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Cu^{2+} AND PERMEABILITY OF ISOLATED FROG SKIN*

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SUMMARY

1. The effects of external copper***, 10^{-5} M, added as $CuSO_4$, were studied with the isolated abdominal skin of *Rana temporaria*.

2. Cu^{2+} causes a decrease in Cl^- permeability and an increase in the transepithelial potential difference (PD); short-circuit current (SCC) may also rise due to Cu^{2+}.

3. These effects are enhanced and more consistent in Cu^{2+}-treated skins obtained from warm-adapted as compared to cold-adapted frogs.

4. The Cu^{2+}-induced PD rise is greater in warm-adapted skins held under short-circuit rather than open-circuit conditions.

5. Low concentrations of Ca^{2+}, at 1 mM, in bathing solutions elicit stronger Cu^{2+} effects; a higher concentration, 8 mM Ca^{2+}, delays and/or inhibits the action of Cu^{2+}.

6. The Cu^{2+} effects on Cl^- permeability, usually seen at about pH 8, are absent at pH values below neutrality (6.6 or lower).

7. In contrast to its depressant effect upon Cl^- influx, Cu^{2+} appears to have little or no effect upon concurrent influxes of Na^+, SO_4^{2-}, sucrose or urea; effluxes of Cl^- and of Na^+, SO_4^{2-} or sucrose, measured simultaneously, are unaffected by Cu^{2+}.

8. The results suggest that Cl^- may move across frog skin (a) by a transcellular route which may be considerably diminished by Cu^{2+} and (b) through an extracellular shunt pathway which is relatively unaffected by Cu^{2+}. The former appears to assume a progressively more important role in the transepithelial transport of Cl^- in frogs maintained at elevated temperatures.

Abbreviations: PD, potential difference; SCC, short-circuit current.

* A preliminary account [1] of some of the phenomena presented here was given at the XIVth Scandinavian Congress of Physiology and Pharmacology in Bergen, Norway, August 5–8, 1973.

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*** Cu^{2+} is used throughout this report primarily as a generic symbol for copper and does not necessarily imply activity in this form.

INTRODUCTION

Reports of the observations by Ussing and his colleagues [2–4] of the increase in voltage across isolated frog skin, following the addition of Cu^{2+} at low concentrations (10^{-5} M) to the external bathing solution, first appeared more than 20 years ago. It was considered that the spontaneous potential across the skin (PD) was largely the result of an active inward transport of Na^+ and the shunting of anions and cations, e.g. of Cl^- in normal Ringer's solution [3, 4]. Thus it seemed reasonable to expect that the rise in PD seen with Cu^{2+} could be due to an alteration in the surface of the skin so as to impede the passive transepithelial movement of anions, particularly Cl^- [3]. Experiments in which the $^{36}Cl^-$ fluxes were measured in the absence and presence of external Cu^{2+} did indeed fulfil this expectation [4]. Since that time a number of workers have noted similar effects upon the open-circuit voltage following Cu^{2+} treatment of isolated skins from several species of frogs, including *Rana pipiens*, *Rana ridibunda*, *Rana temporaria* and *Leptodactylus ocellatus* [1, 5–8].

The effects of Cu^{2+} upon short-circuit current (SCC) and Na^+ fluxes appear to be less consistent. They range from no effect [4] to a decrease [7] or increase [5–8] in one or the other of these parameters. Among the latter instances, the increases in PD and SCC observed in Cu^{2+}-treated skins from *L. ocellatus*, a South American frog, have been taken to indicate a decrease in permeability to Cl^- [5, 6]. This would explain the increase in PD and in SCC, since in this species the current is diminished by active Cl^- transport. However, a recent report describes increases in passive Na^+ and Cl^- fluxes consequent to external Cu^{2+} treatment of isolated skins from *R. ridibunda* [7]. These increases, interpreted as being due to a Cu^{2+}-induced increase in skin permeability, do not seem to be compatible with the earlier observations or with more recent findings [1].

The studies described herein were undertaken in an attempt to obtain more detailed information about the effects of Cu^{2+} upon isolated frog skin. The results (1) specify some of the conditions under which Cu^{2+} is active and they (2) provide more data concerning transepithelial pathways for anions, particularly Cl^-.

