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THE EFFECT OF Cu^{2+} ON ISOLATED FROG SKIN

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SUMMARY

1. The effect of Cu^{2+} in concentrations of 0.1 mM on isolated frog skin was studied.
2. An increase in the short-circuit current simultaneously with an increase in the open-circuit voltages was observed.
3. The Na^+ fluxes increased proportionally with the short-circuit current, suggesting that Cu^{2+} has a stimulating effect on the Na^+ transport by frog skin.
4. Cl^- fluxes increased in both directions, and this fact, together with an increase in Na^+ backfluxes, may point to an overall increase in the permeability of the frog skin.
5. The interference of Cu^{2+} with Ca^{2+} and antidiuretic hormone was also studied, and it was observed that Cu^{2+} acting on the epithelial side interferes with Ca^{2+}, while acting on the corium side it interferes with antidiuretic hormone. Possibly the interference with Ca^{2+} involves a process on the tight junctions and that with the antidiuretic hormone directly on the cell membrane.

INTRODUCTION

Previous studies[1,2] carried out with isolated skins of northern European frogs like *Rana temporaria* have shown that small amounts of Cu^{2+} in the form of $CuSO_4$ (10 μM) added to the solution bathing the outside of the frog skin originate an increase in the open-circuit voltage (o.c.v.). Short-circuit current (s.c.c.) and Na^+ fluxes were not affected, but $^{36}Cl^-$ influxes decreased sharply[3]. The explanation given for these results was that Cu^{2+} acts by reducing the permeability of the frog skin to Cl^-. Another group of investigators[4-6] using Cu^{2+} in the concentration of 20 μM on isolated skins of the South American frog *Leptodactylus ocellatus* again verified an increase in the o.c.v. but also an increase in the s.c.c. These effects could again be explained by a decrease of the permeability of the skin to Cl^-. Good evidence was obtained showing that the s.c.c. in these skins is due to two components, one of Na^+ and another of Cl^-, both actively transported from the outside to the inside of the frog skin. A diminution of the Cl^- component of the s.c.c. would therefore increase its absolute value.

Cu^{2+} in low concentration (10 μM) has thus been used for studying the Na^+ diffusion potentials of the outer skin barrier, as an alternative method to the substitution of Cl^- by SO_4^{2-} in the bathing medium, in order to decrease the shunting

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Abbreviations: s.c.c., short-circuit current; o.c.v., open-circuit voltage; d.c., direct current effect of Cl^-. In a previous work[7] we have obtained results showing that the simple substitution of Cl^- by SO_4^{2-} or other nonpenetrating anions in Ringer's solution systematically induced a decrease in s.c.c. and a corresponding reduction of Na^+ net fluxes. These results were obtained by taking care to maintain an osmolality of 0.220 osM in the Cl^--substituted Ringer solution. Without this precaution the effect is masked in all probability by the now well-established modification of s.c.c. values in preparations bathed in hypotonic solutions[8]. It was then suggested that Na^+ transport is Cl^- dependent. It seemed, therefore, relevant to test Cu^{2+} in our skin preparations in order to see if reducing the permeability of the frog skin to Cl^- would effect a reduction in the s.c.c. and Na^+ transport. The results described in this paper show that this is not the case and that under the influence of Cu^{2+} there is an increase of the o.c.v. together with an increase in the s.c.c. and that this is due to an increase in the Na^+ net fluxes. Finally, good evidence was obtained of a simultaneous increase in $^{36}Cl^-$ fluxes. This confirms our view of the importance of the anion permeability, in this case Cl^-, for the efficiency of the Na^+ transport. In this paper we are furthermore concerned with the mechanism of the action of Cu^{2+}.

METHODS

Frogs of the species *Rana ridibunda Pallas* were used unless otherwise specified. They were kept in a temperature-controlled room at 6–8°, half-immersed in running tap water. Frogs were double-pithed and the abdominal skin dissected and mounted in Ussing-type chambers. The exposed area of the skin was 7.06 cm^2 when two halves were used or 3.14 cm^2 when four pieces of the same skin were needed. The skins were bathed with Ringer's solution on both sides and aerated, and experimental procedures were always performed after an equilibration period of more than 3 h. When experimental processes were performed, the Ringer's solutions were changed either on both sides with the Ringer's solution to be tested or only on one side, the other side being washed with fresh normal Ringer's solution.

