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Mode of Action of Furosemide on the Chloride-Dependent Short-Circuit Current across the Ciliary Body Epithelium of Toad Eyes

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Summary. The effects of furosemide on the chloride-dependent short-circuit current across the toad ciliary epithelium were examined. Under control conditions, the short-circuit current obeyed Michaelis-Menten kinetics against medium chloride concentration, the Michaelis constant (K_m) for chloride being 90 mM and the maximal short-circuit current (V_{max}) 128 μA/cm^2. Furosemide added to the aqueous side of the epithelium rapidly reduced the short-circuit current; the effect was reversible. The effect of furosemide addition to the stromal side was much smaller and slower than that from the aqueous side. The dose-dependent range of furosemide action was from 0.1 μM to 1 mM with 50% inhibition occurring at about 3 μM. Lineweaver-Burk plot of the short-circuit current against the chloride concentration showed that furosemide decreased the value of V_{max} and increased the K_m; the inhibition being of mixed type. A Hill plot of the dose-response curve yielding a slope of unity suggested one furosemide molecule combines with one chloride transport site. Probenecid, a competitive inhibitor of organic acid transport, reduced the effects of furosemide significantly when added simultaneously. The involvement of organic acid transport system in the mechanism of furosemide action on chloride transport was suggested.

The potent diuretic effect of furosemide was first ascribed to its inhibitory action on cation transport and cellular metabolism (*cf.* Burg, 1976; Brazy & Gunn, 1976). Subsequently, the specific inhibitory action of furosemide on the chloride transport mechanism has been clearly shown in a variety of tissues by demonstrating its action at the lower concentration ranges than that required for the inhibition of active cation transport and cellular metabolism. Such an action has been shown in active chloride transport in the diluting segment of rabbit renal tubules (Burg et al., 1973) and frog cornea (Candia, 1973), exchange transport of bicarbonate and chloride in dog pancreatic ducts (Guelrud, Rudick & Janowitz, 1972), and exchange transport of anions in red blood cells (Deuticke & Gerlach, 1967; Brazy & Gunn, 1976). Despite clear and specific action of the drug, the mechanism by which the drug inhibits chloride transport is still poorly understood, particularly in epithelial cells.

Previously, we have reported that toad ciliary epithelium actively transports chloride from the stromal to the aqueous side, while the net transport of sodium in the same direction was not significant. There was a significant correlation between short-circuit current and chloride transport from the stromal to the aqueous side. Many of the characteristics of active chloride transport mechanism in toad ciliary epithelium so far demonstrated (Watanabe & Saito, 1978; Saito & Watanabe, 1979) were common with active anion transport mechanisms in other tissues. In particular, the frog cornea (Zadunaisky, 1966, 1972), the frog choroid plexus (Wright, 1974), the teleost intestine (Field, 1977), and the cat ciliary epithelium (Holland, 1970; Holland & Gipson, 1970) were found to exhibit sodium (and potassium) dependency of active anion transport similar to that seen in the toad ciliary epithelium. Therefore, we think that studies in the toad ciliary body can provide some general understandings of furosemide action on active chloride transport mechanisms.

The present study was undertaken to obtain further information about the mode of action of furosemide and active chloride transport mechanism in the epithelial cells. We analyzed kinetically the effect of the drug on chloride-dependent short-circuit current across the toad ciliary epithelium.

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Materials and Methods

The materials and the methods employed in the present study have been described in detail in our previous paper (Watanabe & Saito, 1978). Briefly, toads *(Bufo vulgaris)* were anesthetized with urethane, and eye-balls were enucleated. After the equatorial incision, the lens, the cornea, and the sclera were removed, and the iris-ciliary body complex was carefully isolated. The tissue was mounted between Lucite flux chambers with a small window, the area being 3.1 mm^2. The standard incubation solution, oxygenated by bubbling with humidified air, was introduced into both sides of the epithelium. The potential difference (PD) across the epithelium was recorded on a pen recorder (TOA EPR-3T). Two sets of agar-KCl bridges, one for PD recording placed 1 mm apart from the surfaces of the tissue, and the other for external current application placed at the both ends of the incubation solutions, were connected to the recorder and DC pulse generator via calomel half cells, respectively. Input resistance of the recorder was 2 MΩ. The DC resistance, consisting of calomel half cells, agar bridges, incubation fluids, and epithelial tissue was about 40 kΩ; the latter two accounted for less than 6 kΩ. Thus various experimental conditions employed in the present study did not affect the accuracy of the recordings. Short-circuit current and transepithelial electrical resistance (*Rt*) were derived from the spontaneous transepithelial PD and the voltage deflections generated by intermittent passages of current pulses across the epithelium. Usually pulses at a current density of 1 μA per 3.1 mm^2 (the exposed epithelial area) and a duration of 10 sec were used. At the duration and current density above described, the transient polarization of the membrane was negligible with respect to the rapid, ohmic response of the membrane (Bindslev, Tormey & Wright, 1974). The resistance of the incubation solution accounted for about 20 to 30% of the resistance between the electrode tips when the tissue was mounted. Therefore, corrections were made for the voltage drops due to the resistance of the incubation solutions between the tissue surfaces and the tip of the PD sensing electrodes.