MATERIALS AND METHODS

Skins

More than 200 isolated abdominal skins of *R. temporaria* were used in the experiments reported here. Frogs were maintained either at 3.1 ± 0.1 °C (mean $\pm$S.E. of the mean), referred to as cold-adapted, or at 12.5 ± 0.1 °C (ambient air temperature), referred to as warm-adapted. The time for adaptation to the warmer environment was not less than 7–10 days. Data obtained with skins from male and female animals were essentially the same.

Frogs were killed by cervical transsection, pithed and the skins, quickly dissected free from underlying abdominal tissues, were placed in the bathing solution, a modified Cl^-–Ringer's solution.

Bathing solutions

The basic solution used throughout was normal frog Cl^-–Ringer's solution with the replacement of 25 mM of NaCl by 25 mM of Na_2SO_4. The SO_4^{2-} present

acted as a carrier for the $^{35}SO_4^{2-}$ flux determinations. The outside and inside solutions were identical.

The composition of the basic solution (in mM) was: NaCl, 90; Na_2SO_4, 25; $KHCO_3$, 2.5; $CaCl_2$, 1; pH 8 (7.8–8.1). In a number of experiments Ca^{2+} was either increased to 8 mM (added as $CaCl_2$) or omitted. Frog skin apparently maintains sufficient Ca^{2+} for normal functioning even when Ringer's solution has no Ca^{2+} in it [9, 10]. Carrier sucrose or urea, 10 mM, was present in the outside and inside solutions for sucrose and urea flux determinations. In all instances Cu^{2+} (as $CuSO_4$) was added only to the outside bathing solution to a final concentration of 10^{-5} M. The osmolarity of the bathing solutions, determined with a Fiske osmometer, ranged between 241 and 250 mosM.

pH studies

The outside and inside bathing solutions were buffered at pH 6.0, 6.6, 7.5 and 7.9 by 10 mM Tris–maleate buffers [11].

Electrical measurements

Trans-epithelial PD and SCC measurements were made with skins mounted in an Ussing–Zerahn chamber [3]. Resistance was calculated as PD/SCC. PD and SCC were intermittently monitored; readings required about 10 s. Corrections were made for the differences between calomel half-cells (< 1 mV) and for the electrical asymmetry between the electrodes and the resistance of the Ringer's solution. When the open-circuit voltage was not fully re-established immediately following a reading of SCC, the data were discarded.

Radioactive substances

Fluxes were measured with tracer amounts (1–10 μCi) of $^{24}Na^+$, $^{35}SO_4^{2-}$, $^{36}Cl^-$ (from Risø, the Danish Atomic Energy Commission); and $^{22}Na^+$, [6,6′-^{3}H]-glucose, [^{14}C]glucose and [^{14}C]urea (from The Radiochemical Centre, Amersham, England). Sample activities were assayed with a Tri-Carb (Packard) spectrometer; $^{24}Na^+$ activities, in a Selectronix (Denmark) spectrometer. Scintillation counts were corrected for residual $^{24}Na^+$ activity.

General procedure

Experiments were carried out at room temperature (20–24 °C). Cold-adapted skins were allowed to stabilize between 1.5 and 2 h; warm-adapted skins, 0.5 h. The equilibration time for radioactive substances was 0.5 h. Cl^- fluxes were determined concurrently with Na^+, SO_4^{2-}, sucrose or urea fluxes. At time zero, and at hourly intervals thereafter, 1-ml samples were removed from the solution into which radioactivity subsequently appeared. Volume replacements were made with non-radioactive solutions; activities were corrected for these dilutions. At the end of a 2-h control period, Cu^{2+} was introduced into the outside bathing solution and readings and sampling were continued for another 3 h.

Mathematical analysis

Permeabilities (P values) have been estimated using the integrated Nernst–Planck equation for the net flux of an ion across the skin, with account taken for the

influences upon the ion of the concentration difference and the electrical field. The latter is assumed to be constant, with the ion able to move freely through a homogeneous membrane and with its mobility and activity coefficient remaining unchanged during its movement. The Nernst–Planck equation may be resolved into two equations, each describing unidirectional fluxes: J_{in}, inward (considered positive) and J_{out}, outward (negative). These are equivalent to Eqns 3a and 4a developed by Mandel and Curran [12].

