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EFFECT OF SILVER ION ON PERMEABILITY PROPERTIES OF FROG SKIN

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SUMMARY

Ag^+ added to the external bathing solution of frog skin causes a marked decrease in potential difference and an increase in conductance. Short-circuit current and net Na^+ flux decrease somewhat. Na^+ outflux and fluxes of SO_4^{2-} and mannitol are increased by Ag^+. The fluxes of these solutes initially increase to 10–15 times control levels and then spontaneously decline to approximately 3 times control levels. These changes appear to be due to alteration of a shunt pathway in the skin. In addition, studies of washout of $^{24}Na^+$ and $^{42}K^+$ indicate that Ag^+ effects the cation selectivity of the outer barrier; movement of K^+ across this barrier increases markedly with minimal change in Na^+ movement.

INTRODUCTION

Heavy metal ions have been shown to have a variety of effects on transport processes in several tissues (see for example Rothstein[1,2] and Clarkson[3]). These effects may be the result of actions on cellular metabolism or of more direct interaction with membrane processes. In the latter case the effects may be useful in providing some insight into the nature of these processes. The present experiments were carried out to examine some of the effects of relatively low concentrations of Ag^+ on transport and permeability properties of frog skin. We were prompted to carry out more detailed studies by the observation that Ag^+ added to the outside solution at concentrations of 10^{-4} M or less caused a very marked drop in potential difference across the skin but a rather smaller change in short-circuit current.

METHODS

Frogs (*Rana pipiens*) were pithed and the abdominal skin was removed and mounted as a flat sheet in a chamber appropriate for the particular experiment. Both sides of the skin were bathed in a solution containing 57 mM Na_2SO_4, 2.5 mM $KHCO_3$ and 1.0 mM $CaSO_4$ and having a pH of approximately 8 when bubbled with air. The potential difference (PD) was determined using calomel electrodes connected to the bathing solutions by Ringer–agar bridges and the skin was short-circuited *via* a second set of bridges connected to Ag–AgCl electrodes and an external battery. In most experiments, the skin was kept short-circuited except for brief intervals to

Abbreviation: PD, potential difference.

determine open circuit PD. Conductance was estimated from short-circuit current ($I_{s.c.}$) and open circuit PD.

Unidirectional fluxes of Na^+ or SO_4^{2-} across the skin were determined by adding $^{24}Na^+$ or $^{35}SO_4^{2-}$ to one bathing solution and measuring the rate of tracer appearance on the opposite side. In some experiments, mannitol fluxes were determined using [^{14}C]mannitol; both bathing solutions then contained 1 mM mannitol. Estimates of Na^+ and K^+ movement across the outer and inner "barriers" in the skin were made by loading the tissue with $^{24}Na^+$ or $^{42}K^+$, mounting it in the chamber and following washout of tracer toward each side using the technique described by Cereijido *et al.*[4]. This involved complete removal of the bathing solutions at specified intervals and replacing them with fresh non-radioactive solution. At the end of the experiment, the amount of tracer remaining in the skin was determined by counting it directly.

Uptake of Ag^+ by the skin was determined using $^{110}Ag^+$ and the technique described by Curran and Cereijido[5]. The skin was mounted on a lucite cylinder with the outside facing outward and the outer surface was exposed to solution containing $^{110}Ag^+$ for a measured time interval. It was then rinsed briefly, blotted and suspended in a reproducible position above the crystal of a gamma counter and counted for one min. The skin was then returned to the bathing solution and the procedure repeated.

In most experiments, Ag^+ was added to the outside solution from a stock solution of $AgNO_3$ following a control period to permit measurement of control fluxes. The usual concentration of Ag^+ was 10^{-4} M since preliminary experiments indicated that this was sufficient to cause fairly reproducible changes in electrical properties of the skin. Ag^+ concentrations as low as 10^{-5} M usually caused detectable changes in PD but the effects appeared to be somewhat variable at concentrations below about $5 \cdot 10^{-4}$ M. Radioactive samples were counted in a crystal scintillation counter or in a liquid scinitillation counter using Bray's solution, depending on the nature of the isotope.