The composition of standard incubation solution was as follows (in mM): NaCl, 100; $KHCO_3$, 2.6; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $CaSO_4$, 1.0; Tris-buffer, 10 (pH 7.4, adjusted by H_2SO_4); and D-glucose, 5.5. All chemicals used were of reagent grade.

About an hour after the start of the incubation, when the electrical parameters became steady, the effects of bathing chloride concentration and furosemide (Lasix, Japan-Hoechst Co.) were studied. Furosemide was added to one or both sides of the epithelium at various concentrations.

The chloride concentration of the incubation solution was changed in the presence or absence of furosemide on both sides of the epithelium by replacing chloride with sulfate equivalently. A desired amount of D-mannitol was added to low-chloride solutions in order to adjust the osmolarity to equal that of the control solution. A potent competitive inhibitor of organic acid transport, probenecid (Weiner, Washington & Mudge, 1960), was used to study the possible involvement of the organic acid transport mechanism in furosemide action. This drug was added to the aqueous side of the epithelium at 1 mM.

The experiments were performed throughout all seasons at room temperature or bath temperature of 20–23 °C. The data obtained from most experiments were given as the mean ± SEM. The data from some series of experiments were expressed as relative values with respect to control values when the variations of the magnitude of control value of short-circuit current were very large among preparations. Also, in experiments which took several hours, the order of experiments was randomized in order to avoid the statistical errors due to the time-dependent decrease in short-circuit current.

Results

Effects of Chloride Concentration on Short-circuit Current

Figure 1 *a* and *b* show the relationship between medium chloride concentration and the magnitude of the short-circuit current. When medium chloride was completely replaced with sulfate, about 9.3 ± 1.5% of the control short-circuit current (75.8 ± 5.7 μA/cm^2, $n=12$) in the standard medium remained. Therefore, in order to elucidate the characteristics of chloride transport, chloride-dependent short-circuit current was derived by subtracting the short-circuit current in the sulfate-medium from those measured in the chloride-containing media.

The short-circuit current increased in a saturable way when medium chloride concentration was in-

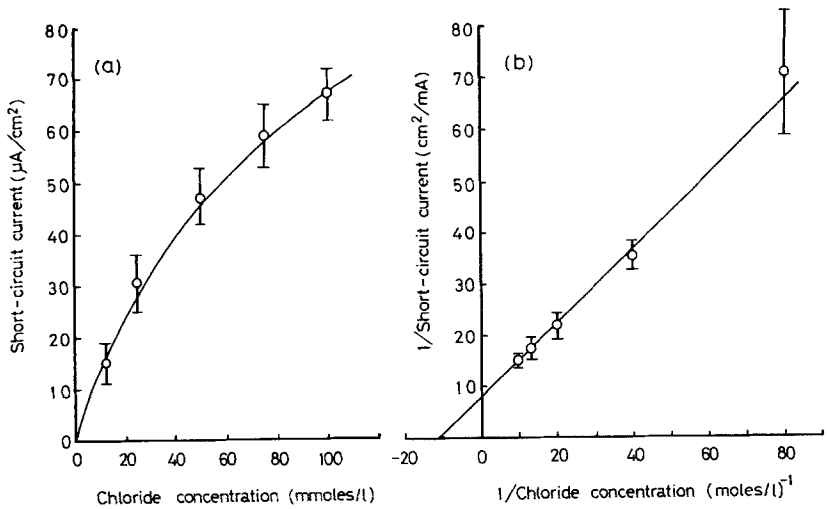

Fig. 1. Relationship between chloride-dependent short-circuit current and bathing chloride concentration (*a*) and Lineweaver-Burk plot (*b*) where the line was drawn by eye. The curve in *a* was drawn from a Michaelis-Menten equation: short-circuit current $= V_{max} \cdot [Cl]/(K_m + [Cl])$, where $V_{max} = 128$ μA/cm^2 and $K_m = 90$ mM; $n=6$