Statistical analysis

Data are presented as the mean $\pm$S.E. Control values represent averages of the data obtained over the two 1-h periods (Periods 1 and 2) prior to the addition of Cu^{2+}. Experimental values represent the data obtained in the second 1-h period (Period 4) after Cu^{2+} had been added to the outside solution. This avoids the inclusion of data representing (a) a series of non-steady states, during which the skin and fluxes were equilibrating to the effects of Cu^{2+} (Period 3) and (b) a subsequent decline or rise in the effects of Cu^{2+} (Period 5). In this way, estimates of both stimulatory and depressive influences of Cu^{2+} may be considered to be conservative.

RESULTS AND DISCUSSION

Effect of Cu^{2+} upon skin resistance

In the studies summarized in Table I Cu^{2+} caused a rise in trans-epithelial resistance (PD/SCC). At the same concentration of Ca^{2+}, the resistance is higher in cold-adapted than in warm-adapted frog skins and Cu^{2+} treatment increases this resistance. Simultaneously, the PD values increased due to Cu^{2+}, especially in skins from warm-adapted frogs, while the corresponding SCC values changed relatively little. Short circuiting increased the resistance in warm-adapted skins. Also, in these skins the resistance was greater at the higher Ca^{2+} concentrations.

In isolated skins from cold-adapted frogs (32 animals), Cu^{2+} induced a rise in PD in 28 experiments (88 %) and a fall in 4 experiments (12 %); SCC values increased in 14 (44 %) and decreased in 18 instances (56 %). Cu^{2+}-evoked changes in the resistance closely followed the PD values, with a rise in 26 (81 %) and a fall in 6 cases (19 %). These observations were noted under both open-circuit and short-circuit conditions.

All the skins from warm-adapted frogs (57 animals) showed a rise in PD following external addition of Cu^{2+}; SCC values increased in 36 (63 %), decreased in 5 (9 %) and showed no change in 16 experiments (28 %). Resistances increased in 45 cases (79 %) and showed no change in the others (12 cases, 21 %). Here, too, the results remained qualitatively unchanged under open- and short-circuit conditions and with Ca^{2+} at 0, 1 or 8 mM in both bathing solutions.

The recent report [1] of consistent increases in PD and SCC due to Cu^{2+} in cold-adapted skins appears to contradict, in part, the findings reported here. However, those experiments were all done between January and July, while the experiments described here extended over the better part of a year. Thus, the more variable findings for cold-adapted frogs reported here seem to reflect seasonal differences in the skin responses to Cu^{2+}. Also, in those studies frogs were warm-adapted to higher temperatures (24–25 °C).

TABLE I

EFFECTS OF TEMPERATURE ADAPTATION AND CONCENTRATION OF Ca^{2+} UPON Cu^{2+}-INDUCED CHANGES IN PD, SCC AND *R*

The number of skins used is in parentheses. Control (before Cu^{2+}) and experimental (after Cu^{2+}) periods were 1-h long. Ca^{2+} concentrations were the same in both bathing solutions. PD values are in mV; SCC, μequiv · h^{-1} · cm^{-2}★; R, ohms · cm^2; each value is expressed as the mean ±S.E.

	Cold-adapted, 3 °C (32)		Warm-adapted, 12 °C (57)					
	1 mM Ca^{2+}		No Ca^{2+}		1 mM Ca^{2+}		8 mM Ca^{2+}	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
Open-circuited skins	(5)		(17)		(8)		(3)	
PD	65.8 ±3.8	69.2 ±3.2	19.5 ±1.0	27.5 ±1.8	30.8 ± 2.0	44.9 ±2.2	38.2 ±2.8	41.8 ±3.4
SCC	0.97±0.06	0.86±0.04	0.66±0.03	0.69±0.03	0.71± 0.06	0.73±0.03	0.63±0.07	0.51±0.06
R	53 ±2	63 ±3	20 ±1	25 ±2	31 ± 1	46 ±2	49 ±2	59 ±2
Short-circuited skins	(27)		(27)		(2)		(–)	
PD	68.8 ±0.4	70.9 ±0.3	25.6 ±1.3	35.4 ±1.6	34.4 ±10.1	57.1 ±8.6		
SCC	1.2 ±0.03	1.1 ±0.01	0.70±0.03	0.77±0.03	0.66± 0.03	0.57±0.06		
R	50 ±1	55 ±1	27 ±1	35 ±1	41 ± 1	76 ±1		

★ Multiply this value by 26.8 to obtain SCC in $\mu A/cm^2$.

