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Submicromolar Ag^+ increases passive Na^+ permeability and inhibits the respiration-supported formation of Na^+ gradient in *Bacillus FTU* vesicles

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The effect of Ag^+ on Na^+ pumping by Na^+-motive NADH-quinone reductase and terminal oxidase has been studied in *Bacillus FTU* inside-out vesicles. Very low concentrations of Ag^+ ($C_{1/2} = 1 \times 10^{-8}$M or 2×10^{-12} g ion · mg protein^{-1}) are shown to inhibit the uphill Na^+ uptake coupled to the oxidation of NADH by fumarate or of ascorbate + TMPD by oxygen but exert no effect on the H^+ uptake by the H^+-motive respiratory chain. Low Ag^+ also induces a specific increase in the Na^+ permeability of the vesicles. HQNO, added before and not after Ag^+, prevents the Ag^+-induced permeability increase, with effective HQNO concentrations being similar to those inhibiting the uphill Na^+-uptake coupled to the NADH-fumarate oxidoreduction. Reduction of terminal oxidase by ascorbate + TMPD in the presence of cyanide sensitizes the Na^+ permeability to Ag^+. It is suggested that low [Ag^+], known as a specific inhibitor of electron transport by the Na^+-motive NADH-quinone reductase, uncouples the electron and Na^+ transports so that the Ag^+-modified NADH-quinone reductase operates as an Na^+ channel rather than an Na^+ pump. This effect is discussed in connection with the antibacterial action of Ag^+.

Bacterial sodium energetics; Silver; NADH-quinone reductase; Terminal oxidase

1. INTRODUCTION

As it has recently been found in our group [1–3], the alkalo- and halotolerant *Bacillus FTU* has two respiratory chains, namely H^+-motive and Na^+-motive. These two chains strongly differ in their sensitivities to inhibitors. The Ag^+ effect was found to be the most demonstrative. As shown by Asano et al. [4], a very low [Ag^+] specifically inhibits the Na^+-motive NADH-quinone reductase of *Vibrio alginolyticus*. As it was shown in our laboratory in the experiments on the *Bacillus FTU* vesicles, Ag^+ strongly suppresses the respiration-supported uphill transport of Na^+ at a concentration as low as 1×10^{-8} M, while that of H^+ is not affected by Ag^+ taken even at a 100-fold higher concentration [3].

Below it will be shown that such small amounts of Ag^+ specifically increase the passive Na^+ permeability of the *Bacillus FTU* membrane. The effect is apparently mediated by an Ag^+ binding to component(s) of the Na^+-motive NADH-quinone reductase, for the Ag^+-induced increase in the Na^+ conductance can be prevented by adding low concentrations of HQNO, specific inhibitor of this enzyme.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DEA, diethylamine; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylene diamine; TPT, triphenyltin acetate

2. MATERIALS AND METHODS

Bacillus FTU, isolated in our laboratory [1,2], was used. The growth medium contained 0.5 M NaCl, 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 5 mM $MgSO_4$, 1×10^{-5} M $FeSO_4$, 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.6, and 60 mM sodium succinate as the only energy and carbon source; $t^o = 37$°C, aerobic conditions.

To obtain ultrasonic inside-out subcellular vesicles, the previously described method [2] was employed.

The Na^+ content in the subcellular vesicles was assayed by means of the gel-filtration and centrifugation procedure according to Penefsky [5]. The process of the respiratory chain dependent Na^+ uptake was initiated by adding TMPD or NADH. To arrest the reaction, 0.04 ml of the incubation mixture was centrifuged in a gel-filtration column ($D = 10$ mm) containing Sephadex G-50 coarse preequilibrated with 0.1 M Tris-H_2SO_4 and 10 mM $MgSO_4$, pH 8.2. The eluate was diluted 10-fold with bidistilled water and [Na^+] was measured with a PFM flame photometer. The above procedure was found to decrease extravesicular [Na^+] by factor 5×10^3. To measure the passive Na^+ permeability of the membrane of *Bacillus FTU* vesicles, 5 mM Na_2SO_4 was added to the vesicles isolated and incubated without added Na^+ (initial [Na^+] in the medium was found to be 0.2 mM).