creased. However, the curve did not reach a plateau even at 100 mM chloride (Fig. 1*a*). A further increase in chloride concentration made the medium hypertonic and usually caused a progressive decrease in the PD, short-circuit current, and *Rt* (data not shown here). At 150 mM NaCl concentration, the PD and short-circuit current became negligibly small. When the tissue was returned in the standard solution, the recovery of these electrical parameters was incomplete, though the *Rt* recovered almost fully. Hypertonic solution made by an addition of D-mannitol to the standard solution caused similar changes in electrical parameters to that caused by hypertonic NaCl solutions. On the other hand, hypotonic solution made by decreasing the NaCl concentration caused an increase in these electrical parameters. This was in contrast to the results in Fig. 1*a* where the chloride concentration was decreased by substituting with sulfate and D-mannitol iso-osmotically. Since the effects of medium tonicity were significant and the underlying mechanism of the effect was unclear, following experiments were performed at iso-osmotic conditions and NaCl concentration range below 100 mM.

The Lineweaver-Burk plot of the chloride-dependent short-circuit current against chloride concentration was linear as shown in Fig. 1*b*, where the line was drawn by eye. From these data, the values of the maximal short-circuit current (V_{max}) and Michaelis constant (K_m) were estimated to be 128 μA/cm^2 and 90 mM, respectively. The curve shown in Fig. 1*a* was drawn using the kinetic parameters described above. These findings indicate that the chloride-dependent short-circuit current conforms to simple Michaelis-Menten kinetics.

Effects of Furosemide on Short-circuit Current and Electrical Resistance

Figure 2 shows the effect on the transepithelial PD of furosemide added at a concentration of 15 μM (Fig. 2*a*) or 300 μM (Fig. 2*b*) on the stromal or aqueous side. At the lower dose, addition of the drug to the aqueous side caused an immediate and marked decrease in the PD, which was followed by a slow and slight decrease in PD. In contrast, the effect from the stromal side was much slower and smaller than those caused by the addition to the aqueous side. The average short-circuit current (percent control) determined at 30 min after the addition either in the aqueous or the stromal side was 37.1±4.5% (n=7) or 84.3±3.8% (n=5), respectively (*see* Fig. 7). The *Rt* (control *Rt* was 59.7±8.6 Ω/cm^2, n=14) decreased slightly by furosemide dose-dependently to 95.8±1.7, 85.8±3.4, 84.0±4.4 and 78.8±4.9% control at the

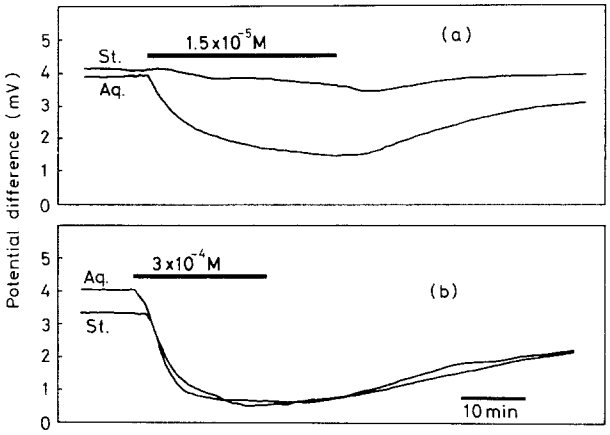

Fig. 2. The time-course of the furosemide action on PD. During the period indicated by horizontal bars, furosemide was added to the stromal (*St*) or aqueous (*Aq*) side. The PD was aqueous side negative. Retouched

furosemide concentrations of 3, 15, 60 and 300 μM, respectively. The addition of 3 μM furosemide to the stromal side caused little effect on PD. When the drug was washed out, the short-circuit current gradually recovered to nearly the same level as that of control (Figs. 2 and 7). However, the time-course of the recovery was considerably slower, often requiring more than 2 hr to reach the steady level. When the supramaximal dose of 0.3 mM was used, the effects from both stromal and aqueous sides were equally profound, and the time-course to reach the maximally inhibited levels was much faster in both cases (Fig. 2). The onset of the effects from the stromal side was, however, delayed a few minutes. Even after the exposure to the supramaximal dose, about 80% of the control short-circuit current was recovered. The recovery of *Rt* was usually incomplete, and sometimes *Rt* remained at a decreased level after washing out, especially when the furosemide dose was higher than 15 μM. When chloride was eliminated from the solution, the effects of furosemide were negligibly small compared to the doses used in the present study.