TABLE II

EFFECTS OF TEMPERATURE ADAPTATION AND CONCENTRATION OF Ca^{2+} UPON Cu^{2+}-INDUCED CHANGES IN Cl^- PERMEABILITY

Permeability values are expressed as $P \times 10^7$ cm · s^{-1} ±S.E.

	Cold-adapted, 3 °C (47)		Warm-adapted, 12 °C (47)					
	1 mM Ca^{2+}		No Ca^{2+}		1 mM Ca^{2+}		8 mM Ca^{2+}	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
Open-circuited skin	(5)		(5)		(8)		(3)	
P_{in}	4.9±0.2	4.7±0.1	8.9±0.1	4.4±0.2	7.2±0.1	4.3±0.1	3.0±0.1	2.1±0.1
Short-circuited skin	(23)		(29)		(2)		(–)	
P_{in}	3.7±0.0	2.9±0.1	5.9±0.1	2.3±0.0	4.9±0.0	1.4±0.0		
	(19)		(–)		(–)		(–)	
P_{out}	3.4±0.0	3.2±0.0						

TABLE III

LACK OF EFFECT OF Cu^{2+} UPON UREA AND SUCROSE PERMEABILITIES

Permeability values are expressed as $P \times 10^7$ cm · s^{-1} ±S.E.

	Cold-adapted, 3 °C (21)		Warm-adapted, 12 °C (20)	
	Control	Experimental	Control	Experimental
Urea				
open-circuited skin				
P_{in}				
1 mM Ca^{2+} (5)	2.4±0.1	2.6±0.1	—	—
1 mM Ca^{2+} (8)	—	—	3.0±0.1	2.9±0.1
short-circuited skin				
P_{in}				
1 mM Ca^{2+} (5)	2.6±0.1	2.6±0.1	—	—
1 mM Ca^{2+} (2)	—	—	2.6±0.2	2.7±0.2
Sucrose				
short-circuited skin				
P_{in}				
0 mM Ca^{2+} (10)	—	—	6.6±0.1	6.9±0.1
1 mM Ca^{2+} (8)	3.2±0.1	3.8±0.1	—	—
P_{out}				
1 mM Ca^{2+} (3)	1.2±0.1	1.6 ±0.1	—	—

From the data in Table I, it appears that a minimum concentration of Ca^{2+}, about 1 mM in the outside solution, may facilitate and increase the Cu^{2+} effect upon resistance*. However, it seems clear that the changes in Cl^- permeability induced by Cu^{2+} are antagonized and considerably attenuated at 8 mM Ca^{2+} in the bathing solution (Table II).

Effect of Cu^{2+} upon Cl^- permeability

Cl^- permeability is markedly reduced by Cu^{2+} in warm-adapted frog skin, more so in short- than in open-circuit preparations (Table II). This increased resistance to trans-epithelial Cl^- movement is consistent with the enhancement in resistance in short-circuited skins noted in Table I. Ca^{2+}, at 8 mM, suppressed but did not eliminate the Cu^{2+} effect (Table II). A smaller Cu^{2+}-induced depression in Cl^- permeability, about 20 %, was observed in short-circuited cold-adapted skins; however, P_{out} values were unaffected, as were P_{in} values under open-circuit conditions. Thus, Cu^{2+} was relatively ineffective with regard to Cl^- permeability in open- and short-circuited skins from cold-adapted frogs. These findings appear to agree with the results of Mandel and Curran [12], from which they concluded that much or nearly all of the movement of Cl^- across the skin may occur via a passive extracellular pathway, such as that followed by urea (vide infra, Table III).

The influence of temperature adaptation upon Cl^- fluxes and permeabilities provides a new variable to be considered concerning the electrochemical properties of frog skin and the effects of Cu^{2+} upon them. In general, there is an increase in Cl^- permeability in warm-adapted skins. Furthermore, with these skins bathed in Ringer's containing 1 mM Ca^{2+}, there is a marked reduction in Cl^- permeability both in short- and open-circuit conditions.

Effect of Cu^{2+} upon urea and sucrose permeabilities

Permeability to urea does not seem to be much affected by Cu^{2+} in cold- or warm-adapted skins, under open- or short-circuit conditions, at 1 mM Ca^{2+} in the outside and inside bathing solutions (Table III). Whether the trans-epithelial movement of urea is essentially intercellular [12, 13], or transcellular in certain circumstances [14], the data here certainly suggest that Cu^{2+}, at 1 mM Ca^{2+}, does not interfere with the movement of urea.

