Actions of External Hypertonic Urea, ADH, and Theophylline on Transcellular and Extracellular Solute Permeabilities in Frog Skin

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ABSTRACT Increases in transepithelial solute permeability were elicited in the frog skin with external hypertonic urea, theophylline, and vasopressin (ADH). In external hypertonic urea, which is known to increase the permeability of the extracellular (paracellular) pathway, the unidirectional transepithelial fluxes of Na (passive), K, Cl, and urea increased substantially while preserving a linear relationship to each other. The same linear relationship was also observed for the passive Na and urea fluxes in regular Ringer and under stimulation with ADH or 10 mM theophylline, indicating that their permeation pathway was extracellular. A linear relationship between Cl and urea fluxes could be demonstrated if the skins were separated according to their open circuit potentials; parallel lines were obtained with increasing intercepts on the Cl axis as the open circuit potential decreased. The slopes of the Cl vs. urea lines were not different from that obtained in external hypertonic urea, indicating that this relationship described the extracellular movement of Cl. The intercept on the ordinate was interpreted as the contribution from the transcellular Cl movement. In the presence of 0.5 mM theophylline or 20 mU/ml of ADH, mainly the transcellular movement of Cl increased, whereas 10 mM theophylline caused increases in both transcellular and extracellular Cl fluxes. These and other data were interpreted in terms of a possible intracellular control of the theophylline-induced increase in extracellular fluxes. The changes in passive solute permeability were shown to be independent of active transport. The responses of the active transport system, the transcellular and paracellular pathways to theophylline and ADH could be explained in terms of the different resulting concentrations of cyclic 3′·5′-AMP produced by each of these substances in the tissue.

INTRODUCTION

The routes of passive solute permeation have been extensively studied in various epithelia. In "leaky" epithelia, such as gallbladder (1), small intestine (2), kidney proximal tubule (3), and choroid plexus (4), it appears that most

of the passive solute permeation occurs through an extracellular (paracellular) pathway. This circumstance has allowed extensive characterizations of paracellular pathways, (e.g., [5]) but the contributions of the transcellular pathway to passive solute permeability have been difficult to separate from those of the high-permeability paracellular pathway. In tight epithelia, such as frog skin and toad bladder, the relative contributions of the transcellular and paracellular pathways to passive solute permeability are comparable and, therefore, more easily separable. In the present study, we utilized the technique described by Mandel and Curran (6), to attempt a separation of the transcellular from the paracellular contributions to passive solute permeability in frog skin under various experimental conditions.

Solute permeation through the paracellular pathway was studied separately at first, by measuring the increases in transepithelial fluxes of Na (passive), K, Cl, and urea produced by external hypertonic urea. This is a condition known to increase paracellular permeability by opening the tight junctions (7), while producing a much smaller change in transcellular permeability (8). When external hypertonic urea was applied to frog skin, all these fluxes were observed to increase substantially while maintaining a linear relationship to each other, indicating that this linearity described a property of the paracellular pathway. Vasopressin (ADH) and theophylline caused clearly different responses from those of external hypertonic urea: linear increases in Na (passive) and urea fluxes, highly nonlinear ones when comparing the fluxes of Cl and urea. The linearity of Na vs. urea was interpreted as an increase in paracellular permeability, whereas the increase in chloride flux appeared to have both a transcellular and an extracellular component. The responses of these two components to ADH and theophylline were separated under various experimental conditions which permitted a study of their interaction with each other and their independence from active transport.

METHODS

The experimental methods used were identical to those previously described (9). Briefly, the skin of *Rana pipiens* was mounted as a flat sheet (3.14 cm^2) between Lucite chambers equipped with solution reservoirs (12 ml each) which were stirred and oxygenated with air. The PD across the skin (expressed with reference to the outside solution) was measured with calomel electrodes and current was passed through the skin via Ag-AgCl electrodes. Both pairs of electrodes were connected to the solution reservoirs with agar bridges having a composition identical to that of the bathing solution in the chamber. An automatic voltage clamp that compensated for the resistance of the solution between the PD bridges was used to pass the appropriate current (short circuit current) to maintain the transepithelial potential at zero. All the experiments reported herein were performed under short circuit conditions except when occasionally the current was interrupted to measure the open circuit potential. Unidirectional fluxes of Na, K, Cl, and urea were determined with ^{22}Na, ^{42}K, ^{36}Cl,

and [^{14}C]urea as previously described; flux periods of 15-min duration were measured for each experimental condition. Transepithelial fluxes from the outside to the inside solution are denoted "influxes," whereas those in the opposite direction are denoted "outfluxes." Some experiments involved paired skins obtained from the same frog by splitting the skin longitudinally along back and abdomen to yield two pieces as nearly symmetrical as possible.