Oxygen consumption was measured with a standard oxygen Clark-type electrode. Intravesicular acidification was monitored by acridine orange fluorescence quenching measured with a MPF-4 Hitachi fluorimeter (excitation at 493 nm, measurement at 539 nm). Fluorimetric measurements were performed by Dr M.L. Vaghina.

3. RESULTS

Fig. 1A shows that very low concentrations of Ag^+ completely inhibit the uphill Na^+ uptake by *Bacillus FTU* vesicles, supported by the oxidation of NADH by

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fumarate ($C_{1/2} = 1 \times 10^{-8}$ M). The H^+ transport, also coupled to the NADH-fumarate oxidoreduction, is resistant to such Ag^+ concentrations. In another experiment, it was found that even 100-fold higher [Ag^+] has no effect on the uphill H^+ uptake (not shown).

Such a specific effect of Ag^+ could be explained by the inhibition of the Na^+-motive NADH-quinone reductase, discovered in *V. alginolyticus* by Asano et al. [4]. Surprisingly, subsequent experiments showed that Ag^+ also interferes with the uphill Na^+ uptake driven by Na^+-motive terminal oxidase. Fig. 1B shows the Ag^+ titrations of the Na^+ uptake supported by the oxidations of NADH by fumarate or of ascorbate + TMPD by oxygen. In fact, the latter system is even slightly more sensitive to Ag^+ than the former one.

To explain the effect of Ag^+ on the ascorbate oxidation-supported Na^+ transport, it might be suggested that Ag^+ inhibits the Na^+-motive oxidoreduction in the terminal segment of the respiratory chain just as it does in its initial segment. Two observations, however, argue against this suggestion.

(i) Low Ag^+ concentrations, used to inhibit the ascorbate oxidation-supported Na^+ transport, were without any measurable effect on the oxidation rate (not shown).

(ii) HQNO (which inhibits the Na^+-motive NADH-quinone reductase but has no effect on the terminal oxidase [3]) was found to prevent Ag^+ inhibition of the Na^+ uptake coupled to ascorbate oxidation (Fig. 1C).

The latter finding suggests that the Ag^+ effect on the Na^+ transport by the terminal oxidase is a consequence of the Ag^+ interaction with the initial step of respiratory chain.

Subsequent experiments showed that Ag^+ increases the passive flux of Na^+ through the *Bacillus FTU* membrane.

To measure Na^+ permeability, the *Bacillus FTU* vesicles were isolated in a Na^+-free medium and added to the incubation mixture containing 5 mM Na_2SO_4. The passive Na^+ equilibration between the outer and intravesicular solutions was shown to take 1–3 min. This value varied in different preparations of the vesicles. The variations seemed to be due to a partial loss of factors F_1 of the membrane ATPase. In preparations showing high Na^+ permeability, the F_0 inhibitors, dicyclohexylcarbodiimide or TPT, were found to suppress the Na^+ equilibration. An example of this kind is shown in Fig. 2A. It is seen that TPT increases the $t_{1/2}$ value of the Na^+ equilibration from 20 to 60 s. In the same experiment, it was shown that Ag^+, added