Figure 3 shows the relationship between short-circuit current and the dose of furosemide added to the both sides of the epithelium. The inhibitory effects were observed at the concentration as low as 0.3 μM. The half maximum inhibition was found at about 3 μM. However, even at the highest dose examined (0.47 mM), more than 10% of control short-circuit current remained uninhibited. Most of the remaining fraction may be the chloride-independent one (9.3±1.5% control), as shown in sulfate-substitution experiments. The midpoint slope of the sigmoid curve in Fig. 3 was 0.56, which was almost identical to the slope 0.575 theoretically predicted from the molecular drug/receptor combining ratio of unity (Goldstein,

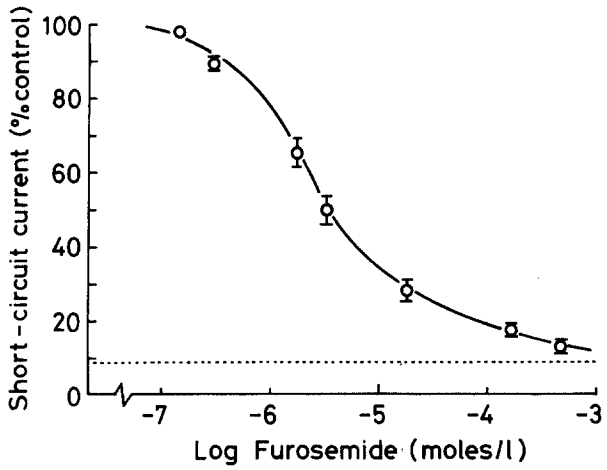

Fig. 3. Dose-response curve for furosemide inhibition of the short-circuit current not corrected for chloride-independent fraction. Relative value (% control) of short-circuit current was plotted against the logarithm of furosemide concentration added to both sides. The dotted line indicates the level of short-circuit current (9.3 ± 1.5% control) in chloride-free, sulfate solution. Data includes only those preparations which exhibited a control short-circuit current of greater than 40 $\mu A/cm^2$. (57.5 ± 10.0 $\mu A/cm^2$ on the average; $n=6$)

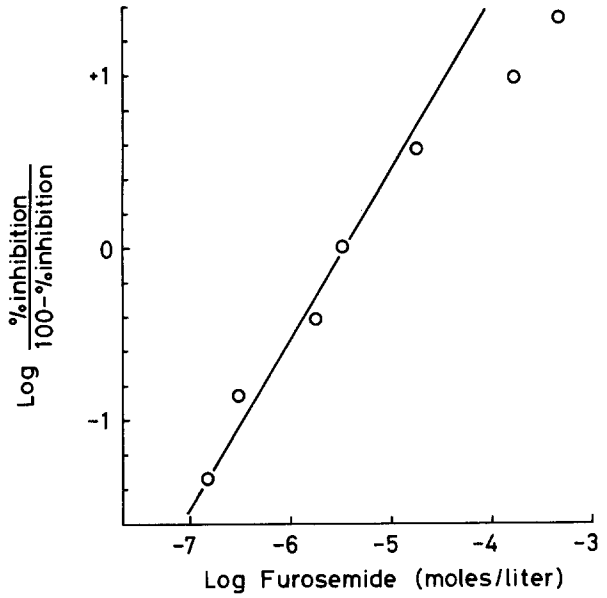

Fig. 4. Hill plot of the dose-response curve in Fig. 3. Logarithm of the ratio % inhibition/(100 − % inhibition) was plotted against the logarithm of furosemide concentration. Chloride-dependent short-circuit current under control conditions was regarded as 100%. Straight line in the figure shows the slope of 1.0 predicted from one for one reaction between furosemide molecule and chloride transport system. *Also see* legend for Fig. 4

Aronow & Kalman, 1974). A Hill plot of the dose-response curve for the chloride-dependent short-circuit current also gave a Hill coefficient of unity (Fig. 4), though a slight deviation from the regression line was seen at the higher doses. The dose-dependent