The composition of regular Ringer solution was 112 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$, and 2 mM urea. In one series of experiments the external solution was KCl Ringer containing 112 mM KCl instead of the NaCl in regular Ringer. In another series, 300 mM urea was added to the external Ringer solution, causing it to be hypertonic to the tissue. Isethionate Ringer, used in one series of experiments, contained 107 mM sodium isethionate, 2.5 mM K_2SO_4, 5 mM NaCl, 1 mM $CaSO_4$, and 2 mM urea. Vasopressin (Pitressin, Parke, Davis & Co., Detroit, Mich.), theophylline, and ouabain were added in specific experiments to the inside Ringer solution; phloretin was added to both sides.

RESULTS

Quantitative Relationships among Na, K, Cl, and Urea Fluxes

The relationships among Na, K, Cl, and urea outfluxes in the presence of hypertonic urea (300 mM) added to the external Ringer solution were tested in three groups of experiments. In each group, four skins were utilized measuring the simultaneous outfluxes of Na and urea, K and urea, and Cl and urea, respectively. A typical experiment from each group is shown in Fig. 1, with the regression lines calculated through all the experimental points of each respective group. After a control period of 30 min in regular Ringer (C), hypertonic urea was added to the external solution and the outfluxes measured during the next five flux periods of 15-min duration each. All the fluxes increased with a similar time-course and retained a linear relationship to each other over a change in permeability encompassing an order of magnitude. This linearity is quite remarkable, because it held even during transient conditions when the overall permeability of the tissue was increasing. The slope of Na outflux (J^o_{Na}) vs. urea outflux (J^o_U) was 34.2 $\pm$ 7.4, not significantly different from that found in regular Ringer by Mandel and Curran (9). The Cl vs. urea fluxes were all measured in skins with open circuit potentials (OCPD) of 20 mV or larger during the control period in order to minimize the magnitude of the Cl flux under control conditions. The importance of this initial screening by OCPD is discussed fully in reference to Fig. 3. The fluxes of Na or K vs. urea under control conditions are not clearly correlated with the open circuit potential; therefore, no screening of skins by OCPD was required when measuring these passive fluxes.

The linearity among these fluxes suggests that all the solutes move through the same permeation pathway under these conditions. External hypertonic urea appears to increase the permeability of this pathway, which is mainly

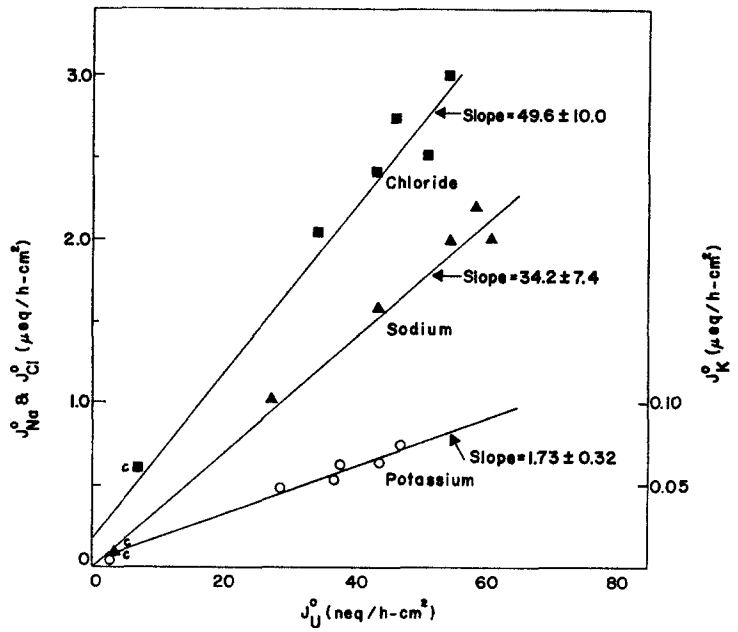

FIGURE 1. Plots of Na, K, and Cl outfluxes vs. the simultaneously measured urea outfluxes. The points marked "C" are controls in regular Ringer; the other experimental points are in hypertonic (0.3 M) external urea. A single experiment is shown for each solute while the lines were calculated by regression analysis from four such experiments, each. The correlation coefficients (P_i) for these are: $P_K = 0.98$, $P_{Na} = 0.96$, $P_{Cl} = 0.94$. Slopes ± SE's.

extracellular (see Discussion) and, therefore, the results shown in Fig. 1 may be utilized as a control describing properties of the extracellular pathway.

The effect of theophylline on the relationship between the passive movements of Na and urea was studied by simultaneously measuring the outfluxes of both solutes under short circuit conditions, first in regular Ringer (control) and then upon addition of 10 mM theophylline to the same skins. The results of experiments in 11 skins are shown in Fig. 2, where the Na outflux is plotted against the urea outflux. The fluxes under control conditions (each point is the average of two 15-min flux periods) appear to be linearly related, as observed by Mandel and Curran (9); a straight line, calculated by regression analysis gives a slope of 41.1 ± 14.0 and a correlation coefficient of 0.98 (this slope is not significantly different from that shown in Fig. 1 for Na vs. urea). Upon stimulation by theophylline, both fluxes increase substantially, but the relationship between them is preserved; a regression line calculated on the basis of the fluxes in theophylline is not significantly different from the control line.