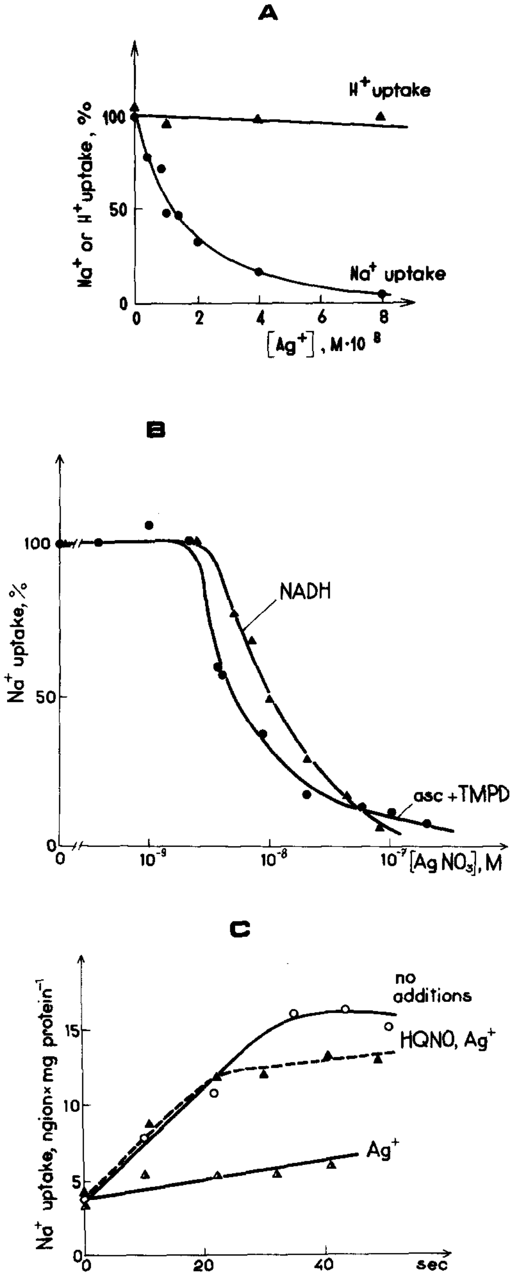

Fig. 1. Effect of Ag^+ upon the respiratory chain-supported Na^+ and H^+ uptakes by inside-out subcellular vesicles of *Bacillus FTU*. (A) NADH oxidation by fumarate. Na^+ uptake: the incubation mixture contained 0.1 M K_2SO_4, 10 mM $MgSO_4$, 2.5 mM Na_2SO_4, 50 mM Tricine-KOH, pH 8.2, 50 mM DEA acetate, 10 mM KCN, 1×10^{-5} M CCCP, 5 mM potassium fumarate and vesicles, 4 mg protein·ml^{-1}. At zero time, 2 mM NADH (potassium salt) was added. H^+ uptake: the incubation mixture contained 0.1 M K_2SO_4, 10 mM $MgSO_4$, 5 mM Na_2SO_4, 50 mM tricine-KOH, pH 8.2, 15 mM KCN, 6×10^{-6} M acridine orange, 4×10^{-7} M valinomycin, 1 mM NADH and vesicles, 0.6 mg protein·ml^{-1}. At zero time, 5 mM potassium fumarate was added. (B) Oxidations of NADH by fumarate or of ascorbate + TMPD by oxygen. NADH oxidation: the incubation mixture contained 0.1 M K_2SO_4, 10 mM $MgSO_4$, 2.5 mM Na_2SO_4, 50 mM tricine-KOH, pH 8.2, 50 mM DEA acetate, 2×10^{-5} M CCCP, 10 mM KCN and vesicles, 4.9 mg protein·ml^{-1}. At zero time, 2 mM NADH was added. Ascorbate oxidation: the incubation mixture, as in the NADH experiment, but the CCCP concentration was 1×10^{-5} M, KCN was absent and 15 mM potassium ascorbate was present. The protein concentration was 5.3 mg·ml^{-1}. The reaction was started by adding 0.5 mM TMPD. 100%, 24 and 20 ngion Na^+·min^{-1}·mg protein^{-1} for NADH and ascorbate + TMPD, respectively. (C) Ascorbate oxidation: the incubation mixture as in B. The protein concentration, 5 mg·ml^{-1}. Additions, 4×10^{-6} M HQNO and 1×10^{-7} M $AgNO_3$. HQNO was added before $AgNO_3$.