The Ringer's solution normally used had the following composition (mM): Cl^-, 119; Na^+, 112; K^+, 2.4; Ca^{2+}, 2.4. The Cu^{2+} (in the form of $CuSO_4$) concentration used was 0.1 mM except in one type of experiment where different concentrations of Cu^{2+} were tested, namely ½, ¼, and $^1/_8$ of the above-mentioned concentration. Ca^{2+} concentrations were also changed in one of the experimental groups, no Ca^{2+}, 2.4, 4.8, and 9.6 mM Ca^{2+} being used. In the Ringer's solution containing no Ca^{2+}, 0.5 mM EDTA was added. All the Ringer's solutions were titrated with Tris buffer so that the aerated Ringer's solution had a pH of 8.2. The composition of the Ringer's solution was frequently checked. Na^+ and K^+ concentrations were measured by flame photometry with an Eppendorf flame photometer and Cl^- by coulometric titration in an Aminco-Cotlove titrator. The osmolality of the Ringer's solution was around 0.220 ± 2 controlled with an Advance osmometer and the pH was checked with a Radiometer pH Meter 4.

O.c.v.'s and s.c.c.'s were measured in the same way as described in a previous paper[7]. They were measured during control and experimental periods every 10 min. The effect of the substance to be studied was measured by taking the values of voltages and currents from the steady-state values of the control and experimental periods. Means and standard deviations were calculated.

Na^+ fluxes were measured by single-labelling techniques with $^{22}Na^+$, using for influxes and outfluxes two symmetrical pieces of the abdominal skin continuously short-circuited. Activities were measured in a well-type scintillation detector. Cl^- fluxes were measured using the same techniques, with $^{36}Cl^-$, assayed with an immersion-type Geiger counter, and using all the fluid collected from the half-chambers (6 ml).

The interference of Cu^{2+} on the well-known effect of oxytocin was studied using two symmetrical pieces of the same skin. In one of these pieces the effect of the oxytocin was measured after a previous addition of Cu^{2+} either to the outside or to the inside of the preparation, while in the other one, used as control, oxytocin alone was studied. Similar experiments inverting the order of the substances were also performed. Oxytocin (Syntocinon, Sandoz S.A.) was used in a concentration of 0.2 units in 6 ml of Ringer's solution.

RESULTS

Effect of Cu^{2+} added to both sides of the frog skin preparation

Eighteen skins were mounted in normal Cl^- Ringer's solution and allowed to equilibrate. Then the chambers were emptied and refilled on both sides with Cl^- Ringer's solution containing $CuSO_4$. Fig. 1 illustrates one such experiment, where s.c.c. and o.c.v. were plotted against time. In the first 20 min there was a sharp increase in the s.c.c. and o.c.v. After these initial changes three different patterns may be observed: (1) the increased values of current and voltages are maintained for several hours, (2) they can decrease slightly and then remain stable, or, finally, (3) currents and voltages decrease slowly but continuously. Table I summarizes the results of these experiments in the form of means of the absolute values of control and experimental periods of currents and voltages. The mean value of the o.c.v. in the experimental period was 140 % of the control mean value, and the mean value of the s.c.c. was 159 %. For each experiment the direct current (d.c.) resistances were calculated by dividing the o.c.v. by the s.c.c. measured in each interval. As shown

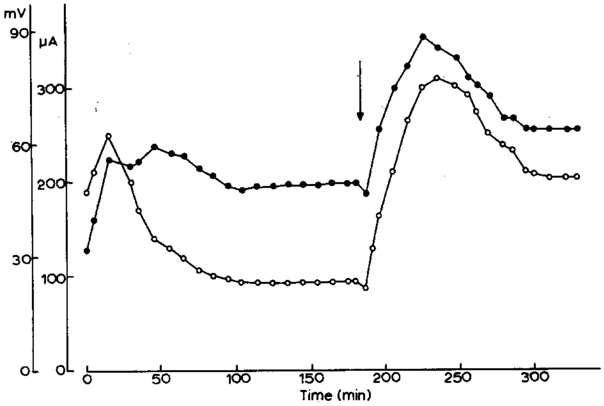

Fig. 1. Skin mounted in Cl^- Ringer's solution. Arrow, Cl^- Ringer's solution containing Cu^{2+} on both sides. ●—●, o.c.v.; ○—○, s.c.c.