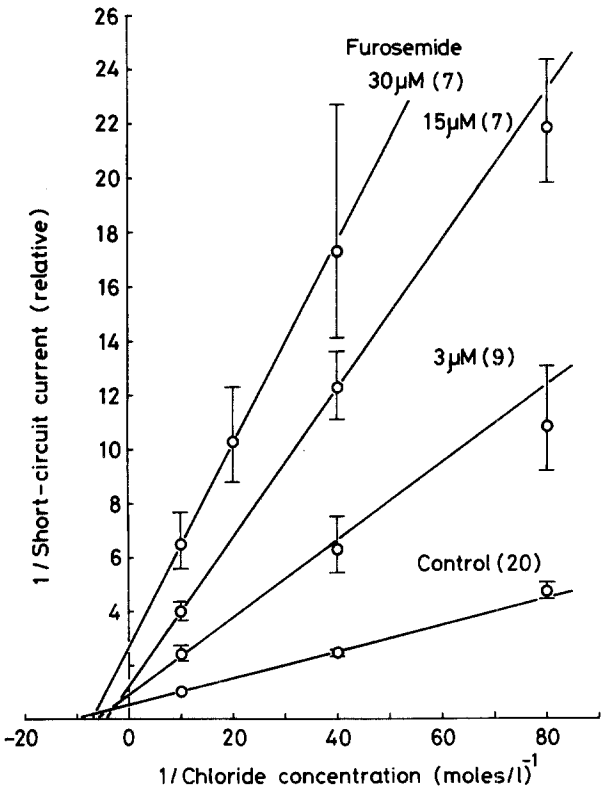

Fig. 5. Lineweaver-Burk plot of chloride dependent short-circuit current (relative to the control value) against chloride concentration under control conditions and in the presence of furosemide at 3, 15 and 30 μM. The straight lines were drawn based on the mean K_m and V_{max} values determined for each experimental condition. Numbers of observations are given in the parentheses

range extended over four orders of the dose, which was about two orders wider than that observed in human red blood cells (Brazy & Gunn, 1976). Among the preparations there was a tendency that the greater the control short-circuit current the greater the percent inhibition caused by a fixed dose of furosemide.

Mechanism of Furosemide Action

The quasi-steady state values of chloride-dependent short-circuit current were measured on a single preparation at various chloride concentrations, both in the absence and presence of 3 μM, 15 μM, or 30 μM furosemide in the bathing media. In this series of experiments, only three chloride concentrations (100, 50, 25 and/or 12.5 mM chloride) were examined in order to avoid time-dependent decreases in short-circuit current. The values obtained were plotted against the chloride concentration double reciprocally. As seen in Fig. 5, the plotted values for each set of experiments fell on a straight line. The regression lines for the control, and at the furosemide concentrations of

3 and 15 μM, intersected at an almost common point above the abscissa to the left of the ordinate, suggesting a mixed type of inhibition (Dixon & Webb, 1964). However, the regression line for the plots at 30 μM furosemide deviated slightly to the left of the above intersection. Table 1 shows the mean value ±SEM of K_m and V_{max} of each experimental condition and the statistical significance of the difference from those of control conditions. V_{max} decreased significantly ($P<0.05$) and progressively as the furosemide concentration was increased. K_m also increased up to 15 μM furosemide; however, the increase in K_m at 30 μM furosemide was smaller than that at 15 μM furosemide, and the difference from the control K_m was statistically insignificant ($0.1>P>0.05$). Therefore, though the intersection of the regression lines in Fig. 5 suggests the kinetic pattern as a mixed type inhibition, statistical significance of the changes in K_m could not confirm the type to be a mixed type except for the data obtained at 15 μM furosemide.

The inhibitor constant K_i for furosemide can be derived from the dose-response curves in Fig. 3 and Fig. 5 following the method of Dixon (Dixon & Webb, 1964). Using the same data in Fig. 5, the reciprocal of short-circuit current was replotted against the furosemide concentration (Fig. 6). The intersections of the lines on the horizontal axis of Fig. 6 converged almost at the same point, giving the K_i of about 3 μM. Dixon replot of the data in Fig. 3 also gave a K_i value of 3 μM.

Again from this plot in Fig. 6, we could not determine the type of inhibition; either mixed type or noncompetitive type was possible. As seen in Fig. 6, there was a significant deviation from the linearity of the lines when furosemide concentration exceeded 3 μM.

Table 1. Effects of furosemide on kinetic parameters K_m and V_{max}[a]

Conditions	K_m (mM)	V_{max} (μA/cm²)	n
Control	101±11	112±13	20
Furosemide			
3 μM	174±34	54±12	9
	$0.1>P>0.05$	$P<0.01$	
15 μM	229±22	35± 5	7
	$P<0.001$	$P<0.001$	
30 μM	153±21	26± 5	7
	$0.1>P>0.05$	$P<0.001$	

[a] Mean ±SEM. For each sample of each condition, the K_m and V_{max} were determined by a graph of Lineweaver-Burk plot. Statistical significance of the difference in the mean values between control conditions and in the presence of furosemide was estimated by nonpaired t test.