RESULTS AND DISCUSSION

The effect of 10^{-4} M Ag^+ added to the outside solution on the electrical properties of the skin is illustrated for one experiment in Fig. 1. Almost immediately after the addition of Ag^+, there was a very rapid drop in PD. The short-circuit current ($I_{s.c.}$) usually rose transiently and then fell but less rapidly than the PD. The time course of change in $I_{s.c.}$ was somewhat variable; in many experiments, it fell less rapidly than in the skin shown in Fig. 1. The total skin conductance rose rapidly and then declined slowly toward control levels. In most skins in which all electrical properties were followed for 2 h or more, conductance remained 2–3 times above control levels. However, in some skins it returned to control levels 1–2 h after addition of Ag^+. In a number of experiments in which PD was observed, a gradual recovery was observed beginning 30–60 min after addition of Ag^+, but the PD never approached control levels. Although we have not systematically examined effects of varying Ag^+ concentration, preliminary experiments suggested that recovery of electrical properties was more obvious at Ag^+ concentrations below 10^{-4} M.

As shown in Fig. 2, Ag^+ caused a very marked increase in Na^+ outflux across the short-circuited skin. The peak outflux, observed approximately 40 min after addition

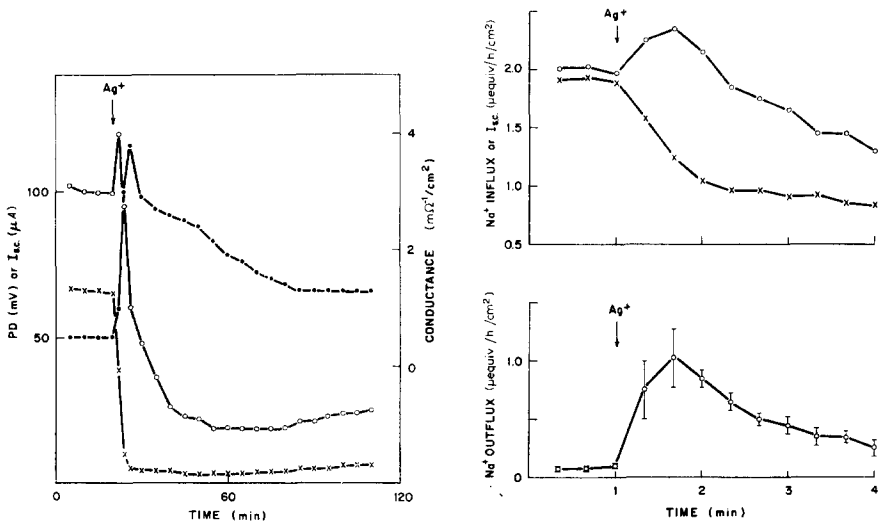

Fig. 1. Effect of Ag^+ on PD (×), $I_{s.c.}$ (○) and skin conductance (●). Ag^+ (10^{-4} M) was added to the outside solution only.

Fig. 2. Effect of Ag^+ (10^{-4} M, outside) on unidirectional Na^+ fluxes and $I_{s.c.}$ (×). Results are averages of 7 experiments on influx and 5 experiments on outflux. The S.E. values for outflux were ± 0.2–0.3 μmole/h per cm^2.

of Ag^+, was 15 times the control level. The outflux then decreased but appeared to stabilize at approximately 3 times the control flux. Na^+ influx also increased initially after addition of Ag^+ and then declined slowly and continuously over the course of the experiment. This fall in influx is approximately parallel to the decline in $I_{s.c.}$ observed in these skins and the difference between Na^+ influx and $I_{s.c.}$ is quite similar to the pattern observed for Na^+ outflux. Thus although double-label experiments were not done to test the identity of $I_{s.c.}$ and net Na^+ flux after treatment with Ag^+, the major portion of $I_{s.c.}$ still appears to represent Na^+ flux. Additional experiments would, however, be necessary to prove an identity because on the basis of the averaged results available, net Na^+ flux tends to be slightly larger than $I_{s.c.}$ in the presence of Ag^+.

The rather marked increase in Na^+ outflux suggests that Ag^+ increases passive permeability of the skin. To test this possibility further, influxes of SO_4^{2-} and mannitol were determined. As shown in Fig. 3, influxes of both solutes increased significantly and showed a pattern very similar to that observed for Na^+ outflux. The fluxes reached a peak 40 min after addition of Ag^+ and then declined to a level approximately 3 times the control flux.