TABLE I

ABSOLUTE VALUES OF O.C.V., S.C.C. AND d.C. RESISTANCES OF CONTROL AND EXPERIMENTAL PERIODS UNDER THE EFFECT OF COPPER ON BOTH SIDES OF THE FROG SKIN PREPARATION

Means and standard deviations. Number of experiments is eighteen.

	o.c.v. (*mV*)	*s.c.c.* ($\mu A/7.06$ *cm*^{2})	*Resistance* ($\Omega/7.06$ *cm*^{2})
Control	43 ± 9	158 ± 75	336 ± 138
Cu^{2+}	61 ± 16	252 ± 95	253 ± 35
Increase (%)	42	59	—
Decrease (%)	—	—	25

in Table I the mean value of the d.c. resistance in the experimental periods was 75 % of the control value. When lower concentrations of Cu^{2+} were used (10 μM), the effect was not always present, but when present the type of effect described above was observed.

Another group of skins was mounted from the beginning in Cl^- Ringer's solution containing Cu^{2+}, and the time evolution of the short-circuit current was studied for several hours. The results were compared with the results obtained by a control group mounted in normal Cl^- Ringer's solution. The results are plotted in Fig. 2. The results of s.c.c. measurements in each group were pooled and running time averages calculated according to the technique described in a previous paper[7]. It can be seen that the s.c.c. values of the skins bathed by Cl^- Ringer's solution containing Cu^{2+} are higher and steadier than those of the control group. $^{22}Na^+$ flux measurements were done in six skins of each group. The results are given in Table II. Values are expressed in μequiv/7.06 cm^2 per 30 min. In both groups of skins there is a good agreement between s.c.c. and Na^+ fluxes, the latter being around 100 % of the s.c.c., showing that the effect of Cu^{2+} on the s.c.c. can be accounted for by the increase in the net Na^+ fluxes.

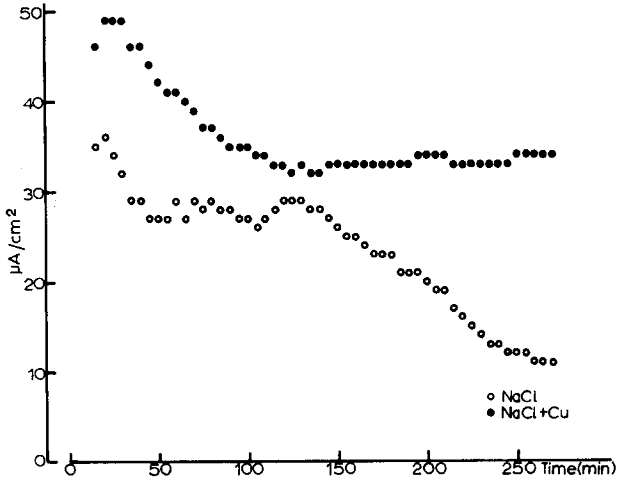

Fig. 2. Time evolution of s.c.c. of skins mounted in Cl^- Ringer's solution (○) and in Cl^- Ringer's solution containing Cu^{2+} (●). Plot of a moving average of five adjacent mean values of s.c.c. expressed in $\mu A/cm^2$.

TABLE II

$^{22}Na^+$ FLUX MEASUREMENTS IN SKINS BATHED IN Cl^- RINGER'S SOLUTION OR Cl^- RINGER'S SOLUTION WITH $CuSO_4$

Results are expressed in µequiv/30 min per 7.06 cm^2. Means and standard deviations. Number of periods in parentheses.

	Influx			*Backflux*			*Net flux* (%)
	s.c.c.	*Na^+ flux*	%	*s.c.c.*	*Na^+ flux*	%	
Control group: NaCl Ringer's solution	2.447 ± 1.2 (25)	2.594 ± 1.25 (25)	108.7 ± 17.3	2.479 ± 0.79 (26)	0.267 ± 0.11 (25)	11.3 ± 4.8	97.4
NaCl Ringer's solution with 0.1 mM $CuSO_4$	4.182 ± 0.75 (30)	4.780 ± 1 (30)	114.1 ± 10.5	4.499 ± 1 (30)	0.80 ± 0.24 (30)	16.8 ± 6	97.3

TABLE III

$^{36}Cl^-$ FLUX MEASUREMENTS IN SKINS BATHED IN Cl^- RINGER'S SOLUTION OR Cl^- RINGER'S SOLUTION WITH $CuSO_4$

Results are expressed in µequiv/30 min per 7.06 cm^2. Means and standard deviations. Number of periods in parentheses.