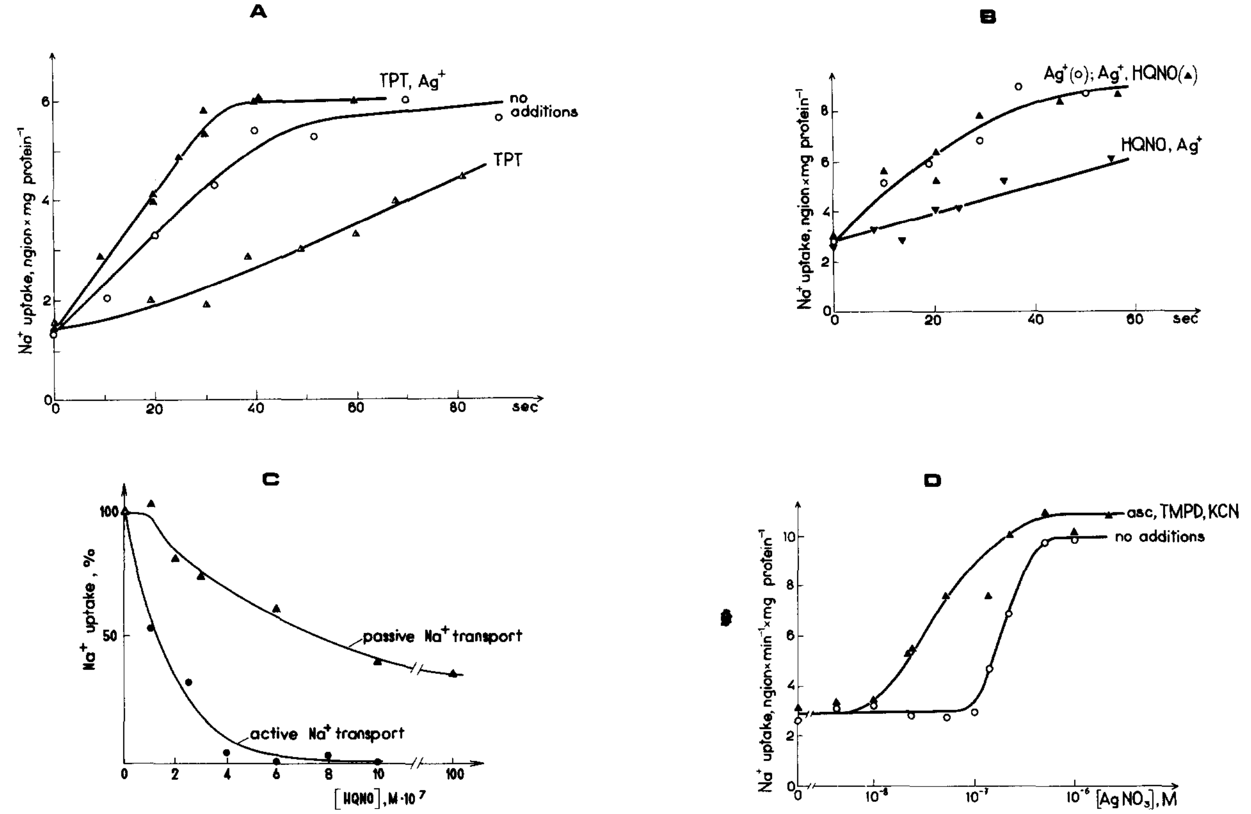

Fig. 2. Effects of Ag^+ and HQNO upon the passive Na^+ permeability of *Bacillus FTU* vesicles. (A) The incubation mixture contained 0.1 M Tris-H_2SO_4, pH 8.2, 10 mM $MgSO_4$, 50 mM DEA acetate, 1×10^{-5} M CCCP, 0.2 mM Na^+ and vesicles, 5.6 mg protein·ml^{-1}. At zero time, the mixture was supplemented with 5 mM Na_2SO_4. Other additions were 5×10^{-6} M TPT and 1×10^{-6} M $AgNO_3$. (B) The incubation mixture was as in A but 5×10^{-6} M TPT is present in all the samples. Additions: (○) 1×10^{-6} M $AgNO_3$; (▲) $AgNO_3$ and then (after 1.5 min) 4×10^{-6} M HQNO; (▼) HQNO and after 1.5 min $AgNO_3$. (C) Comparison of the inhibitory effects of HQNO on the active Na^+ transport coupled to NADH-fumarate oxidoreduction and preventing HQNO action on the Ag^+-stimulated passive Na^+ transport in *Bacillus FTU* vesicles. Active transport, incubation mixtures: 0.1 M K_2SO_4, 10 mM $MgSO_4$, 2.5 mM Na_2SO_4, 50 mM tricine-KOH, pH 8.2, 50 mM DEA acetate, 10 mM KCN, 2 mM NADH, 5 mM fumarate, 1×10^{-5} M CCCP and vesicles, 5.2 mg protein·ml^{-1}. 100%, 36 ng ion Na^+ · min^{-1}·mg $protein^{-1}$. Passive transport, as in Fig. 2A but 5×10^{-6} M TPT was added to all the samples. Vesicles were preincubated with various HQNO concentrations for 1.5 min, and then 1×10^{-6} M $AgNO_3$ and 5 mM Na_2SO_4, were added. Na^+ concentration before the Na_2SO_4 addition was 0.2 mM. Protein concentration, 4.5 mg·ml^{-1}, 100%, 8 ngion Na^+·min^{-1}·mg $protein^{-1}$. (D) Incubation mixture as in Fig. 2A. Additions: 15 mM ascorbate, 0.5 mM TMPD and 10 mM KCN. Protein concentration: 4.9 mg·ml^{-1}.

after TPT, strongly facilitates the passive Na^+ flux ($t_{1/2}$ becomes smaller than 20 s).

