Biochimica et Biophysica Acta, 510 (1978) 298—304

BBA 78062

THE EFFECT OF COPPER ON FROG SKIN

THE ROLE OF SULPHYDRYL GROUPS

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(Received November 29th, 1977)

Summary

1. Cu^{2+} at a concentration of 10^{-4} M, when applied to the external side of the frog skin produces an increase in the short-circuit current (I_{sc}).

2. This effect was studied in skins of *Rana temporaria* adapted to cold (5°C) and room temperature (20°C), skins of *Rana pipiens* adapted to cold, and the results compared with those obtained previously with *Rana ribibunda*.

3. The observed effect is less dependent upon the adaptation to cold than upon the functional state of the skin: skins with low short circuit currents have a bigger response to Cu^{2+} than skins with high I_{sc}.

4. A species difference cannot be ruled out since skins of *Rana ribibunda* exhibiting high I_{sc} give good responses to Cu^{2+}.

5. 5,5′-dithiobis(2-nitrobenzoic acid), a sulphydryl-oxidizing reagent, produces an effect similar to that of Cu^{2+}, and dithiothreitol an SH-reducing agent, reverses the effect of this ion.

6. Cu^{2+} also induces an increase in the unidirectional K^+ fluxes and unmasks a net outward potassium flux.

7. The outward K^+ flux induced by Cu^{2+} is sensitive to ouabain.

8. It is concluded that Cu^{2+} increases the permeability of the external barrier of the frog skin to Na^+ and K^+, probably by reacting with SH groups.

Introduction

Cu^{2+} as copper sulphate at a concentration of 10^{-5} M was originally reported by Ussing [1] to increase the frog skin's open circuit potential without affect-

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This work is part of a Ph.D.thesis submitted to the University of Cambridge.

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

ing the short circuit current (I_{sc}) the effect resulting from a decrease in the overall Cl^- permeability of the preparation (*Rana temporaria*). These observations were later confirmed by Ussing and Zerahn [2] and by Koefoed-Johnsen and Ussing [3]. Zadunaisky et al. [4] using *Leptodactylus ocelatus*, were able to show a decrease in Cl^- fluxes with a simultaneous increase in I_{sc} (in this preparation, in presence of NaCl, the I_{sc} is composed of a net inward flux of Na^+ minus a net inward Cl^- flux). Ferreira [5] showed in *Rana ribibunda* an increase in the I_{sc}, which could be entirely accounted for by an increase in the net Na^+ flux, using Cu^{2+} at a concentration of 10^{-4} M. Less consistent results were reported by Cuthbert et al. [6] who with *R. temporaria*, obtained an increase in the I_{sc} in 2 out of 5 experiments and a decrease in the other 3.

More recent work done by Koefoed-Johnsen et al. [7] and Lyon [8] in *R. temporaria* showed that this discrepancy might be due to the way in which frogs were kept before being used for experimental purposes; frogs kept in cold rooms responded to copper ions with an increase in the I_{sc} and no change in Cl^- permeability, while animals adapted to room temperature showed changes in Cl^- permeabilities, with no effect on the I_{sc}.

In this study we attempted to confirm the observations made by these authors, and investigated whether there is a species difference.

As Cu^{2+} is a sulphydryl-oxidizing reagent [9] and since sulphydryl-oxidizing reagents have been used extensively on red blood cells [10—12] and on squid axon [13] to increase the cation permeability (specially to K^+), we decided to investigate the hypothesis that the effect of copper could be due to its interaction with the SH groups of the skin [14].

Therefore we studied the effect of Cu^{2+} on the I_{sc} together with another oxidizing reagent (DTNB) and a reducing agent (Cleland reagent), and also its effect on the K^+ fluxes across the frog skin.