The striking similarity in relative changes of Na^+ outflux, SO_4^{2-} influx and mannitol influx suggest that one effect of Ag^+ is to increase the permeability of a shunt pathway in the skin. Mandel and Curran[6] have recently examined properties of such as shunt in parallel with the active Na^+-transport system of the skin. They found that this pathway was permeable to Na^+, K^+, Cl^-, urea and mannitol and that changes in properties of this shunt caused by depolarizing potentials led to parallel changes in fluxes of all these solutes. The parallel changes in Na^+, SO_4^{2-} and mannitol fluxes following treatment with Ag^+ thus suggest an effect on such a shunt. It should

be of interest to compare further the three treatments that appear to increase shunt permeability, depolarizing potentials[6], addition of urea to the outside[7,8] and Ag^+ and to determine whether the same pathway is involved in all three cases. Ag^+ also appears to affect the active Na^+ transport pathway since Na^+ influx declines significantly. However this effect develops rather more slowly than changes in other fluxes (see Fig. 2) and may be due to inhibition of metabolism or direct action on the Na^+ pump by Ag^+ as it penetrates slowly into deeper layers of the epithelium.

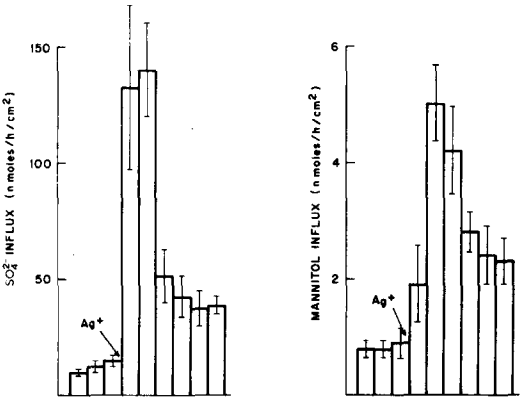

Fig. 3. Effect of Ag^+ (10^{-4} M, outside) on SO_4^{2-} and mannitol influxes. Average values from 7 experiments on SO_4^{2-} and 6 experiments on mannitol. Each bar represents a 15-min flux period.

Although Ag^+ caused a substantial increase in shunt permeability, there was some question as to whether this effect was sufficient to account for the large decrease in PD. We therefore carried out a series of experiments to examine effects on the exit of $^{24}Na^+$ and $^{42}K^+$ from the skin toward the two bathing solutions. A typical experiment showing $^{42}K^+$ efflux (cpm/min) from the skin is shown in Fig. 4. Addition of 10^{-4} M Ag^+ to the outside solution caused a 10-fold increase in efflux toward the outside solution but the effect diminished with time. Efflux toward the inside changed only slightly. Results of five such experiments are summarized in Fig. 5 in terms of rate coefficients (percent/min) for efflux in the two directions. Movement of $^{42}K^+$ toward the inside was little affected by Ag^+ although a small increase in the rate coefficient in the first 5 min period was observed in each experiment. The coefficient for movement toward the outside reached a maximum of 15 times the control value and then declined. However, after 40 min it was still 3 times the control level. These experiments were carried out under open-circuited conditions. This should minimize the changes in the coefficient for $^{42}K^+$ movement toward the outside since the PD across the outer barrier probably decreases, an event that would lead to a decrease in the rate coefficient for movement of a cation in this direction. Three additional experiments were carried out under short-circuit conditions. The rate coefficients for $^{42}K^+$ washout followed a pattern identical to that shown in Fig. 5 but the effect on movement toward the outside was somewhat greater. This coefficient reached a maximum of 19 times the control and was approximately 6 times the control value at 40 min after addition of Ag^+.

Effects on $^{24}Na^+$ washout from the skin under short-circuited conditions were

relatively minimal. No change could be detected in washout toward the inside solution following treatment with Ag^+. As shown in Fig. 6, the rate coefficient for $^{24}Na^+$ movement toward the outside increased transiently and returned to the control level fairly rapidly. This rise is statistically significant and was observed in each of the eight experiments. However, the scatter is appreciable due to the relatively few counts appearing in the outside solution.