As shown in Fig. 2, 10 mM theophylline causes a two- to fourfold increase in the outfluxes of Na and urea. Lesser increases in these fluxes are obtained

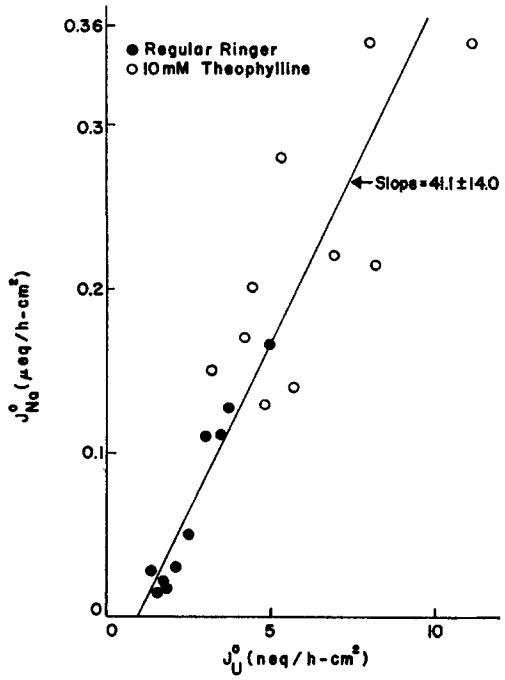

FIGURE 2. Plot of Na outflux (J^o_{Na}) vs. the simultaneously measured urea outflux (J^o_U) in regular Ringer and theophylline (10 mM). The line was calculated by regression analysis using the experimental points in regular Ringer; the same line is seen to fit the theophylline data. ± SE's.

with lower theophylline concentrations and also with vasopressin (ADH); the latter, at a dose of 20 mU/ml produces a stimulation (maximal) of 28 ± 5% in both Na and urea fluxes, similar to that observed by Helman and Miller (15). The linearity between Na and urea outfluxes is also maintained with vasopressin.

The effects of theophylline on the relationship between chloride and urea can be best understood by initially studying the response to a low concentration of theophylline (0.5 mM). The influxes of Cl and urea were simultaneously measured under short circuit conditions, first in regular Ringer (control) followed by the application of 0.5 mM theophylline (experimental) to the inside solution. Influxes of these passively transported solutes are equal to outfluxes under short circuit conditions in regular Ringer (9, 10). The results are shown in Fig. 3, where the numbers in parentheses are the respective open circuit voltages recorded by momentarily interrupting the short circuit current passing through the skins. If a regression line were calculated through all the *control* points it would give a slope of 57 ± 20 and an intercept on the ordinate of 0.32 μeq/h-cm². However, better correlations are obtained if separate regression lines are calculated through control and experimental points

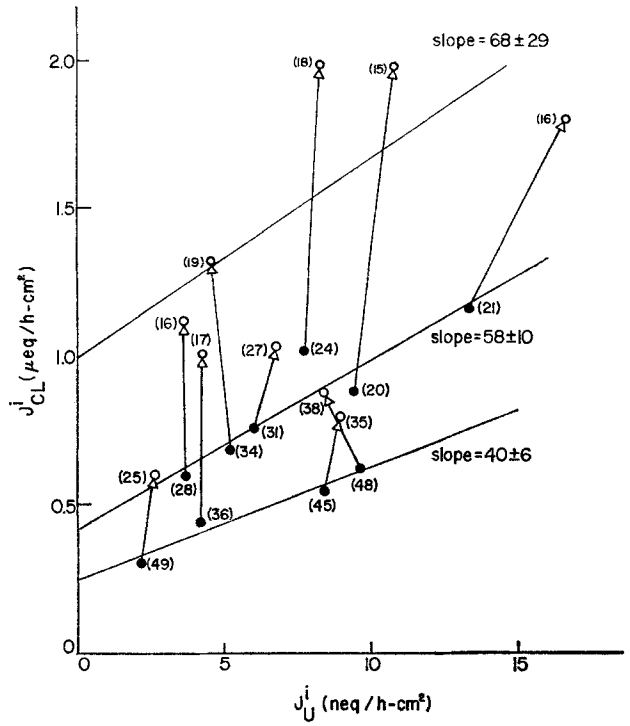

FIGURE 3. Plot of Cl influx (J^o_{Cl}) vs. the simultaneously measured urea influx (J^o_U) in regular Ringer (●) and with 0.5 mM theophylline (○). The regression lines were calculated through three groups of experimental points separated by open circuit potential (numbers in parentheses) as follows: 36–50 mV, 20–35 mV, and less than 20 mV. The arrows join each control point to its experimental counterpart in each skin. ± SE's.

pooled together on the basis of their open circuit potentials: those skins between 36 and 50 mV, those between 20 and 35 mV, and those below 20 mV, as shown in Fig. 3. The three lines have essentially the same slope, with increasing intercepts on the ordinate as the open circuit potential decreases.