	Influx		*Backflux*		*Net flux* $O \leftarrow I$
	s.c.c.	*Cl^- flux*	*s.c.c.*	*Cl^- flux*	
Control group: NaCl Ringer's solution	2.54 ± 0.82 (14)	0.426 ± 0.36 (14)	1.85 ± 0.56 (14)	0.455 ± 0.19 (14)	0.029
NaCl Ringer's solution with 0.1 mM $CuSO_4$	4.8 ± 1.4 (7)	1.024 ± 0.12 (7)	3.18 ± 0.4 (7)	1.249 ± 0.32 (7)	0.225

These results also show that the backfluxes of Na^+ increase in the presence of Cu^{2+}. In five skins Cl^- fluxes were measured using $^{36}Cl^-$. In three of these, normal Cl^- Ringer's solution was used throughout the experiment, while in the other two, the skins were mounted and maintained in Ringer's solution containing Cu^{2+}. The results of these experiments are given in Table III. In the absence of Cu^{2+} a negligible net outflux of Cl^- was detected; in the presence of Cu^{2+} the fluxes in both directions increase and a slight net outflux was measured. Even if the small difference between both fluxes (0.225 μequiv/30 min per 7.06 cm², t test, $P < 0.10$) corresponds to a Cl^- net flux, this would account for only 5 % of the overall effect of Cu^{2+} on the s.c.c. The increase in influxes and outfluxes of Na^+ and Cl^- are consistent with the fall of the d.c. resistance under these conditions. To check if this effect was due to a species difference, Na^+ fluxes and s.c.c. were also measured under the same condition in one frog of the species *R. temporaria*. The results agree with those obtained in *R. ridibunda*. An increase in s.c.c. (28 %) with a parallel increase in Na^+ fluxes was again observed.

The possibility that the results could be due to the fact that our frogs were Cu^{2+} depleted was tested, using a batch of eleven frogs kept for 11–21 days in water into which small bits of Cu^{2+} tubing were added. Although no determinations of Cu^{2+} were made, an effect on o.c.v. and s.c.c. was observed. The time-course of the s.c.c. differs from the pattern we normally observe. S.c.c.'s and o.c.v.'s tend to increase and reach a first plateau in the first 2 h, and this plateau is higher than the values found in frogs maintained as usually in running tap water. After this period s.c.c.'s and o.c.v.'s decrease and tend to a new steady value still higher than the values of the control group. Adding Cu^{2+} at this moment gives the usual effect. The results are summarized in Table IV and can be compared with those of Table I.

TABLE IV

ABSOLUTE VALUES OF VOLTAGES, CURRENTS AND RESISTANCES OF CONTROL AND EXPERIMENTAL PERIODS UNDER THE EFFECT OF Cu^{2+} ON BOTH SIDES OF THE FROG SKIN PREPARATION OBTAINED FROM FROGS KEPT IN WATER CONTAINING A COPPER TUBING FOR 7–21 DAYS

Means and standard deviations. Number of experiments is eleven.

	o.c.v. *(mV)*	*s.c.c.* *(μA/7.06 cm²)*	*Resistances* *(Ω/7.06 cm²)*
Control	57 ± 15	215 ± 135	308 ± 40
Cu^{2+}	68 ± 20	380 ± 120	183 ± 37
Increase (%)	19	77	—
Decrease (%)	—	—	41

Effect of Cu^{2+} added separately either to the outside or to the inside of the frog skin

Table V shows the effect of $CuSO_4$ when added either to the outside or to the inside of the frog skin preparation. When in contact with the epidermal side, Cu^{2+} produces an increase in both o.c.v.'s and s.c.c.'s and a slight decrease in d.c. resistance. When Cu^{2+} acts on the corium side, an increase in the s.c.c.'s is again evident but the effect on the o.c.v.'s is only very slight. Resistances were also measured in short-circuited preparations using a low frequency (0.06 cycles/sec) a.c. signal and the results agree with the d.c. measurements. Again in this situation there is a good agreement between s.c.c.'s and Na^+ fluxes as shown in Table VI.