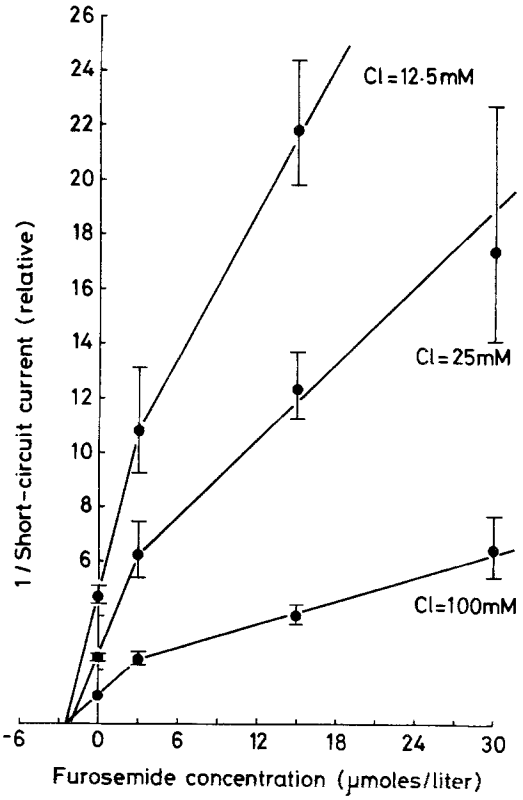

Fig. 6. Dixon plot of the dose-response curve for the data presented in Fig. 5. The reciprocal of the chloride-dependent short-circuit current was plotted against furosemide concentration. Intersection of the line on the base line indicates the inhibitor constant *Ki* which is about 3 μM in this case

Effects of Probenecid on Furosemide Action

It was shown that organic acids are accumulated by the ciliary body and transported out of the rabbit eye (Becker, 1960; Forbes & Becker, 1960). Also in the renal tubule, furosemide is secreted at the proximal tubule into the lumen by a organic acid transport mechanism (Hock & Williamson, 1965*a*); then, at the diluting segment of the tubule it blocks active chloride reabsorption from the luminal side (Burg et al., 1973; Burg, 1976). Therefore, in order to explore the possible involvement of organic acid transport mechanism in the ciliary body of the toad, the effect of probenecid, a potent competitive inhibitor of organic acid transport (Weiner et al., 1960), on furosemide action was examined.

In Fig. 7 the time-course of change in short-circuit current after the addition of furosemide (15 μM), probenecid (1 mM), or both, to the aqueous side was shown. As seen in the figure, the addition of probenecid alone caused a slight, reversible decrease in short-circuit current. The presence of probenecid greatly reduced the inhibitory effects of furosemide. Also, probenecid caused a retardation of appearance of furosemide action. The treatment by probenecid also

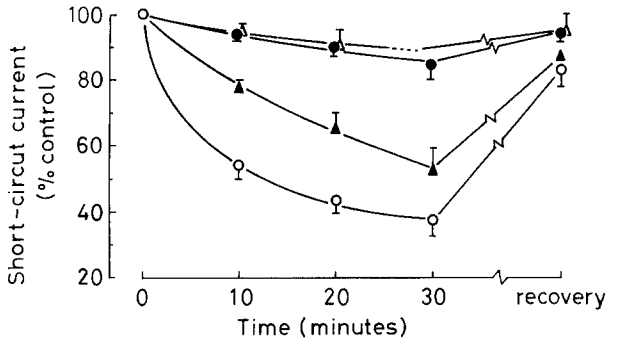

Fig. 7. Changes in short-circuit current (% control) after addition of 15 μM furosemide to the aqueous side (○) or stromal side (●), 1 mM probenecid (△) or both (▲) to the aqueous side. The recovered levels were given as the levels maximally recovered after washing out of the drug or drugs; $n=5$–7

slowed the time-course of recovery to the control level after the removal of both furosemide and probenecid from the incubation solutions.

Discussion

Present study on the effects of furosemide on short-circuit current across the toad ciliary epithelium made clear the specific action of the drug on active chloride transport. Furthermore, kinetic analyses of the data revealed some details of the furosemide reaction with the chloride transport site.

Our previous study on the relationship between sodium chloride fluxes and short-circuit current across the toad ciliary epithelium showed that the short-circuit current was linearly related to the difference between net fluxes of chloride (J_{Cl}^{net}) and sodium (J_{Na}^{net}) from the stromal to the aqueous side under short-circuit conditions (Saito & Watanabe, 1979). The ratio, short-circuit current/($J_{Cl}^{net}-J_{Na}^{net}$) (in μA/cm^2), was 0.89. However, the J_{Na}^{net} was statistically insignificant, while J_{Cl}^{net} was significant and several times greater than J_{Na}^{net}. Also the reduction of short-circuit current caused by ouabain or theophylline was explained mostly by the inhibition of active chloride transport toward the aqueous side. Therefore, in the present study, the magnitude of short-circuit current can be regarded as a valid measure of the rate of active chloride transport. Also, it was shown in the present study that Michaelis-Menten kinetics was applicable to the short-circuit current. Thus, analyses of the present data were made based on the kinetics. The double reciprocal plot of the short-circuit current against the chloride concentration conformed to straight lines both in the absence and presence of furosemide (Figs. 1*b* and 5). The V_{max} and K_m determined under control conditions were 128 μA/cm^2 and 90 mM, respectively. The V_{max} was several times greater than that found in the frog cornea (Zadunaisky, 1966), and the K_m was very similar to that reported for frog cornea (Zadunaisky, 1966) and for human red blood cells (Brazy & Gunn, 1976).

