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STIMULATION OF TRANSEPITHELIAL SODIUM AND CHLORIDE TRANSPORT BY ASCORBIC ACID

INDUCTION OF Na^+ CHANNELS IS INHIBITED BY AMILORIDE

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Ascorbic acid increases the short circuit current (I_{sc}) across the amphibian cornea when it is present at either surface of this epithelium. These effects were additive. The effect was greater when it was on the tear side. The response returned to baseline levels when the ascorbic acid was washed from the bathing media. The effect of ascorbic acid on I_{sc} when it was on the aqueous humor side of the cornea could be blocked by bumetanide but that due to the vitamin's presence on the tear side was unchanged. The ascorbic acid could enter the tissue and crossed the cornea at similar rates in either direction. When the cornea was bathed by a Cl^--free solution or exposed to bumetanide, the rise in I_{sc} observed with ascorbic acid on the tear side was equivalent to an increased Na^+ flux from the tear to the aqueous humor side. In normal (Cl^- present) Conway solution the rise in the I_{sc} seen with ascorbic acid on the aqueous humor side was equal to an increased flux of Cl^- from the aqueous to the tear surface. However, when ascorbic acid was present on the opposite, tear, side the increased I_{sc} reflected a rise in both Cl^- and Na^+ transport, aqueous-to-tear side, and tear-to-aqueous side, respectively. Thiol reagents (tear side), including reduced glutathione (10^{-5} M), blocked the effect of ascorbic acid (10^{-3} M) providing they were added to the bathing solution prior to the vitamin. However, they had no effect once the response had been established. The effect of the reduced glutathione appeared to be of a non-competitive nature. Oxidized glutatione (10^{-4} M) (and cystamine) blocked the effect of ascorbic acid (10^{-3} M) when present on the tear side prior to the vitamin. However, they also increased the rate of decline of the response when added subsequently to the ascorbic acid. Amiloride (as low as $5 \cdot 10^{-9}$ M), on the tear side but not the aqueous humor side, prevented the response to ascorbic acid but could not reverse it, once it was established. The possible nature of the effect of ascorbic acid is discussed in relation to its pharmacological interactions with thiol and disulfide reagents and amiloride.

Introduction

Chloride ion is actively transported across the amphibian cornea, in vitro, from solutions bathing the aqueous humor side to the tear side [1]. There is in addition an active transport of sodium in the opposite direction, which in the toad's cornea contributes about 20% of the observed short-circuit current (I_{sc}) [2]. Ascorbic acid stimulates the I_{sc}

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and Cl^- transport across the toad's cornea [3,4]. This effect is of special interest as this vitamin is accumulated in the ocular fluids, in which the concentration may be as high as 1 mM [5,6].

While studying the interactions of ascorbic acid and bumetanide (which inhibits Cl^- transport in the cornea [7]), we were surprised to observe that when ascorbic acid was on the tear side of the cornea the diuretic did not inhibit its effect. Ascorbic acid even stimulated the I_{sc} when the cornea was bathed with Cl^--free solutions. This increment in the I_{sc} was found to reflect a stimulation of active Na^+ transport and the response could be inhibited by amiloride.

Materials and Methods

Toads (*Bufo marinus*) were obtained from the Dominican Republic and kept in the laboratory at 20°C.

