag⁺-ISE.

Instruments. An Olympus inverted microscope (Olympus Co.) was used to observe the cell coverage and growth status of the fibroblast cells. Micropipets were prepared with a model-2000 laser puller (Sutter Instrument Co.) as

described elsewhere (42). The Olympus microscope was used to examine the quality and measure the diameter of the micropipet. Ag wire with a diameter of 100 μ m was anodized in DCE solution with 5 mM BTTPATPBCl overnight to form a layer of AgTPBCl. The AgTPBCl-coated Ag wire was inserted into a micropipet as the reference electrode in the DCE phase. To avoid Ag⁺ contamination, polypyrrole-coated stainless steel was used as reference electrode in the aqueous solution (43). All of the EC measurements were performed with a commercial SECM workstation (CH Instrument Co.).

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