TABLE V

ABSOLUTE VALUES OF VOLTAGES, CURRENTS AND RESISTANCES OF CONTROL AND EXPERIMENTAL PERIODS UNDER THE EFFECT OF Cu^{2+} EITHER ON THE OUTSIDE OR ON THE INSIDE OF THE FROG SKIN PREPARATION

Means and standard deviations. Number of experiments in parentheses.

	Cu^{2+} on the outside			*Cu^{2+} on the inside*		
	Voltages (mV)	*Currents ($\mu A/7.06\ cm^2$)*	*Resistances ($\Omega/7.06\ cm^2$)*	*Voltages (mV)*	*Currents ($\mu A/7.06\ cm^2$)*	*Resistances ($\Omega/7.06\ cm^2$)*
Control	28.4 ± 16 (14)	112.6 ± 65 (18)	266 ± 165 (14)	30.5 ± 15.8 (14)	129.8 ± 65 (18)	218 ± 138 (14)
Cu^{2+}	44.4 ± 24.5 (14)	173.9 ± 74 (18)	256 ± 163 (14)	33.6 ± 15.8 (14)	176.4 ± 51 (18)	193 ± 124 (14)
Increase (%)	54.2	54.4	—	10.2	35.9	—
Decrease (%)	—	—	3.8	—	—	11.5

TABLE VI

$^{22}Na^+$ MEASUREMENTS IN SKINS FIRST BATHED IN Cl^- RINGER'S SOLUTION AND THEN IN Cl^- RINGER'S SOLUTION CONTAINING Cu^{2+} EITHER ON THE OUTSIDE OR ON THE INSIDE

Results are expressed in μequiv/30 min per 7.06 cm². Means and standard deviation. Number of periods in parentheses.

	Influx			*Backflux*			*Net flux (%)*
	s.c.c.	*Na^+ flux*	%	*s.c.c.*	*Na^+ flux*	%	
Control	1.82 ± 0.44 (9)	2.15 ± 0.50 (9)	116.2 ± 2.87	1.38 ± 0.17 (9)	0.30 ± 0.18 (9)	20.7 ± 11.4	95.5
Cu^{2+} outside	2.08 ± 0.38 (8)	2.46 ± 0.42 (8)	118.5 ± 3.34	2.25 ± 0.13 (8)	0.54 ± 0.23 (8)	23.5 ± 9.2	95
Control	2.22 ± 0.43 (8)	2.35 ± 0.25 (8)	107.8 ± 10.2	1.68 ± 0.10 (8)	0.12 ± 0.02 (8)	7.2 ± 1.12	100.6
Cu^{2+} inside	3.19 ± 0.36 (8)	3.17 ± 0.19 (8)	100.1 ± 6.0	2.62 ± 0.28 (8)	0.16 ± 0.05 (8)	6.1 ± 1.4	94.0

Finally, when we used Cu^{2+} on both sides of the same skin successively first on one and then on the other, we can see that the presence of Cu^{2+} on one side does not interfere with its action on the opposite side. This is the case independent of the order in which the experiment is performed.

Concentration dependence of the Cu^{2+} effect

Different concentrations of Cu^{2+} were tested using four pieces of the same skin mounted in chambers of 3.14 cm^2 area. Cu^{2+} was used on the outside of the frog skin preparation in the usual concentration of 0.1 mM of CuSO$_4$ and in ½, ¼ and $^1/_8$ of this concentration. These were chosen after a preliminary study in which lower concentrations were tested and very often did not have any effect. Higher concentrations produced a very sharp and transitory increase of currents and voltages, followed by a drastic change to very low values, as if there was a poisoning effect with these higher doses. Fig. 3, where percent increase in current is plotted against concentration of Cu^{2+}, shows the results of these experiments. It is seen that there is a concentration-dependent effect for low concentrations of Cu^{2+} and that the effect reaches saturation with concentrations near 0.1 mM. When these studies were performed with Cu^{2-} added to the inside half-chambers, a similar but less evident relation could be observed.