The corneas were prepared in vitro by mounting them in an Ussing-type chamber, the cross sectional surface area of the corneal membrane being 0.5 cm^2. The aqueous humor side (endothelial or stromal side) was bathed with 6 ml of an amphibian Conway solution while the tear (or epithelial) side was bathed with 5 ml. The difference in volume results in a hydrostatic pressure of about 2 cm of water, which is routinely applied to maintain the normal curvature of the cornea. The amphibian Conway solution had the following composition (mM): PO_4^-, 2.9; Na^+, 104; K^+, 2.5; Mg^{2+}, 1.2; SO_4^{2-}, 1.0; HCO_3^-, 25; gluconate, 1.0; Cl^-, 74.5 and glucose, 20. The Cl^--free Conway had Na_2SO_4 and K_2SO_4 substituted for the Cl^- salts with sucrose added to maintain the osmotic concentration. The solutions were aerated with room air and the pH was about 8.4. The experiments were performed at 21 ± 1°C. The electrical potential difference (p.d.) and short-circuit current (I_{sc}) were measured with an automatic voltage clamp and potentiometric recorder connected the each side of the cornea by Ag-AgCl cells (for short-circuit current) and calomel cells (for p.d.) [8]. Unidirectional fluxes of Na^+, Cl^- or ascorbic acid were measured using the isotopes $^{22}Na^+$, $^{36}Cl^-$ or [^{14}C]ascorbic acid (about 0.2 μCi/ml) added to the solution bathing one side of the membrane preparation. Samples were taken at 20 min intervals from the opposite side. The $^{22}Na^+$ was measured in a gamma counter (Beckman, Biogamma 2) and the $^{36}Cl^-$ and ^{14}C in a scintillation counter (Beckman, LS-9000). The I_{sc} was allowed to stabilize before adding the isotope (about 1 h) and the samples were collected until a stable movement of isotope was observed. Usually four such periods were measured followed by another four periods after adding a drug. The individual results for each membrane preparation are the means for the three periods before adding the drug and the three subsequent periods.

The following drugs were gifts: bumetanide from Hoffman-La Roche Inc., Nutley, NJ; and amiloride from the Merck Institute for Therapeutic Research, West Point, PA. Dehydroascorbic acid was obtained from Pfaltz and Bauer, Stamford, CT. The ascorbic acid (reduced form), oxidized and reduced forms of glutathione, cystamine and cysteine, were from Sigma Chemicals, St. Louis, MO. The $^{22}Na^+$ and [^{14}C]ascorbic acid were obtained from New England Nuclear, Boston, MA, and $^{36}Cl^-$ from ICN, Irvine, CA.

Results

Effects of ascorbic acid on I_{sc} across the amphibian cornea

Ascorbic acid in its reduced form can stimulate the I_{sc} (as well as the transepithelial p.d.) when it is present in the solution bathing either side of the toad's cornea. The response is, however, much

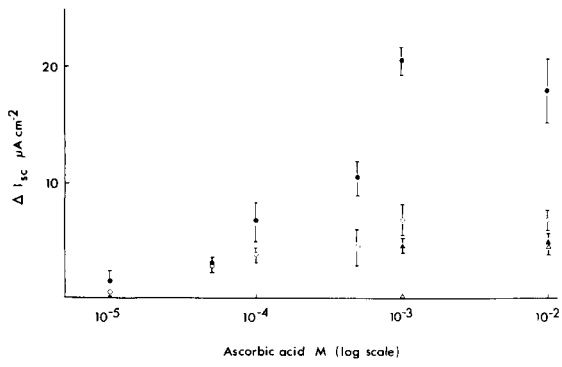


Fig. 1. Effects of ascorbic acid on I_{sc} across the amphibian cornea. Ascorbic acid on the tear (●) and aqueous humor (○) sides. Effects of reduced glutathione (GSH), 10^{-5} M, on the response to ascorbic acid on the tear (▲) and aqueous humor (△) sides. Each point is the mean ±S.E. for six corneas.

TABLE I

THE ADDITIVE EFFECTS OF ASCORBIC ACID (10^{-3} M) ON THE I_{sc} ACROSS THE AMPHIBIAN CORNEA, WHEN ADDED, IN TURN, TO EACH SIDE OF THE MEMBRANE PREPARATION

The responses represent the maximal increases. The results are as means and mean differences ±S.E. for six corneas.

I_{sc} ($\mu A \cdot cm^{-2}$)				
I (Initial)	II (Peak response) plus ascorbic acid	II−I	III (Peak response) plus ascorbic acid	III−II
	on tear side		on aqueous side	
8.3	32.4	24 ±2.0	36.1	3.7±0.8
	on aqueous side		on tear side	
7.7	12.0	4.3±0.5	35.6	23.6±1.2

greater when it is present in the solution bathing the outer, tear, as compared to the inner, aqueous humor, side of the membrane (Fig. 1). The effect was greatest when the concentration of ascorbic acid was about 1 mM on either surface. Concentrations as low as $5 \cdot 10^{-5}$ increased the I_{sc} from either side. The effects from either side were additive (Table I).