When 0.5 mM theophylline is applied to frog skin there is, on the average, a doubling of the Cl flux and no significant change in the urea flux (Fig. 4). These effects of theophylline are reflected in Fig. 3 as an almost vertical shift of the control points to the next parallel line with a higher intercept on the ordinate (see arrows). In addition, theophylline causes a significant decrease in the open circuit potential occurring in conjunction with the increase in Cl flux, suggesting that this portion of the flux may be diffusional in nature and serve to shunt the preexisting potential.

The Cl vs. urea slopes calculated in Fig. 3 are not significantly different from the Cl vs. urea slope shown in Fig. 1, indicating that this slope may represent the relationship existing between the movement of Cl and urea through the extracellular pathway. On the other hand, the flux calculated

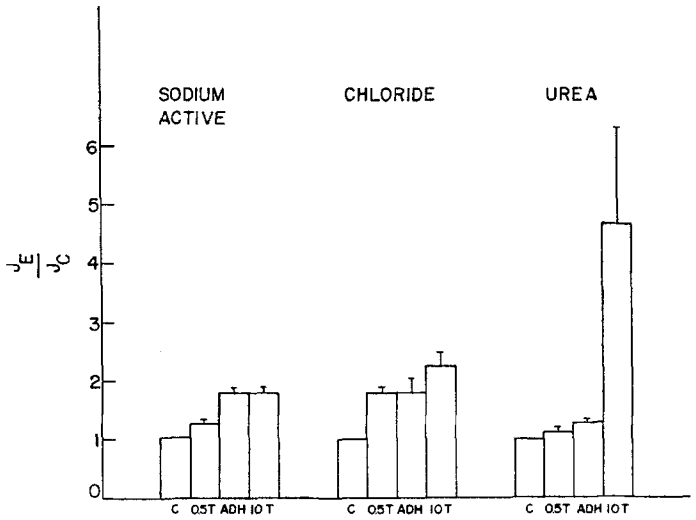

FIGURE 4. Response of the short circuit current and the transepithelial influxes of chloride and urea to 0.5 mM theophylline, 20 mU/ml of ADH, and 10 mM theophylline. Each set of experiments is normalized to its respective control condition (C). J_E and J_C are the experimental and control fluxes, respectively. Mean values of eight experiments; vertical bars are SE's.

from the intercept on the ordinate would appear to traverse a separate pathway, since this portion of the flux is independent of the urea flux. The main action of 0.5 mM theophylline is, therefore, to increase the chloride flux through the urea-independent pathway, while leaving the urea flux unchanged.

The effects of 20 mU/ml of ADH added to skins in regular Ringer under short circuit conditions are very similar to those of 0.5 mM theophylline on the Cl and urea fluxes. As shown in Fig. 4, ADH causes almost a doubling of chloride flux and a small but significant increase in urea flux, indicating that most of the chloride flux increase is through the urea-independent pathway. When 10 mM theophylline is added to skins in regular Ringer large increases are observed in both fluxes although the increases in urea flux are variable under these conditions, it appears that the increased chloride flux traverses both the urea and the urea-independent pathways. The average control values for chloride and urea fluxes were 1.23 $\pm$ 0.19 μeq/h-cm^2 and 2.6 $\pm$ 0.35 neq/h-cm^2, respectively. Dividing the increase in Cl flux stimulated by 10 mM theophylline (ΔJ_{Cl}) in each skin by its respective increase in urea flux (ΔJ_U) gives an average of

$$\frac{\Delta J_{Cl}}{\Delta J_U} = 520 \pm 200; \qquad (n = 12).$$

(The large SE is due to the large span of values, from 70 to 3,300; the average slope is significantly different from 50.) Since the Cl vs. urea slope for the extracellular chloride movement is about 50 (Figs. 1 and 3), it appears that the increase in chloride flux produced by 10 mM theophylline is 10 times larger than the flux calculated to be moving through an extracellular pathway. These considerations suggest that about 90% of the increase in chloride flux is through the urea-independent pathway.

Separation between Increases in Chloride and Urea Fluxes

The increases in the fluxes of chloride and urea produced by theophylline can also be separated in other ways. A temporal separation can be obtained by measuring the time-course for the increase in the outfluxes of chloride and urea after the application of 10 mM theophylline to the inside solution. Fig. 5 shows a typical experiment which starts with two control flux periods of 15 min each, followed by the addition of theophylline at about 35 min. A maximal increase in chloride flux is already observed in the next flux period, remaining approximately constant for 45 min. This response, which could be characterized by a first order function, is quite different from that of the urea outflux, which exhibits the delay and slow rise characteristic of a second or higher order response. Similar experiments, simultaneously measuring Na and urea outfluxes, show that both of these solutes exhibit a similar slow increase in flux after the addition of theophylline, in contrast to the rapid rate of increase displayed by chloride.