These results indicate that Ag^+ also has a rather striking effect on the cation selectivity of a barrier located at the outer side of the skin. According to the hypothesis first suggested by Koefoed-Johnsen and Ussing[9], this barrier is normally permeable to Na^+ and nearly impermeable to K^+ and because of these properties contributes significantly to generation of the PD across the skin. The present results

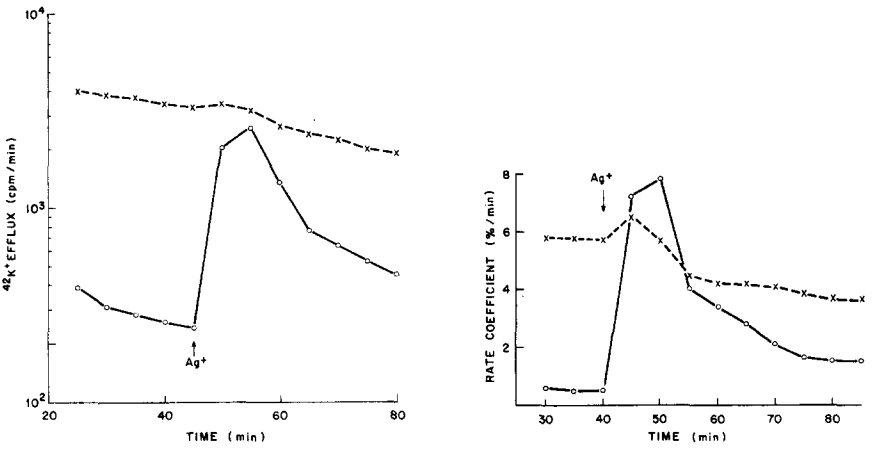

Fig. 4. Effects of Ag^+ (10^{-4} M, outside), on $^{42}K^+$ efflux toward the outside (○) and inside (×) solutions.

Fig. 5. Effect of Ag^+ (10^{-4} M, outside), on rate coefficients for $^{42}K^+$ efflux toward outside (○) and inside (×) solutions. Average values for 5 experiments.

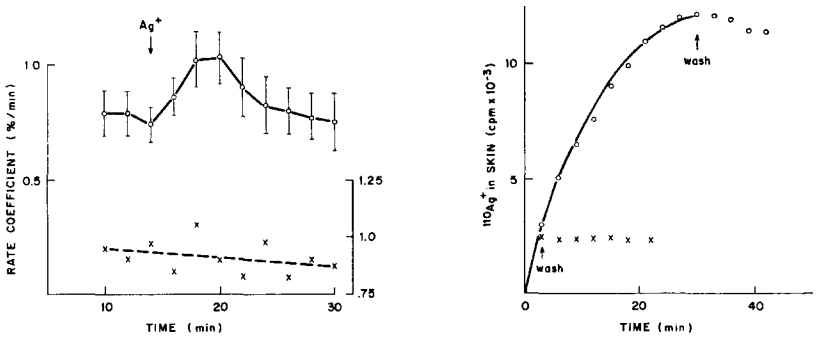

Fig. 6. Effect of Ag^+ (10^{-4} M, outside) on rate coefficients for $^{24}Na^+$ efflux toward the outside solution. Average of 8 experiments. A single control experiment in which Ag^+ was not added is shown (×) with scale displaced as shown on the right.

Fig. 7. Uptake of $^{110}Ag^+$ by skins as a function of time. The outer surface only was exposed to 10^{-4} M Ag^+. At the arrows, the skins were washed in Ringer containing no Ag^+ and counting was continued.

indicate that Ag^+ causes a marked increase in K^+ permeability of the outer barrier with relatively little change in Na^+ permeability. Since the PD across this barrier is normally oriented with the cell interior positive relative to the outside solution and since the cells contain a relatively high concentration of K^+, an increase in K^+ permeability of the barrier should cause a decrease in PD across it. Thus, this change in cation selectivity of the outer barrier may contribute significantly to the decrease in overall PD caused by Ag^+. The present data do not permit us to make quantitative estimates of relative Na^+ and K^+ permeabilities of the outer barrier following treatment with Ag^+. They do, however, suggest that further studies of this type could provide some insight into factors contributing to the cation selectivity of this barrier.

A puzzling observation in this study was the finding of a spontaneous recovery of the skin from treatment with Ag^+, an effect observed to a greater or lesser degree in all properties measured. In an effort to obtain some insight into this phenomenon, uptake of Ag^+ by the skin from the outside solution was measured directly using $^{110}Ag^+$. Fig. 7 shows the uptake of $^{110}Ag^+$ as a function of time from a solution containing 10^{-4} M Ag^+. The amount of Ag^+ in the skin appears to be approaching saturation after 30 min. During this period, no $^{110}Ag^+$ appeared in the inside solution. The $^{110}Ag^+$ taken up cannot be easily washed off the skin since there was virtually no decrease in cpm in the skin during a 12-min exposure to solution containing no Ag^+. In four such experiments, the total amount of Ag^+ taken up in 30 min averaged 0.17 μmole/cm^2. Apparently only a small fraction of the total Ag^+ taken up is involved in the observed effects because changes in PD occur within 1–2 min after exposing the outer surface to Ag^+. As shown in Fig. 7, the Ag^+ taken up during a brief (3 min) exposure is also tightly bound.