The responses to ascorbic acid, at 10^{-3} M, commenced about 1 min after adding the vitamin to the bathing media and attained a maximum increase after about 45 min. If the ascorbic acid was then washed from the solution at this latter time the response rapidly declined to the baseline level. If it was left in the media the I_{sc} slowly declined and reached this value about 4 h later. Repetition of the stimulus after washing the first dose of ascorbic acid from the media resulted in a response which was reduced by about 50%.

Effects on bumetanide on the response of the cornea to ascorbic acid

Bumetanide inhibits the Cl^--dependent I_{sc} across the amphibian cornea [7]. When ascorbic acid was present in the solution bathing the inner, aqueous humor, side of the membrane no increase in I_{sc} was observed in the presence of bumetanide; inhibition was complete. However, if ascorbic acid was present on the outer, tear, side bumetanide failed to block to effect, suggesting that another

TABLE II

EFFECTS OF ASCORBIC ACID (10^{-3} M ON THE TEAR SIDE) ON THE UNIDIRECTIONAL FLUX OF $^{22}Na^+$ ACROSS THE AMPHIBIAN CORNEA IN VITRO, IN THE PRESENCE OF BUMETANIDE AND Cl^--FREE CONWAY BATHING MEDIA

For details of the experiments see Methods. The values are as mean and mean differences ±S.E. for six corneas. *P* for mean differences **<0.01, ***<0.001.

	I	II plus ascorbic acid	II−I
a. Bumetanide, 10^{-5} M both sides in I&II			
I_{sc} ($\mu A \cdot cm^{-2}$)	3.1	18.0	14.9±1.8**
$^{22}Na^+$, tear→aqueous side ($\mu A \cdot cm^{-2}$)	9.4	22.8	13.1±2.1***
b. Cl^--free Conway soln, both sides in I&II			
I_{sc} ($\mu A \cdot cm^{-2}$)	2.8	20.6	17.8±3.4**
$^{22}Na^+$, tear→aqueous side ($\mu A \cdot cm^{-2}$)	6.4	20.9	14.5±2.4**

type of ion transport, apart from Cl^-, may be occurring (Table IIa). When corneas were bathed in solutions with no chloride present the response to the ascorbic acid, on the tear side, was also observed to persist (Table IIb). Under these conditions the increase in the I_{sc} could be accounted for by an increased flux of Na^+ from the tear to the aqueous humor side of the membrane (Table IIa, b).

Effects of ascorbic acid on Na^+ and Cl^- transport across the cornea

In order to further elucidate the nature of the ion transport stimulated by ascorbic acid we measured the unidirectional fluxes of $^{22}Na^+$ and $^{36}Cl^-$ across the cornea when it was bathed by normal Conway solution, and exposed to ascorbic acid from either side of the membrane (Table III). With ascorbic acid present on the aqueous humor side the increment in I_{sc} could be completely accounted for by an increase in the flux of Cl^- from the aqueous humor to the tear side of the cornea. However, when present on the tear side of ascorbic acid increased both the flux of Cl^-, from aqueous humor to tear side, and that of Na^+, in the opposite direction. The sum of the changes in the fluxes of Na^+ and Cl^- were found to be equivalent to the increase in I_{sc}.

Fluxes of ascorbic acid across the cornea

Ascorbic acid is known to be present in high concentrations in the aqueous humor but there appears to be no information about its access into the epithelial cells or to the tear film. The unidirectional fluxes of ascorbic acid across the cornea in either direction, were therefore measured. The vitamin was able to cross the cornea, at similar rates in either direction.

The unidirectional fluxes of ascorbic acid (nmol $\cdot cm^{-2} \cdot h^{-1}$ in six corneas), with a concentration of 1 mM in the solutions, were 3.06 ± 0.35 (from the aqueous humor to the tear side) and 2.56 ± 0.62 in the opposite direction. It appears that ascorbic acid can enter the corneal tissue from either side and it does not accumulate in the cornea, as tissue: medium ratios greater than 1 were not observed.

Effects of thiol and disulfide reagents on the response to ascorbic acid

We investigated the nature of the effect of ascorbic acid on Cl^- and Na^+ transport across the cornea by examining the responses in the presence of some thiol and disulfide reagents (Table IV).