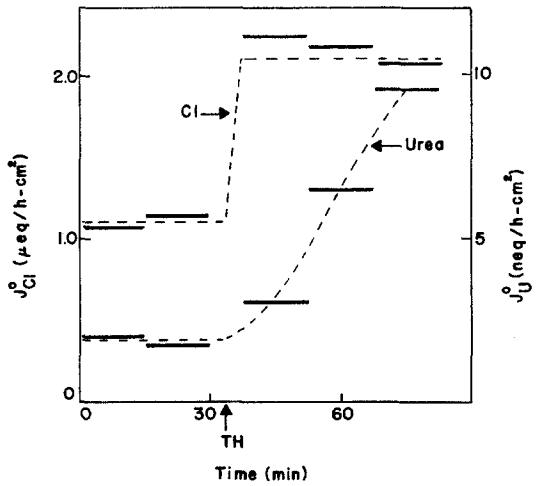

FIGURE 5. Typical experiment showing the time-course for the increase in chloride and urea outfluxes after the addition of theophylline (arrow points to time of addition of 10 mM). The chloride response is first order, whereas the urea one is second or higher order. J^o_{Cl} and J^o_U are chloride and urea outfluxes, respectively.

Another way to separate the responses in the fluxes of chloride and urea produced by theophylline, is to decrease the chloride concentration of the bathing media. This was accomplished by measuring chloride and urea fluxes in isethionate Ringer solutions containing 5 mM NaCl. Paired skins were utilized to measure influxes in one half of the skin and outfluxes in the other half, with results shown in Table I. During the control periods, while both skins were in isethionate Ringer under short circuit conditions, both the chloride and urea influxes were about twice the value of the outfluxes. This observation is easily explained for chloride, since a small component of inward active chloride transport has been described in frog skin at low chloride concentrations (11). The simultaneously measured urea fluxes were also significantly different from each other; this is a puzzling observation with no satis-

TABLE I

EFFECTS OF 10 mM THEOPHYLLINE IN ISETHIONATE RINGER CONTAINING 5 mM NaCl (n = 7) ± SE

Experimental condition	J^i_{Cl}	J^o_{Cl}	J^i_U	J^o_U
	neq/h-cm²	*neq/h-cm²*	*neq/h-cm²*	*neq/h-cm²*
Isethionate Ringer	82±10	36±10	5.4±1.1	2.2±0.5
10 mM Theophylline	160±20	150±20	5.5±0.9	3.2±0.4
Regular Ringer "cold" side	270±50	190±20	8.0±1.0	6.5±0.9

factory explanation since other evidence presented in this paper and others (9, 15) overwhelmingly favors purely passive movement of urea through frog skin. When 10 mM theophylline was added to the inner bathing solutions of both halves (still in isethionate Ringer), both the outfluxes and influxes of chloride increased significantly to give an influx to outflux ratio close to unity. At the same time, there was no significant theophylline-stimulated increase in the urea fluxes at this low chloride concentration. Chloride was subsequently introduced by changing the "cold" (*trans*) side solutions to regular Ringer (112 mM NaCl); that is, the insides (with theophylline) for the influxes and the outsides for the outfluxes. Additional increases in the chloride fluxes were observed in both directions and, in addition, the urea fluxes in both directions almost doubled. Hence, it appears that the presence of chloride is necessary to obtain the stimulation of urea fluxes elicited by 10 mM theophylline.

Passive Transport, Active Transport, and Theophylline

The effect of 0.5 mM theophylline after virtually complete inhibition of active transport by ouabain (10^{-4} M) is shown in Fig. 6. No increase in the inhibited active transport is observed, whereas chloride influx almost doubles when

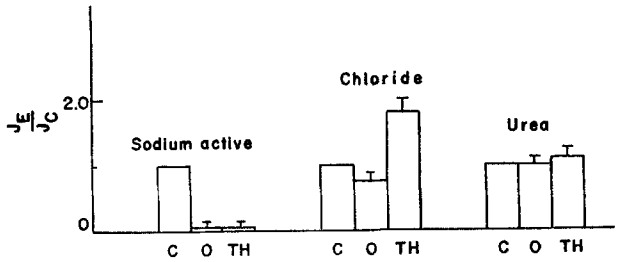

FIGURE 6. Response of the short circuit current and the transepithelial influxes of chloride and urea to ouabain (10^{-4} M) followed by theophylline (0.5 mM). The chloride and urea responses to theophylline are not impaired by the poisoning of the Na pump. All values are normalized to the control period (C). Ouabain (O), theophylline (TH). All symbols as in Fig. 4 (n = 6).

compared to the control, or triples when compared to the flux in ouabain. The urea influx shows the same small increase as in Fig. 4. The response of chloride influx to theophylline when active transport is nonexistent demonstrates that this response is independent of the operation of the Na pump. The reduction of chloride influx in the presence of ouabain appears to affect mainly the urea-independent pathway, since the urea flux remains unchanged.