In the present study, the addition of furosemide to the incubation medium caused a significant decrease in short-circuit current. The dose, time-course, and reversibility of the furosemide action in the present study were comparable with those in the diluting segment of the rabbit renal tubule (Burg et al., 1973) and the frog cornea (Candia, 1973). However, there are several points to be discussed in relation to the mode and site of furosemide action. In the frog cornea, furosemide was effective only when added to the endothelial side (Candia, 1973). In the diluting segment of renal tubule (Burg et al., 1973), furosemide inhibited active chloride transport immediately when added to the luminal side. The addition to the peritubular side, even at the high dose (10^{-4} M), caused a delayed decrease in the transtubular potential difference in some tubules. However, the change was considerably less than that elicited by the additions to the luminal side. The mode of furosemide action was speculated to be competitive inhibition.

In the ciliary body of the toad, the effect from the stromal side was almost two dose orders of magnitude smaller than that from the aqueous side. However, the effects from the stromal side were significant when compared with the effects from peritubular and epithelial sides of the diluting segment (Burg et al., 1973) and frog cornea (Candia, 1973), respectively. Since the ciliary body epithelium has a very low *Rt* (Watanabe & Saito, 1978), it is possible that furosemide added to the stromal side diffused into the aqueous side and acted from this side.

The mode of action of furosemide on the chloride self-exchange flux across the human red blood cell membrane was analyzed precisely by Brazy and Gunn (1976). They found that chloride efflux out of the red blood cell was inhibited by furosemide rapidly and reversibly, and was inhibited 50% at 2×10^{-4} M of furosemide and almost completely at 5×10^{-3} M furosemide. The kinetic pattern of the furosemide effects was mixed-inhibition. From these observations, including the effects of pH on furosemide action, they concluded that the anionic form of furosemide interacts primarily at the outer aspect of plasma membrane with the chloride transport mechanism at a site separate from both the transport site and the halide-reactive modifier site.

As to the kinetic pattern of furosemide action at the site of the chloride transport system of the toad ciliary body, the results in Figs. 5 and 6 can be interpreted as a mixed type inhibition, since the

statistical significance of the effect of furosemide on both V_{max} and K_m was confirmed at 15 μM furosemide (Table 1). However, while a mixed type inhibition is a combination of competitive and noncompetitive inhibition, the results of the present study would appear to be weighted in favor of the latter inhibitory mechanism rather than the former.

One-for-one reaction between furosemide molecule and chloride transport unit was suggested from the slopes of the dose-response curve in Fig. 3 and the Hill plot of it in Fig. 4. However, as discussed later, if furosemide action is intracellular, the site stoichiometry for furosemide should be reevaluated in view of the uncertainties involved in the rate-determining step of furosemide action.

The K_i value of 3 μM in the ciliary body was more than one order of magnitude lower than the K_i of 50 μM found in the red blood cells. In the latter the K_i was determined at 0 °C, though there was no unusual temperature sensitivity of the inhibition of the chloride flux by furosemide (Brazy & Gunn, 1976).

The deviation of the plot from the linearity in the Dixon plot of the dose-response curve (Fig. 6) was also noticed in the red blood cells (Brazy & Gunn, 1976) at the lower chloride concentration and at the higher furosemide concentration. They interpreted this deviation as a reflection of the presence of the furosemide-insensitive chloride flux. In the ciliary body, the deviation is obvious at the higher chloride concentration and at the lower furosemide concentration above 3 μM than those in red blood cells. The presence of a furosemide-insensitive fraction is possible in the present study. However, the fraction should be very small (Fig. 3) and would be insufficient to explain the significant deviation in Fig. 6. Rather, it seems probable to assume another low affinity site of the K_i being more than 10 μM for furosemide.

The site of furosemide reaction with chloride transport system in the red blood cells was assumed to be the outer aspect of the plasma membrane since a steady level of inhibitory effect was attained within 6 sec. In the ciliary body the onset of decrease in short-circuit current after furosemide addition was within several sec. However, the time necessary to reach half maximal, steady, inhibition was about 4 min. The time-course of recovery of short-circuit current after the wash-out of furosemide was more than several times longer than that after the addition of furosemide (Fig. 2). Such time-courses were also considerably slower than those observed in the previous experiments (*cf.* Figs. 3, 5, 6, and 7 in Watanabe and Saito, 1978) which suggests the site responsible for furosemide action is not in direct contact with bathing solutions. In addition, the competitive inhibitor of organic acid transport, the probenecid (Weiner et al., 1960; Hook & Williamson, 1965*a*), inhibited the action of furosemide (Fig. 7). The probenecid effects were mainly on the rapid phase of the time-course of furosemide action. It suggests that the rapid reaction of furosemide with chloride transport system was inhibited, but not the slow reaction.