The oxidized form of ascorbic acid, dehydroascorbic acid, (10^{-3} M on either side of the cornea) had only a small effect on the I_{sc} (Table IV)

TABLE III

EFFECTS OF ASCORBIC ACID (10^{-3} M) ON I_{sc} AND UNIDIRECTIONAL FLUXES OF $^{36}Cl^-$ AND $^{22}Na^+$ ACROSS THE AMPHIBIAN CORNEA IN VITRO

For details of the experiments see Methods. Results are as means and mean differences ±S.E. for six corneas. *P* for mean differences *<0.05, **<0.01, ***<0.001.

	I	II (plus ascorbic acid)	II−I
Ascorbic acid on aqueous side in II			
$^{36}Cl^-$, aqueous→tear side ($\mu A \cdot cm^{-2}$)	17.4	22.2	4.8±0.8*
I_{sc} ($\mu A \cdot cm^{-2}$)	10.1	15.0	4.9±0.8**
^{22}Na, tear→aqueous side ($\mu A \cdot cm^{-2}$)	11.8	13.1	1.3±0.8
I_{sc} ($\mu A \cdot cm^{-2}$)	12.2	16.9	4.7±0.06***
Ascorbic acid on tear side in II			
$^{36}Cl^-$, aqueous→tear side ($\mu A \cdot cm^{-2}$)	16.3	21.2	4.9±1.6*
I_{sc} ($\mu A \cdot cm^{-2}$)	8.8	20.7	11.9±1.5***
$^{22}Na^+$, tear→aqueous side ($\mu A \cdot cm^{-2}$)	7.0	13.1	6.1±1.6*
I_{sc} ($\mu A \cdot cm^{-2}$)	8.5	19.6	11.1±1.9**

TABLE IV

EFFECTS OF DEHYDROASCORBIC ACID AND VARIOUS THIOL AND DISULFIDE REAGENTS ON THE RESPONSE (INCREASE IN I_{sc}) TO ASCORBIC ACID

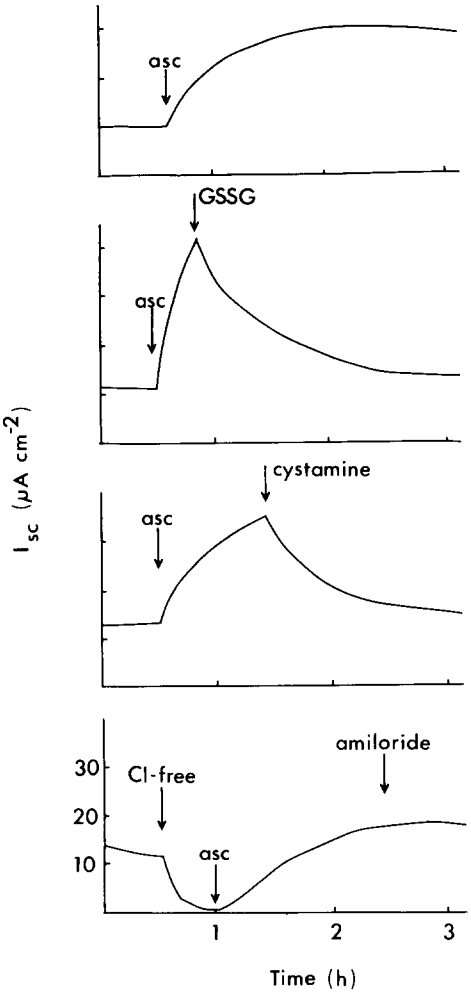
Results are as means and mean differences ±S.E. for six corneas. The preparations were exposed to the dehydroascorbic acid for 30 min and the other reagent for 2 min (period II) before adding the ascorbic acid in III. *P* for mean differences *<0.05, **<0.01 and ***<0.001.