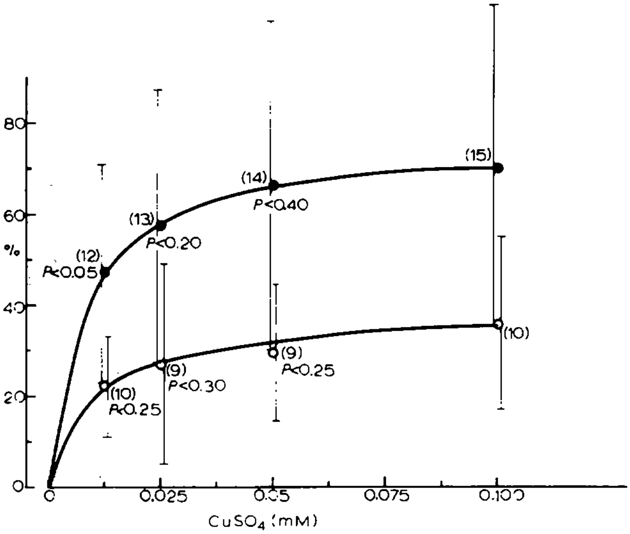

Fig. 3. Effect of different concentrations of Cu^{2+} on s.c.c. Plot of means and standard deviations of percent increases of currents against Cu^{2-} concentrations. ●—●, Cu^{2+} acting on the outside; ○—○, Cu^{2+} acting on the inside. Number of experiments for each concentration in parentheses. The results of the lower concentrations were statistically analyzed against the higher one. *P* values greater than 0.05 considered not significant (N.S.).

Interference of Cu^{2+} with Ca^{2+} and antidiuretic hormone

As considerable evidence exists of the effect of Ca^{2+} and antidiuretic hormone on frog skin, experiments were made to see if either of these chemicals shares a common site of action with Cu^{2+}. The interference of Cu^{2+} with Ca^{2+} was studied by using one concentration of Cu^{2+} (0.1 mM) and four different concentrations of Ca^{2+}, namely 0, 2.4, 4.8 and 9.6 mM. To begin with, the skins were mounted in the Ringer's solution containing the Ca^{2+} concentrations to be tested. Those ones with no Ca^{2+} contained 0.5 mM EDTA, and the Ringer's solutions were changed several times during 4 h before experimental periods were performed. After a steady preparation was obtained Cu^{2+} was added, and the increase in s.c.c. was expressed as percentage of the control value. In Fig. 4, where percent increases in currents are plotted against concentrations of Ca^{2+}, it can be seen that there is a linear relationship between the effect of Cu^{2+} and the concentrations of Ca^{2+}, so that the higher the Ca^{2+} concentration the lower the effect of Cu^{2+}. This interference of Ca^{2+} on the effect of Cu^{2+} is almost nonexistent when Cu^{2+} is added to the inside half-chamber.

Oxytocin was tested using two symmetrical pieces of skin. The skins were mounted and allowed to reach steady values. To one of them, Cu^{2+} was added either to the outside or to the inside, while the other remained as control. After a new steady state has been reached, oxytocin was added to both of them. The same procedure was repeated inverting the order of these two substances; that is, oxytocin was first added to one half-skin, and Cu^{2+} was added afterwards to both half-skins. The results are summarized in Table VII.

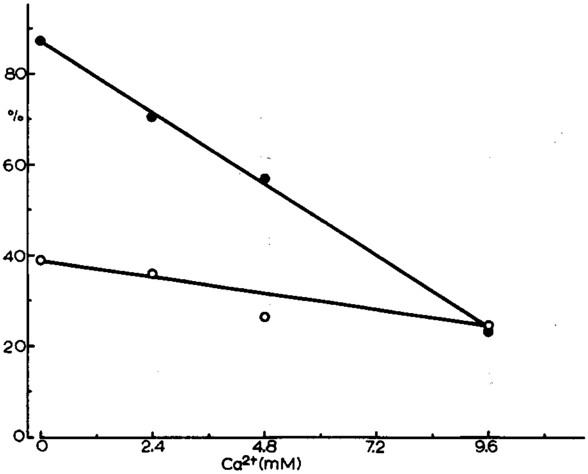

Fig. 4. Interference of Ca^{2+} on the effect of Cu^{2+}. Plot of percent increases of s.c.c. against Ca^{2+} concentrations. ●—●, Cu^{2+} acting on the outside; ○—○, Cu^{2+} acting on the inside.