Methods

Frogs of the species *R. temporaria* or *Rana pipiens* were used. They were kept either at room temperature (20°C) or in a cold room for 2 weeks (4—6°C). Frogs were double pithed and skins mounted in Ussing type chambers between two neoprene O-rings to avoid edge damage. The area of the chambers was 3.14 cm^2. Whenever necessary two symmetrical pieces from the ventral skin were used. Short circuit current were measured by an automatic voltage clamp device and conductances by means of a current deflection produced by a known voltage pulse. $^{42}K^+$ fluxes were measured after skins reached a steady state of current and conductance, and after 2 h of equilibration time with the isotope. The chamber was emptied completely after each period and the Ringer collected directly into the counting vials, and replaced by means of a calibrated syringe. The volume used was 4 ml. Samples were counted in a γ-counter with a preset count of 10^4 and counts were all corrected for decay to the same initial time. The Ringer's solution used had the following composition (in mM): Na^+, 112; Cl^-, 119; K^+, 3; Ca^{2+}, 1; Mg^{2+}, 1. The aerated Ringer's solution were titrated with Tris buffer to a pH of 8. The osmolality of the Ringer was 220 mOsM. $^{42}K^+$ was obtained from Amersham, U.K. The copper sulphate, DTNB and dithiothreitol Ringers were used at a concentration of 10^{-4} M and the pH

kept at 8. All solutions were prepared just before use. Results are expressed as means and standard errors of the mean.

Results

(1) Dependence of Cu^{2+} effects on adaptation to cold

The effect of copper on the external side of the skin of *R. temporaria* is very irregular. From 27 cold-adapted animals 18 responded to copper with an increase in I_{sc} (67%) and from 27 warm-adapted animals 14 skins responded with an increase in I_{sc} (52%). The magnitude of the effect on both currents and conductances can be seen in Table I. Only the skins that responded to copper are reported in the table. Furthermore the skins that did not respond to Cu^{2+} had much higher currents (mean value of 19 skins: 26.2 ± 2.2 $\mu A/cm^2$), than the ones that responded to Cu^{2+} (16 ± 2 $\mu A/cm^2$).

In *R. pipiens* the effect of Cu^{2+} was apparent in almost all skins. Only cold adapted animals were used due to the high mortality rate of these animals at laboratory room temperatures. These skins had in general much lower currents and higher conductances. The overall results can be seen in Table I. We added for comparison the results obtained with *R. ridibunda* at 5°C [5].

(2) The effect of sulphydryl reagents

The interference of Cu^{2+} and DTNB was studied in paired half-skins. For one of them Cu^{2+} was added first, and when the I_{sc} reached a steady level DTNB was tested. In the second half-skin the reverse order was used (Fig. 1). It can be seen that both Cu^{2+} and DTNB stimulate the I_{sc} in a similar way (first part of the curves). However when one of the substances is added after the other its effect is less evident (Table II). Independently of the order in which the substances are applied the final effect is very much the same. It was also observed that those skins that do not respond to copper do not respond to DTNB.

In our experience the effect of Cu^{2+} on the I_{sc} is not reversed by washing the chambers several times with Cu^{2+}-free Ringer. Yet as can be seen in Fig. 2 and Table III, when under the same conditions dithiothreitol is added the effect is reversed, bringing the I_{sc} to the control value. In 4 of the half skins used as controls, where dithiothreitol was tested alone, it had only a slight effect. Since the pK_a of the SH groups is between 7 and 10 [15] and the pH at which

TABLE I

THE EFFECT OF Cu^{2+} ON THE I_{sc} AND G_t OF DIFFERENT FROG SPECIES

Results expressed as means and standard errors of the mean. Number of experiments in brackets.

I_{sc} ($\mu A/cm^2$)			G_t ($mohm^{-1}/cm^2$)		
Control	Cu^{2+}	Δ	Control	Cu^{2+}	Δ
R. temporaria (30)					
15.9 ± 2.0	23.6 ± 2.2	48%	0.292 ± 0.029	0.363 ± 0.032	23.9%
R. pipiens (18)					
6.85 ± 0.72	19.04 ± 1.32	178%	0.732 ± 0.089	0.796 ± 0.08	8.7%
R. ridibunda (18)					
22.4 ± 2.5	35.7 ± 3.2	57%			