	I_{sc} ($\mu A \cdot cm^{-2}$)				
	I	II (plus reagent)	II−I	III (plus ascorbic) acid (10^{-3} M)	III−II
a. Control preparations (see Fig. 1)					
Tear side	–	10.8	–	31.3	20.5±1.2***
Aqueous side	–	11.5	–	18.3	6.8±1.4
b. Dehydroascorbic acid (10^{-3} M)					
Aqueous side	9.4	11.4	2.0±0.5**	13.8	2.4±0.7*
Tear side	6.6	8.5	1.9±0.5**	15.3	7.0±2.1*
c. Reduced glutathione (10^{-5} M)					
Aqueous side	9.5	9.5	0.0	14.0	4.5±0.9*
Tear side	11.0	11.0	0.0	12.3	1.3±1.1
d. Cysteine (10^{-4} M)					
Tear side	9.4	10.0	0.6±0.2	10.2	0.2±0.2
e. Oxidized glutathione (10^{-4} M)					
Tear side	8.3	8.5	0.2±0.3	8.5	0.0
f. Cystamine (10^{-4} M)					
Tear side	8.3	8.3	0.0	8.3	0.0

suggesting that the reducing action of ascorbic acid could be involved or its effect could be indirect due to accumulated hydrogen peroxide. (Oxidation of ascorbic acid results in the formation of H_2O_2 [9].) Dehydroascorbic acid also decreased the response of the cornea to its reduced form.

Reduced glutathione (GSH) (10^{-5} M), as well as cysteine (10^{-4} M), when placed in the bathing media on the tear side (but not aqueous side) prior to ascorbic acid (10^{-3} M) completely blocked the latter's action (Table IV, c and d). They had no effect on the basal level of the I_{sc} (exposure for 30 min). These thiol reagents could be acting directly on the tissue or be blocking the formation of H_2O_2. In the latter respect, however, it was observed that GSH had no effect when placed in the media at the time when the response had reached its peak, which is in contrast to the effect of washing the ascorbic acid from the bathing medium. Hence it is unlikely that an indirect effect due to changes in the formation of H_2O_2 can account for the interaction of GSH and ascorbic acid. Supramaximally effective doses of ascorbic acid were unable to overcome the effects of 10^{-5} M GSH (tear side) suggesting that the inhibition is of a non-competitive nature (Fig. 1).

Disulfide reagents, oxidized glutathione (GSSG) and cystamine, 10^{-4} M (on the tear side), also inhibited the effects of ascorbic acid (10^{-3} M) (Table IV, e and f). It is possible that this effect is due to a conversion of the oxidized form of the compound to its reduced form; for instance GSSG to GSH, which is more active. Neither GSSG nor cystamine had an effect on the basal I_{sc}. When placed in the media (tear side) at the time of the peak response to ascorbic acid (also on the tear side) they initiated an increase in the rate of normal decline to the vitamin's effect (Fig. 2). This observation is in contrast to the lack of effect of the thiol reagents.



Effects of amiloride on the response to ascorbic acid

Amiloride blocks Na^+ channels in many epithelia [10] but it did not alter the basal I_{sc} across the amphibian cornea (Table V). However, when this diuretic drug was placed in the solution bathing the tear side of the cornea it reduced the subsequent response to ascorbic acid. It had no effect when present on the aqueous humor side. Amiloride (on the tear side) completely blocked the effects of ascorbic acid (also on the tear side) when the cornea was bathed with a Cl^--free Conway solution. Extremely low concentrations of amiloride were effective; $5 \cdot 10^{-9}$ M reduced the response by about 50%. Amiloride did not antagonize the effect of ascorbic acid if placed in the bathing media after the response had been established (Fig. 2). It was only effective when present in the solution prior to the addition of the ascorbic acid.

Fig. 2. Effects of oxidized glutathione (10^{-4} M), cystamine (10^{-4} M) and amiloride (10^{-5} M) on ascorbic acid-stimulated I_{sc} across the amphibian cornea. The experiment using the amiloride was performed in Cl^--free Conway solution. Each curve is an individual representative from a group of at least five such experiments.

TABLE V

EFFECTS OF AMILORIDE (10^{-5} M) ON I_{sc} ACROSS THE AMPHIBIAN CORNEA (IN VITRO) IN THE ABSENCE AND PRESENCE OF ASCORBIC ACID (10^{-3} M)

Results are as means and mean differences ±S.E. for six corneas. *P* for differences; *<0.05, **<0.01, ***<0.001. Amiloride was added after I and remained in contact with the tissue for 20 min when reading II was taken. III is the maximal response after adding ascorbic acid.