It can be seen from these results that when Cu^{2+} is added to the outside the effects of both Cu^{2+} and oxytocin are fully obtained, but that is not the case when Cu^{2+} is added to the inside of the preparation. Here, the overall effect is as if oxytocin is acting on its own. In one type of experiment, the addition of the effect of Cu^{2+} and the effect of oxytocin is similar to the effect of oxytocin at the control skin. In the second one, oxytocin gives the full effect and Cu^{2+} added afterwards has almost no effect. This may suggest that Cu^{2+} acting at the inside of the frog skin has a similar site of action as oxytocin.

DISCUSSION

In 1949–1951 USSING and co-worker[1,2] described the use of $CuSO_4$ in concentrations of 10 μM in the Ringer's solution bathing the outside of frog skin. They

TABLE VII

INTERFERENCE IN THE ACTION OF Cu^{2+} AND OXYTOCIN

Absolute values of currents from control, Cu^{2+} and oxytocin periods. Means and standard deviations. Number of experiments in parentheses.

	Experimental skin (Cu^{2+} outside)			*Control skin*	
	Control	*Cu^{2+}*	*Oxytocin*	*Control*	*Oxytocin*
Current (μA/7.06 cm^2)	102.0 ± 39.2 (9)	162.4 ± 38.6 (9)	255.4 ± 80.6 (9)	111.0 ± 47 (9)	222.3 ± 55.1 (9)
ΔIncrease		60.4	93		111.3
%		59	91		100
	Control	*Oxytocin*	*Cu^{2+}*	*Control*	*Cu^{2+}*
Current (μA/7.06 cm^2)	148 ± 62 (13)	245 ± 65 (13)	314 ± 68 (13)	152 ± 82 (13)	222 ± 85 (13)
ΔIncrease		97	69		70
%		66	46		46
	Experimental skin (Cu^{2+} inside)			*Control skin*	
	Control	*Cu^{2+}*	*Oxytocin*	*Control*	*Oxytocin*
Current (μA/7.06 cm^2)	159.9 ± 48 (9)	195 ± 67 (9)	244.4 ± 80.8 (9)	159.9 ± 33.2 (9)	269.1 ± 91 (9)
ΔIncrease		35	49.4		109.2
%		22	31		68
	Control	*Oxytocin*	*Cu^{2+}*	*Control*	*Cu^{2+}*
Current (μA/7.06 cm^2)	141 ± 39 (12)	240 ± 67 (12)	234 ± 66 (12)	135 ± 52 (12)	179 ± 59 (12)
ΔIncrease		99	−6		44
%		70	−4		33

observed an increase in the o.c.v. Measurements of s.c.c. and Na^+ fluxes did not reveal any effect of Cu^{2+} on these parameters. This effect was interpreted as being due to a diminution of the permeability of the frog skin to Cl^- (from the outside to the inside) reducing therefore the shunt path of the skin to these anions. The lowering of the Cl^- influx was confirmed by KOEFOET-JOHNSEN AND USSING[3] using $^{36}Cl^-$. The effect of Cu^{+2} or the substitution of the Cl^- by a nonpenetrating anion-like SO_4^{2-} in the Ringer's solution bathing the outside of the frog skin was frequently used to get a better idea of the true electromotive force of the Na^+ pump or to study the Na^+ diffusion potentials across the outside barrier.

ZADUNAISKY *et al.*[4] using $CuSO_4$ (20 μM) on the outside of the frog skin preparation also observed an increase in o.c.v. and a simultaneous increase in s.c.c. These authors used a different species of *R. leptodactylus ocellatus*, which differs in some characteristics from the frogs of the northern European countries used by USSING and others (*R. temporaria sculenta*). In these South American frogs the s.c.c. has two components, one the Na^+ transport and the other the Cl^- transport from the outside to the inside of the frog skin. Na^+ fluxes determined when the skins were mounted in Cl^- Ringer's solution do not agree with the s.c.c. There is an excess of Na^+ fluxes as much as 40 %. When the same Na^+ fluxes were made with skins bathed in Ringer's solution where the Cl^- was substituted by SO_4^{2-} or methyl sulphate, this agreement is very good. On the other hand, Cl^- fluxes reveal a net Cl^- transport from the outside to the inside of the frog skin of an order of magnitude sufficient to explain the discrepancy of Na^+ fluxes and s.c.c. Under these conditions, the effect of Cu^{2+} was interpreted as being due to a decrease in the permeability of the frog skin to Cl^- which explained the increase in the o.c.v. and s.c.c. by decreasing the negative component of this same current. Cl^- fluxes under these conditions were not performed.