Similarly, the response of the active transport system to theophylline is independent of the presence of chloride in the media. The short circuit current increased an average of 79 ± 11% when the skins were stimulated with 10 mM theophylline in isethionate Ringer (n = 10).

Effects of Phloretin on Chloride and Urea Transport

Phloretin is a compound which has been shown to inhibit the transepithelial flux of urea in toad bladder and, especially, to abolish the increase in this flux normally observed with ADH (12). It also inhibits the fluxes of urea and chloride (13) in human red cells. These observations prompted the investigation of the effect of phloretin on the fluxes of chloride and urea in frog skin under control and stimulated conditions. Addition of phloretin in concentrations of 2×10^{-5} or 10^{-4} M to both sides of the skin did not change the influxes of either chloride or urea measured in regular Ringer under short circuit conditions. Stimulation of these influxes with ADH (20 mU/ml) was not affected by phloretin, since paired experiments (n = 7) with or without phloretin showed identical behavior. Similarly, stimulation of these fluxes by 10 mM theophylline or external 0.3 M (hypertonic) urea was unimpaired by the presence of phloretin. These experiments demonstrate that the chloride and urea transport systems in frog skin exhibit strikingly different behavior than the phloretin-sensitive transport found in other tissues.

DISCUSSION

The linear relationship among the fluxes of Na, Cl, K, and urea observed in external hypertonic urea (Fig. 1) is consistent with other observations which indicate that an extracellular (paracellular) pathway is the main contributor to the increased solute permeability produced by this experimental condition. These observations are: First, lanthanum penetrates through the tight junctions of frog skin when the tissue is bathed in external hypertonic urea, whereas no such penetration is observed in regular Ringer (7, 14). Second, the extracellular component of the transepithelial resistance shows a large decrease when toad bladder is bathed in external hypertonic urea whereas the transcellular resistance changes little in comparison, when measured with intracellular microelectrodes (8). Third, the slope of the J^o_{Na} vs. J^o_U regression line in external hypertonic urea is the same as under control conditions in regular Ringer, indicating that the same permeation pathway is involved under both of these conditions; since passive Na and urea appear to move mainly extracellularly under control conditions (9), the same is expected to be true in hypertonic urea.

The experimental evidence, therefore, strongly suggests that the main increase in solute permeability produced by external hypertonic urea occurs via an extracellular transepithelial pathway through the tight junctions and the intercellular canals. The tight junctions appear to open under the influence of the external hypertonic urea, thereby increasing the permeability to all solutes through the tissue. The ratio of the chloride vs. urea slope to that of Na vs. urea is 1.45, not very different from the ratio of the free-solution conductances of Cl to Na of 1.48. These two ions, therefore, behave as if they moved through a free-solution channel. The K vs. urea slope cannot be directly compared to the Na and Cl ones because the K concentration was only 2.5 mM; since a linear extrapolation to 112 mM may not be valid, no quantitative conclusions are drawn from these data.

No deviations from linearity have been observed for the Na vs. urea fluxes measured under a variety of experimental conditions. It is particularly remarkable that the slope between these fluxes is the same in regular Ringer, ADH, theophylline, and external hypertonic urea, encompassing almost two orders of magnitude change for these fluxes. This linearity confirms the existence of a predominantly extracellular route for the passive permeation of Na and urea; it also justifies the use of urea flux as a measure of permeability for the extracellular pathway in frog skin, as introduced by Mandel and Curran (9).

The possible contributions of edge damage to the outfluxes of Na and urea have been discussed by Helman and Miller (15). These authors calculated that about 70% of the outfluxes of Na and urea in regular Ringer were due to edge damage in preparations such as the present one, in which the tissue

was clamped between two Lucite chambers. On this basis, the linear Na vs. urea fluxes under control conditions (Figs. 1 and 2) would be mainly measuring the properties of the edge-damaged portion of tissue. When these fluxes are stimulated with 10 mM theophylline or external hypertonic urea, morphological changes are observed everywhere in the tissue: the tight junctions open sufficiently to allow lanthanum penetration and precipitation between the cells (7, 14; also, Mandel and Zampighi, unpublished observations). Correspondingly, the extracellular permeability of the tissue as a whole increases and the contribution due to the damaged edge to the *increased* fluxes would be small, since the damaged area is less than 1% of the total tissue area. The slope of the increased fluxes obtained under stimulated conditions would, therefore, measure the properties of the extracellular pathway of the whole tissue with little error due to the damaged edge. Actually, the similarity in Na vs. urea slopes between control and stimulated conditions may provide some information about the nature of the damaged edge. The simplest explanation for the similar slopes would be that the damaged edge might represent an area of skin with higher than normal extracellular permeability; this would be true if the damage to the skin consisted in forcing the tight junctions apart. On this basis, the main contribution of the damaged edge would be an overall increase in extracellular permeability.