	I_{sc} (μA·cm^{-2})				
	I (basal value)	II (plus amiloride)	II−I	III (plus ascorbic acid)	III−II
Amiloride and ascorbic acid on tear side	9.4	9.4	0.0	15.0	5.6±0.07***
Control, (no amiloride in II)	10.1	10.1	0.0	27.8	17.7±1.3***
Amiloride and ascorbic acid on aqueous side	6.8	6.8	0.0	13.2	6.4±0.05***
Control, (no amiloride in II)	9.3	9.5	0.2±0.2	16.7	7.0±0.9***
In Cl^--free Conway solution (added in II)					
Amiloride and ascorbic acid on tear side	10.8	1.6	9.2±1.6	4.3	2.7±0.6*
Control, (no amiloride in II)	11.0	2.2	8.8±0.9	13.0	10.8±1.5***

Discussion

Ascorbic acid not only increases active Cl^- transport across the amphibian cornea, in vitro, but also active Na^+ transport. The latter effect is, however, only seen when it is present on the tear side of the tissue. Under these conditions it stimulates both Cl^- and Na^+ transport. However, the effect of Na^+ is independent of that on Cl^-, as when the anion is not present in the incubation medium or if its transport is blocked by bumetanide the response persists.

The nature of the effects of ascorbic acid on ion transport are unknown. It has been suggested that the increase in active Cl^- transport results from a partial inactivation of tissue phosphodiesterase [4]. Others, however, favor the possibility that it may act more generally and influence tissue metabolism by functioning as a proton donor for cytochromes [3]. It is possible that the latter type of role may also explain the vitamin's effect on Na^+ transport. However, a common intracellular site of action affecting transport of both ions appears to be unlikely as ascorbic acid can only influence Na^+ transport when it is present on the tear side of the membrane, though it increases Cl^- transport from either side. It is thus possible that a specific site of action on the cell membrane, such as involving the formation of Na^+ channels on the tear surface, is involved. This possibility is supported by the observation that this response to ascorbic acid is blocked in the presence of amiloride on this, but not the opposite side of the cornea. The effects of ascorbic acid on each side of the cornea were additive, suggesting that two distinct sites mediating the increase of Cl^- transport are also present.

The response to ascorbic acid on the tear side can be blocked by relatively low concentrations of thiol and disulfide reagents, also on the tear side, suggesting that such reactive groups may be involved in responses that influence both Na^+ and Cl^-. Ascorbic acid can reduce disulfide bonds to sulfhydryl groups and it is possible that such a reaction is mediating its effects on ion transport. This possibility would be consistent with the observed inhibition of the response to ascorbic acid by exogenous disulfide reagents, which could be mending such broken disulfide bridges. Such substances may also, however, be converted by ascorbic acid to their more active reduced forms. Thiols may also be interacting with such sites on the cell so as to exclude the action of the ascorbic acid. They may be acting like non-competitive antagonists. The failure of GSH, in contrast to GSSG, to reverse the response to ascorbic acid once it has been established is intriguing, and difficult to explain. As the response to ascorbic acid is readily reversible, when it is washed from the bathing solutions, it seems unlikely that it is binding covalently to its active site. Possibly its presence there is maintained even in the presence of added GSH, by a preferential self-exchange process.

The effect of amiloride, which is a classical blocker of Na^+ channels in epithelia [11] did not influence the normal basal I_{sc} across the cornea, even though a part of this current reflects transepithelial Na^+ transport. However, this diuretic drug is effective in the presence of ascorbic acid, suggesting that the vitamin may be activating or forming Na^+ channels in the corneal epithelium. These Na^+ channels, however, have some unique features as they apparently cannot be blocked by amiloride once they have been 'opened' by the as corbic acid (this effect of amiloride is also like that of GSH). In other amphibian epithelia Na^+ channels, including those which can be induced or activated by aldosterone and vasopressin, can be blocked by subsequently exposing them to amiloride. The present results suggest that amiloride may be able to bind to inactive Na^+ channels and 'paralyze' them but once opened by ascorbic acid an effective interaction with the diuretic no longer occurs. Possibly amiloride and ascorbic acid have the same or neighbouring bindings sites from which they can block the effect of the other substance.

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