In this connection it is interesting to compare the toad ciliary epithelium with the other epithelia which perform electrogenic chloride transport with respect to the site of furosemide action. In frog cornea (Candia, 1973) and the diluting segment of rabbit renal tubule (Burg et al., 1973), furosemide was effective on the side from where chloride was removed (Zadunaisky, 1966; Burg & Green, 1973; Rocha & Kokko, 1973). Also it was suggested in the intestines that furosemide inhibits coupled, neutral NaCl transport processes located at the luminal border of the rat ileum (Humphreys, 1976) and the basolateral plasma membrane of rabitt colonic mucosa (Frizzell & Heintze, 1979); the former is responsible for the NaCl absorption and the latter for the electrogenic chloride secretion. In contrast to these studies, where the site of furosemide action was on the import of chloride transport, in the present study furosemide action was stronger from the aqueous side, the export of chloride transport. Therefore, the outer aspect of aqueous-facing plasma membrane can be assigned as the most probable site of furosemide reaction. However, the other possibilities cannot be disregarded. In particular, the inhibitory effect of probenecid (Fig. 7) on the effect of furosemide, together with the slow time-course of furosemide action, may suggest a possible involvement of the organic acid transport mechanism with furosemide action since furosemide is known to be transported into the lumen of the renal proximal tubule via the organic acid transport system (Hook & Williamson, 1965*a*, *b*; Deetjen, 1966) and the ciliary epithelium has an ability to transport various kind of organic acids out of the eye (Becker, 1960; Forbes & Becker, 1960). Thus, a possibility cannot be ruled out that furosemide may be taken up by the epithelial cells and exert effects on some transport processes responsible for electrogenic chloride transport.

The underlying mechanisms of electrogenic chloride transport in the toad ciliary epithelium have not been elucidated yet; however, our previous studies suggested that a few processes are involved, though the location is uncertain. These are Na-K exchange mechanism, coupled NaCl transport mechanism, and/or active chloride transport mechanism (Watanabe & Saito, 1978; Saito & Watanabe, 1979). Among them, Na-K exchange mechanism can be ruled out from the possible site for the reaction with furosemide since the dose used in this study was lower than that

required to inhibit Na-K ATPase (Hook & Williamson, 1965*b*; Sachs, 1971; Ferguson & Twite, 1975). With respect to the Na-dependent characteristics of the short-circuit current (Watanabe & Saito, 1978), either a coupled NaCl transport mechanism or an active chloride transport mechanism energized by a Na-dependent metabolic process might be plausible. If the former were responsible in this tissue, the mechanism should have to be located at the stromal border of the epithelial cells since no significant net Na transport was observed (Saito & Watanabe, 1979). With a favorable sodium gradient and the coupled NaCl transport mechanism, chloride accumulated within the cells would be expelled down the electrochemical potential gradient across the aqueous-facing membrane (Field, 1977; Schultz, 1979; Frizzell & Heintze, 1979). Alternatively the latter mechanism can be supposed at either the aqueous-facing or stromal-facing plasma membrane. At present we don't know whether furosemide acts directly on a coupled NaCl transport mechanism or on an active or passive chloride permeation mechanism. In every case discussed above, however, changes in the series, passive chloride permeability should have profound effect on the rate of chloride transport (Field, 1977). In the present study, the *Rt* decreased significantly and dose-dependently after furosemide treatment. If we assume the ciliary epithelium as a single, homogeneous membrane, and this conductance increase is attributable solely to an increase in the chloride conductance, an increase in the short-circuit current would be caused when the electromotive force for chloride was unchanged (Ussing & Zerahn, 1951). However, the present result was the opposite. In the actual epithelium used in the present study, this consideration cannot be applied directly since the epithelium is composed of two membranes in series (if we neglect the role of pigmented epithelium as a diffusion barrier) and a leaky paracellular shunt. In such a system, it is impossible to evaluate the effect of *Rt* change on the short-circuit current (Ginzburg & Hogg, 1967; Field, 1977) based on the data obtained so far. Since we have no direct evidence whether the epithelial cells accumulate furosemide or not, it is too soon to ascertain whether or not furosemide exerts an effect from inside the cell on either the aqueous-facing or stromal-facing plasma membrane. Further studies on these points are required.

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