The same reasoning could be applied to the chloride vs. urea relationship since the slope obtained with external hypertonic urea (Fig. 1) is the same as that obtained under control conditions (Fig. 3). The linear relationship could be attributed to chloride movement through the extracellular pathway, whether it is through the tight junctions opened by the external hypertonic urea or those opened by edge damage. The chloride vs. urea slopes obtained in Figs. 1 and 3 are about one-half the magnitude of the average slope obtained by Mandel and Curran (9) under similar experimental conditions. A slope of approximately 50 was obtained in Fig .3 by utilizing skins exhibiting open circuit potentials larger than 20 mV. The procedure used by Mandel and Curran (9) involved no separation of skins by open circuit potential and, consequently, skins with a larger range of open circuit potentials were used. It could be easily seen, with reference to Fig. 3, that if the points with open circuit potentials lower than 20 mV were used in conjunction with those of higher potentials, a steeper regression line would be obtained. It appears, therefore, that the slope of 126 calculated by Mandel and Curran (9) for the chloride vs. urea fluxes does not accurately describe the properties of the extracellular pathway. A slope closer to 50 appears to be the correct one, giving a chloride to sodium permeability ratio of about 1.4 in the present investigation. Hence, the extracellular pathway in frog skin displays the properties of a free-solution shunt, whether it is in regular Ringer or stimulated by external hypertonic urea or theophylline.

The effect of 0.5 mM theophylline (Figs. 3 and 4) clearly dissociates the chloride movement from that of urea, since it produces a doubling of the chloride fluxes without a significant change in the urea fluxes (or the passive Na fluxes which are linearly related to the urea fluxes). Since this theophylline concentration causes mainly an increase in the intercept on the ordinate, while maintaining a constant chloride vs. urea slope, it appears that the properties of the extracellular pathway remain constant under these conditions. The main action of 0.5 mM theophylline seems to be the increase in chloride flux through a parallel, but separate pathway. It is difficult to determine the nature of this pathway without additional experimental evidence; however, one can speculate whether it is transcellular or extracellular. The pathway would have to be separate from the urea and passive Na pathway and, in addition, it would have to be almost ideally permselective for chloride (or anions) because the cationic permeability does not change with 0.5 mM theophylline in the presence of a twofold increase in chloride permeability. The possibility of an extracellular pathway permselective to chloride cannot be excluded but seems rather unlikely; a transcellular pathway appears to be more probable because of the following considerations:

(*a*) The extracellular pathways which have been investigated in other epithelia have either cationic selectivity (2, 3, 5) or the characteristics of a free-solution shunt (5). An anionic permselective shunt is possible, but would be a rare occurrence.

(*b*) The transcellular pathway fulfills the requirement of almost ideal permselectivity to chloride because the sodium permeability of the inner border is extremely small (16, 17, 18). Even if the outer cellular border is Na permeable, the transepithelial permeability of the transcellular pathway to Na is extremely small because of the properties of the inner membrane. On the other hand, both cellular borders are permeable to chloride (18) and, therefore, an appreciable transepithelial movement of chloride would be expected.

(*c*) Theophylline and ADH increase the water permeability of the mucosal border in a variety of epithelia (19, 20) and ADH increases the permeability of lipophilic substances (presumably traversing the transcellular pathway) through toad bladder (21). If these observations are indicative of a general increase in cellular permeability, an increase in the transcellular chloride fluxes would also be expected.

On the basis of these considerations, it appears that chloride flux increases not accompanied by corresponding changes in urea flux can be considered to be transcellular. The main action of 0.5 mM theophylline on these fluxes would, therefore, be to increase the transcellular Cl flux while leaving the extracellular fluxes unchanged. ADH appears to have a similar action to that of 0.5 mM theophylline (Fig. 4), whereas 10 mM theophylline appears to in-

crease both the transcellular and the extracellular fluxes. The contribution of the damaged edge to the transcellular chloride flux is difficult to assess because a large amount of spontaneous variation is observed in the intercept on the ordinate, from near zero to more than 2 μeq/h-cm^2. Since an approximately constant contribution could be expected from a mechanically compressed area of skin, it is unlikely that the large range in variation is due to the damaged edge; rather, the contribution from the edge is probably small and most of the variation is probably due to biological circumstances.

The relationship between the increases in Cl flux (mainly transcellular) and the increases in urea flux (mainly extracellular) obtained with theophylline may be summarized as follows: (*a*) An increase in urea flux under the action of theophylline has never been observed without an increase in Cl flux, although the reverse is often observed. (*b*) The increase in Cl flux precedes the increase in urea flux. (*c*) The increase in urea flux does not occur in low Cl concentrations, although the increase in Cl flux is observed.