The Ag^+ effect proved to be sensitive to HQNO added before Ag^+. But the addition of HQNO after Ag^+ was ineffective (Fig. 2B). $C_{1/2}$ of the above-mentioned HQNO effect was found to be of the same order of magnitude as that of the inhibiting action of HQNO on the active Na^+ uptake supported by NADH-fumarate oxidoreduction (Fig. 2C). The HQNO action cannot be attributed to the Ag^+ binding to HQNO since a 10-fold excess of Ag^+ over HQNO, added after HQNO, failed to cause any measurable increase in the Na^+ permeability (not shown).

Reduction of the terminal step of the respiratory chain by ascorbate + TMPD in the presence of cyanide proved to be favourable for the Ag^+-induced increase in the passive Na^+ permeability (Fig. 2D). Cyanide without ascorbate and TMPD was ineffective (not shown).

4. DISCUSSION

The above data indicate that Ag^+ increases the Na^+ permeability of the *Bacillus FTU* membrane. This effect may explain the inhibition of the respiratory chain-linked Na^+ accumulation in inside-out vesicles. Apparently, H^+ permeability is not affected by low Ag^+ since it does decrease the H^+ pumping (Fig. 1A).

The Ag^+-induced Na^+ permeability increase is prevented by very low [HQNO] (which is known to specifically inhibit the Na^+-motive NADH-quinone reductase [6,7]), the fact pointing to involvement of this enzyme in the Ag^+ effect on Na^+ permeability. One more piece of evidence in favour of this suggestion is that Ag^+ specifically inhibits the electron transfer by the Na^+-motive NADH-quinone reductase of *Vibrio alginolyticus* [4].

One may propose that the combination of Ag^+ with NADH-quinone reductase not only inhibits its redox activity but also modifies its Na^+-transporting part in such a way that it becomes capable of transmembrane Na^+ transport non-coupled to electron transport.

Apparently HQNO, another inhibitor of the Na^+-motive NADH-quinone reductase, prevents the abovementioned modification of this enzyme by Ag^+. This is why HQNO abolishes the Ag^+-induced inhibition of the active Na^+ uptake supported by the Na^+-motive terminal oxidase (Fig. 1C).

It is interesting that the reduction of the cyanide-inhibited terminal oxidase by ascorbate + TMPD proved to be favourable for the Ag^+-induced Na^+ permeability increase. This may be due to complexation between the Na^+-motive NADH-quinone reductase and the reduced terminal oxidase; this complex, for some reason, is, apparently, more sensitive to Ag^+ than is the reductase per se or its complex with oxidized oxidase.

It should be noted that half-maximal concentrations of Ag^+ decreasing the *active* Na^+ pumping (Fig. 1) are somewhat lower than those increasing the *passive* Na^+ permeability (Fig. 2) or inhibiting the activity of NADH-quinone reductase [4]. This is not surprising since complete inhibition of Na^+ accumulation because of the increase in the Na^+ permeability may occur when only a small portion of the NADH-quinone reductase pool combines with Ag^+.

One should stress that the above-described inhibition of the Na^+ pumping, regardless of its mechanism, proceeds at very low Ag^+ concentration ($C_{1/2}$ at about 1×10^{-8} M or about 2×10^{-12} gion $Ag^+ \cdot mg\ protein^{-1}$; see Fig. 1B). It means that traces of Ag^+ in the medium can, in fact, be inhibitory for bacteria employing the Na^+ cycle, i.e. the system which is assumed to be responsible for the adaptation of microorganisms to unfavourable conditions where the H^+ cycle is not operative [8]. This fact may explain the well-known bactericidal effect of Ag^+ and must be helpful in a search for new methods of antibacterial therapy in the case of *Vibrio cholera, Vibrio parahaemolyticus, Klebsiella pulmoniae, Salmonella typhimurium*, etc., in which the Na^+ cycle has already been shown, and also with respect to some other pathogenic bacteria thus far not studied in this regard.

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