These observations might suggest a possible explanation for the tendency of the Ag^+ effect to reverse spontaneously. Assume that Ag^+ is initially bound to relatively external sites causing the observed permeability change and then is slowly transferred to deeper sites in the epithelium. Since the total uptake of Ag^+ is sufficient to cause a substantial reduction in external Ag^+ concentration, under our usual conditions for flux measurement it may become impossible to keep the external sites saturated so that some recovery occurs. Such an effect could also be enhanced by release from the skin of substances that bind Ag^+ in the external solution. A slow recovery of the increase in K^+ permeability of red cells caused by *p*-chloromercuriphenyl sulfonate has been ascribed to such an effect[10]. This explanation appears untenable, however, in view of results obtained in experiments studying K^+ washout from the skin. In these experiments, new outside solution containing 10^{-4} M Ag^+ was introduced into the chamber every 5 min and in each experiment some recovery of the PD was noted 20–40 min after beginning treatment with Ag^+. We therefore have no explanation at present for the spontaneous partial reversibility of the Ag^+ effect. Perhaps the initial reaction with Ag^+ causes structural changes that reduce the susceptibility of certain groups to react further with external Ag^+.

These effects of Ag^+ appear to be quite different from those of other heavy metal ions that have been tested on frog skin. Martinez-Palomo *et al.*[11] found that La^{3+} added to the outside solution caused an increase in PD and $I_{s.c.}$. La^{3+} did not cross the skin and La^{3+} uptake by the skin was relatively similar to the Ag^+ uptake shown in Fig. 7. In contrast, however, most of the La^{3+} taken up was released when the skin was incubated in solution containing no La^{3+}. We have noted effects of

UO_2^{2+} on PD and $I_{s.c.}$ similar to those reported for La^{3+} by Martinez-Palomo *et al.*[11] but have not studied them in any detail. Cu^{2+} appears to reduce passive permeability of the skin since it causes an increase in PD with little change in $I_{s.c.}$; a decrease in Cl^- permeability was demonstrated[9,12]. However, a recent report by Ferreira[13] indicates that Cu^{2+} added to the outside solution causes an increase in both PD and current together with a significant increase in Cl^- permeability in a different species of frog. On the other hand, Clarkson[3] has reported effects of Hg^{2+} on rat intestine that are similar in certain respects to those shown here. For example, 1 mM Hg^{2+} in the mucosal solution caused a large transient loss of tissue K^+ and a delayed inhibition of active Na^+ transport. Heavy metals are also known to cause increases in cation permeability of red blood cells, but both Na^+ and K^+ permeability are altered[2,14]. Sulfydryl reagents such as *p*-chloromercuriphenyl sulfonate have similar effects[10]. It is also of interest to note the results of Nielsen[15] indicating that amphotericin B causes a marked increase in K^+ permeability of the "outer barrier" of frog skin. In fact several aspects of the effect of amphotericin on the skin seem somewhat similar to that of Ag^+.

The mechanism by which Ag^+ causes changes in permeability is also unknown. The most likely sites of reaction with Ag^+ are sulfydryl groups but other possibilities such as the imidazole moiety of histidine cannot be excluded. We have at present no information on the nature of the changes in the membranes resulting from reaction with Ag^+. It does appear however that two different barriers are involved in the response. There appears to be an increase in permeability of a shunt pathway that permits passage of a variety of solutes across the skin. In addition, the data suggest an effect on the cation selectivity of the outer barrier of the skin. This barrier may be in parallel with the less specific shunt pathway but detailed evidence on this point is lacking. Janacek[16] has reported that relatively low concentrations of *N*-ethylmaleimide and *p*-chloromercuribenzoate cause a rise in PD while higher concentrations of these agents cause a rapid fall in PD, presumably similar to that observed here with Ag^+. Clearly it would be of interest to examine effects on the skin of other sulfydryl reactive agents and to explore further the apparent changes in selectivity and shunt permeability.

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