On the basis of these observations it is possible to hypothesize that the theophylline-caused increase in extracellular permeability, which presumably occurs by opening the tight junctions, could be controlled intracellularly. The mechanism behind such a process is unknown, but it is possible to speculate on its origin. Since the increase in transcellular chloride permeability *and* the presence of chloride in the media are both required to observe the increase in extracellular permeability, this effect may be mediated by the entry of KCl from the inside solution, and/or Cl from the inside and Na from the outside solution, causing an accumulation of solute in the cells which would produce an increase in their volume. A slight swelling of the outermost layer of the stratum granulosum has been observed in the presence of 10 mM theophylline accompanied by an opening of the tight junctions, as visualized by lanthanun precipitation (Mandel and Zampighi, manuscript in preparation). This same cell layer shows a rough correlation between cellular swelling and an increase in extracellular permeability (14). A mechanism by which cellular swelling causes a disruption of the tight junctions would explain why the relationship between the increases in Cl and urea fluxes due to theophylline are so variable. Two complex steps would follow the increase in transcellular chloride permeability whose effects are difficult to predict quantitatively: increase in cellular volume and, subsequently, the disruption of the tight junctions. Complete ignorance over the relationships among these steps makes quantitative prediction extremely difficult. Variability among skins may explain why, in some instances, no increase in urea flux is observed even if a large increase in Cl flux is obtained, since some skins may not show a volume increase with no disruption of the tight junctions. Nevertheless, despite these quantitative problems, the concept of intracellular control of extracellular permeability is viable and worthy of further investigation.

The responses of the active and passive transport systems to theophylline appear to be independent of each other. The increase in active transport in isethionate Ringer is not different from the response in regular Ringer; this result is in agreement with that of Kristensen (22), but is in conflict with Cuthbert et al. (23), who observed no increase in short circuit current under similar conditions. The increase in Cl flux caused by theophylline appears to be independent of active transport, as shown in Fig. 6. In these experiments, it appears that the inhibition of active Na transport decreases the Cl permeability of frog skin. The urea fluxes remain unchanged when active transport is inhibited and, therefore, the decreased Cl fluxes seem to be purely transcellular.

Phloretin does not appear to have any effect on the unstimulated Cl or urea fluxes in frog skin or on the increased fluxes observed in the presence of ADH, theophylline, or external hypertonic urea. This result contrasts with observations made on toad bladder demonstrating a clear inhibition of urea fluxes by phloretin (12) and in red cells demonstrating inhibition of both urea and chloride fluxes by this compound (13). All the transport systems found to be inhibited by phloretin appear to be saturable and, otherwise, have properties identified with carrier systems. In contrast, neither the urea nor chloride fluxes in frog skin exhibit carrier-mediated properties such as exchange diffusion (10) or saturation, except for chloride at low concentrations (24). These observations, therefore, confirm the possibility that phloretin interacts primarily with carrier-mediated systems and does not seem to affect transport systems involving simple diffusion.

The responses of the chloride and urea fluxes to 0.5 mM theophylline are almost identical to those of 20 mU/ml of ADH, while 10 mM theophylline is seen to increase both of these fluxes further (Fig. 4). In contrast, the active transport system does not appear to be maximally activated by 0.5 mM theophylline, but ADH and 10 mM theophylline produce maximal activation. All these results are consistent with the hypothesis that both ADH and theophylline converge on the same effector mechanism, namely, 3′-, 5′-cyclic AMP (25). The quantitative differences between the actions of ADH and theophylline could be interpreted in terms of the resulting 3′-, 5′-cyclic AMP (cAMP) tissue concentrations elicited by each of these substances, since 10 mM theophylline has been shown to produce a higher increase in tissue cAMP than large ADH concentrations in toad bladder (26). On this basis, the active Na transport system appears to saturate at a rather low cAMP concentration, whereas the chloride fluxes continue to increase. The lesser response of the active transport system to 0.5 mM theophylline than to ADH could be similar to the series of different responses observed with ADH analogs in toad bladder (27); these experiments lead to the postulation of separate cellular cAMP pools for the natriferic than the hydro-osmotic effects. It is also possible that

such a separation in cAMP pools be present in frog skin, isolating the responses of the active from those of the passive transport systems.

The responses of the active and passive transport systems to ADH and 10 mM theophylline are also observed electrically. Since ADH slightly increases the open circuit voltage and the resistance of frog skin, while theophylline decreases both of these parameters, Cuthbert and Painter (28) concluded that the two pharmacological agents acted through different mechanisms. However, as described above, the quantitative differences in the resulting tissue cAMP concentrations could result in the differences in electrical responses. The action of theophylline in decreasing the open circuit voltage and transepithelial resistance could be due to the larger increase in passive permeability that it produces as compared to ADH; when this increase in passive permeability is largely prevented by a chloride-free medium, the electrical responses of the tissue to 10 mM theophylline are identical to those in ADH.

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