As with urea, Cu^{2+} seems to have little effect upon the sucrose movements across frog skin (Table III), movements which are generally assumed to occur extracellularly. If anything, Cu^{2+} may have increased the permeability to sucrose slightly. Sucrose permeability ratios, P_{in}/P_{out}, about 2.5–3, suggest that sucrose may be subjected to drag effects of considerable magnitude, probably due to the active transport of Na^+. Sucrose permeability exceeds that of urea, particularly in warm-adapted skins. There is no simple explanation for this.

Effect of Cu^{2+} upon SO_4^{2-} permeability and Na^+ efflux

Sulfate permeability, too, is practically unaffected by Cu^{2+} (Table IV). In warm-adapted skins the inward permeability to SO_4^{2-} is lower than in cold-

* It is possible, of course, that the stimulatory concentration of Ca^{2+} may be somewhat greater than 1 mM, since concentrations between 1 and 8 mM were not tested.

TABLE IV

LACK OF EFFECT OF Cu^{2+} UPON SO_4^{2-} PERMEABILITY AND Na^+ EFFLUX

Permeability values are expressed as $P \times 10^7$ cm · s^{-1} ±S.E. For SO_4^{2-}, $z = -2$

	Cold-adapted, 3 °C (28)		Warm-adapted, 12 °C (26)	
	Control	Experimental	Control	Experimental
SO_4^{2-}				
open-circuited skin				
P_{in}				
0 mM Ca^{2+} (5)	—		0.86±0.03	0.75±0.03
8 mM Ca^{2+} (3)	—	—	0.26±0.01	0.35±0.02
short-circuited skin				
P_{in}				
0 mM Ca^{2+} (18)	—	—	2.2 ±0.0	2.2 ±0.0
1 mM Ca^{2+} (10)	4.6±0.1	5.2±0.1		
P_{out}				
1 mM Ca^{2+} (4)	2.0±0.1	2.0±0.1	—	—
Na^+				
short-circuited skin				
P_{out}				
1 mM Ca^{2+} (14)	2.3±0.0	2.4±0.0	—	—

TABLE V

IS THE SHUNT PATHWAY THROUGH DAMAGED CELLS?

Sylgard 186, an electrical sealant, was applied to the outside periphery of the skin in contact with the circular edge of the outer lucite half-chamber. Open-circuited warm-adapted skins were used, in the absence of Ca^{2+} in the bathing solutions. PD values are in mV; SCC, μequiv · $h^{-1} \cdot cm^{-2}$; R, ohms · cm^2; each value, except the Cl^-/SO_4^{2-} ratio, is expressed as the mean ±S.E. $^{36}Cl^-/^{35}SO_4^{2-}$ ratios were calculated from simultaneously measured Cl^- and SO_4^{2-} influxes expressed as μmoles · $h^{-1} \cdot cm^{-2}$.

	With Sylgard (6)		Without Sylgard (6)		With/Without ratio	
	Control	Experimental	Control	Experimental	Control	Experimental
PD	22.8 ±3.8	25.1 ±4.3	21.8 ±4.6	23.9 ±5.8	1.04	1.05
SCC	0.65±0.05	0.73±0.05	0.66±0.03	0.62±0.06	0.98	1.19
R	22 ±3	23 ±3	22 ±4	22 ±5	1.00	1.03
$^{36}Cl^-/^{35}SO_4^{2-}$ ratio	16.0	12.9	11.9	9.0	1.34	1.43

TABLE VI

EFFECT OF pH UPON Cu^{2+}-INDUCED CHANGES IN Cl^- AND SO_4^{2-} INFLUXES

Warm-adapted short-circuited skins were incubated in identical outside and inside bathing solutions. The pH values studied were maintained with 10 mM Tris–maleate buffers, with no Ca^{2+}. Influx values are expressed as $J \times 10^{10}$ moles · $cm^{-2} \cdot s^{-1}$ ±S.E.