When we used $CuSO_4$ in the concentration of 0.1 mM on both sides of the frog skin preparation, we got a systematic increase in the s.c.c. and o.c.v. Na^+ fluxes measured under these experimental conditions showed that the increase in s.c.c. is due to a proportional increase of the Na^+ fluxes. Cl^- fluxes also increase in both directions but more in the outward direction giving a net Cl^- transport that can be responsible for 5 % of the s.c.c., under these conditions. These observations together with an increase in the backflux of Na^+ may be interpreted as being due to an increase of the permeability of the skin, facilitating therefore all the passive movements of these ions. The effect of Cu^{2+} stimulating the Na^+ transport seems to be a new finding not described until now. On the other hand, the increase in the passive movements of the Na^+ and Cl^- is not in agreement with the facts described in the literature.

The possibility that this effect might be a specific one of our frog species or due to the conditions under which the frogs were kept was tested with the same results as described before. We had the opportunity to study one animal of the species *R. temporaria* (kindly supplied by Dr. Zerahn), and we could observe the same increase in s.c.c. and Na^+ fluxes as observed in our frogs. Na^+ fluxes agreed very well with the s.c.c. before and after the addition of Cu^{2+}.

To test the possibility that the frogs might be depleted of Cu^{2+}, some experiments were done using a batch of frogs kept in water containing bits of Cu^{2+} tubing. The response to Cu^{2+} *in vitro* of the skins of frogs kept under these conditions was similar.

Cu^{2+} concentrations used in our experiments were higher than those utilized by other authors, but they were chosen after some preliminary experiments in which some other concentrations were tested, namely 1 μM, 10 μM, 1 mM and 10 mM. The lower concentrations did not always have an effect, but when it existed the effect was simultaneous on currents and voltages. Higher concentrations gave a sharp positive response followed by a drop to values lower than the control values.

The results might be due to the fact that we used Cu^{2+} on both sides of the frog skin preparation. Therefore, Cu^{2+} was used separately on the outside and on the inside of the preparation. Although the overall results were similar, some differences were observed. The effect on the s.c.c., Na^+ fluxes and the o.c.v. is evident on both sides but more intense on the outside. The o.c.v. is very little affected by Cu^{2+} added to the inside; therefore, a decrease in the d.c. resistance is more marked under these conditions.

The concentration dependence in a range of 10–100 μM of $CuSO_4$ is more evident when tested on the outside of the frog skin, and so is the dependence of the effect on Ca^{2+} concentration between a range of 0–9.6 mM, using a fixed concentration of Cu^{2+}.

This suggests a different mechanism of action when Cu^{2+} is acting on the outside or on the inside, as if different sites of action were available. In order to test this hypothesis further, the interference of Cu^{2+} with oxytocin was also studied.

No interference with oxytocin could be detected when Cu^{2+} was acting from the outside, but it was marked when Cu^{2+} was acting from the inside. It cannot be excluded that when both these substances are added to the same Ringer's solution bathing the inside of the preparation they may interfere with each other; however, it may be possible that the effect of Cu^{2+} acting on the inside of the frog skin is at the same site as oxytocin, while it is mainly at the same site as Ca^{2+} when acting on the outside of the frog skin. The existence of two parallel sites of passage of Na^+ in the frog skin, one sensible to the concentration of Ca^{2+} in the Ringer's solution and Cl^- dependent, and the other sensible to oxytocin and Cl^- independent, was already mentioned by HERRERA AND CURRAN[9].

The possibility that copper is acting from the outside on one site and from the inside on another site is a complicated mechanism of action for a substance, but using the knowledge of the ultrastructure of the frog skin from studies of FAQUARD AND PALLADE[10, 11] and the model proposed by CEREIJIDO AND ROTUNO[12], it may be possible that Cu^{2+} on the outside acts directly by competing with Ca^{2+} on the tight junctions of the epithelium, and when acting on the inside it must penetrate the skin (the time constant of the action on this side is in fact much longer) and acts directly on the permeability of the cell membranes, as has already been postulated for oxytocin.

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