	pH 6.0 (8)		pH 6.6 (8)		pH 7.5 (4)		pH 7.9 (4)	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
Cl^-	0.45±0.01	0.47±0.02	0.48±0.01	0.44±0.01	1.3 ±0.1	1.6 ±0.1	1.8 ±0.1	1.1 ±0.1
SO_4^{2-}	0.04±0.00	0.04±0.00	0.03±0.00	0.04±0.00	0.10±0.01	0.13±0.00	0.09±0.00	0.08±0.00

adapted skins and under open- compared with short-circuit conditions. There is no obvious explanation for these findings, both contrary to what might be anticipated. Cu^{2+} also appears to have little influence on the SO_4^{2-} influxes under open-circuit conditions. However, the absolute values of these influxes are about one-third of the corresponding short-circuit values. Furthermore, they are depressed about 60% by raising the concentration of Ca^{2+} in the bathing medium to 8 mM. No effect of Cu^{2+} on SO_4^{2-} influx or permeability was observed under short-circuit conditions in warm-adapted skins. This finding is not unexpected if the trans-epithelial movement of SO_4^{2-} is passive and confined to an extracellular shunt, as may be supposed for sucrose.

The Na^+ efflux was unaffected by Cu^{2+} in cold-adapted skins (Table IV).

Do damaged cells constitute the shunt pathway?

A number of the flux measurements indicated rather low rates of movement of the various radioactive species of ions and non-electrolytes used in these studies. The question thereby arose as to whether some significant fraction of these fluxes, and thus of the shunt itself, might be attributable to leakage through damaged cells located around the periphery of the skin. Sylgard 186 (Ringsted and Semler, A/S, Copenhagen), an electrical sealant which may also cushion the skin against excessive compression, was used to determine the possibility of such damage and to quantify it.

The data in Table V indicate that the shunt is not likely to be through damaged cells, since there is no evidence for such damage from the influx data or from the electrical parameters. As far as the latter are concerned. no significant differences were observed with Sylgard compared with data obtained in its absence. Although the absolute values of Cl^- and SO_4^{2-} influxes were higher in the absence of Sylgard, the differences, again, were not significant. Moreover, changes in the Cl^-/SO_4^{2-} influx ratios induced by Cu^{2+} seem to occur in a parallel fashion with and without Sylgard. These results suggest that a valid assessment of the effects of Cu^{2+} upon Cl^- fluxes may be made from the data obtained without Sylgard. The basis for the apparent contradiction between these results and those of Helman and Miller [15] is obscure at present. As those workers point out, a number of factors might be involved, including species differences, chamber characteristics, mounting technique, etc.

pH and the effect of Cu^{2+} upon Cl^- and SO_4^{2-} influxes

There is no observable Cu^{2+} effect upon Cl^- and SO_4^{2-} influxes at pH values below 7.9 (Table VI). However, as the pH is raised, Cl^- and SO_4^{2-} influxes increase and a distinct Cu^{2+} effect, typical of those noted earlier, is seen at pH 7.9. Anion permeability would be expected to decrease as the pH rose if the shunt pathway was analogous to a simple charged membrane. Therefore, the nature and distribution of charges lining the shunt must be more complex than this, since the opposite relationship was observed.

A comparison of the Cl^- influxes at pH values 7.5 and 7.9 before Cu^{2+} treatment with the post-Cu^{2+} influx of Cl^- at the higher pH, suggests that the difference, about $0.6 \cdot 10^{-10}$ moles $\cdot$ cm$^{-2} \cdot$ s^{-1}, may represent that fraction of Cl^- moving across the skin through a pathway other than the extracellular shunt. This finding, of course, coincides with others noted above and lends support to the main conclusions of this report.

CONCLUSIONS

The effect of Cu^{2+} upon Cl^- influx is different from its effects upon Na^+, SO_4^{2-}, urea and sucrose influxes: the latter remained unchanged or increased slightly; Cl^- influxes showed changes ranging from very little to a definite decrease. The net effect was a fall in the ratio of Cl^- influx to the influxes of the other substances, measured concurrently. The conclusion seems inescapable: Cu^{2+} influences a transepithelial Cl^- influx pathway other than the shunt channels followed by Na^+ (passive), SO_4^{2-}, urea and sucrose. A reasonable interpretation of these findings is that Cl^- follows two routes across frog skin: (1) an extracellular one, probably in common with other substances moving passively through the system of intercellular spaces, which is relatively unaffected by Cu^{2+}, and (2) a transcellular route which is definitely influenced by Cu^{2+}. In the absence of warm-adaptation, the shunt would probably provide the main route for Cl^- movement [12]. Also, Ca^{2+} concentration may be a factor in anion preference between these routes. Thus, to some extent, different skin responses to Cu^{2+} may reflect differences in Ca^{2+} concentration.

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