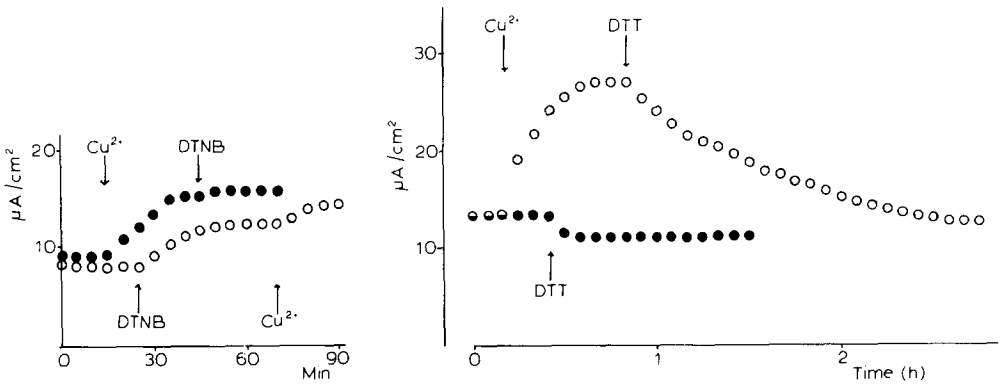

Fig. 1. The effect of copper and DTNB on the I_{sc} of two paired half-skins. In one of them copper was added first; in the second one DTNB was tested first; After the effect on the I_{sc} reached a steady state the Ringer was removed and the second drug was tested, in the reversed order.

Fig. 2. The effect of Cu^{2+} and dithiothreitol on the I_{sc} of two paired half-skins. In one of them Cu^{2+} was added first and when the I_{sc} reached a steady state the Ringer was removed and dithiothreitol was tested. In the second half skin dithiothreitol was tested by itself.

the experiments were performed is 8 there are always some oxidized forms of the SH groups that might be available to the reducing agent.

(3) The effect of Cu^{2+} on $^{42}K^+$ fluxes

$^{42}K^+$ was added either to the outside or to the inside of two paired half-skins. After an equilibration time of 2 h, fluxes were measured during 4 periods of 15 min each. Cu^{2+} was then added to the external solution and after 20 min another four periods of fluxes were measured. The results reported in Table IV correspond to 4 influx and 7 outflux experiments. Before the addition of Cu^{2+} the average influx of $^{42}K^+$ was slightly larger than the backflux but the difference between these averages is less than 1% of the I_{sc}. Copper induces an increase both in influx and outflux of $^{42}K^+$ but while the effect on the influx is small (although statistically significant $P < 0.01$) the effect on the outflux is quite substantial. This asymmetric stimulation of the $^{42}K^+$ fluxes suggests that

TABLE II

THE INTERACTION OF Cu^{2+} AND DTNB ON THE I_{sc}

Results expressed as means and standard errors of the means (%, 5 experiments). (a) The effect on I_{sc} ($\mu A/cm^2$) of Cu^{2+} followed by the effect of DTNB.

Control	Cu^{2+}	DTNB
8.54 ± 0.84	14.97 ± 1.50	15.99 ± 1.37
	76.8 ± 12.8	14.6 ± 7.9
(b) DTNB followed by Cu^{2+}		
Control	DTNB	Cu^{2+}
8.28 ± 0.41	13.38 ± 0.80	15.6 ± 1.53
	63.6 ± 14.6	31.8 ± 8.6

TABLE III

THE EFFECT OF DITHIOTHREITOL ON THE I_{sc} PREVIOUSLY STIMULATED BY Cu^{2+} (11 EXPERIMENTS)

In 4 half-skins the effect of dithiothreitol by itself was tested on the I_{sc} ($\mu A/cm^2$). Results expressed as means and standard errors of the means.

Control	Cu^{2+}	Dithiothreitol
21.28 ± 3.9	30.98 ± 4.1	20.47 ± 4.0
20.0 ± 5.1	—	17.9 ± 4.7

TABLE IV

THE EFFECT OF Cu^{2+} ON ^{42}K FLUXES IN FROG SKINS

Results expressed as means and standard errors of the mean. Number of experiments in brackets. Each period is of 15 min. After Cu^{2+} was added 20 min elapsed before the next steps were performed. (a) K fluxes in (μequiv./cm^2 · h) × 10 (control).

Period	Influx (4)	Backflux (7)
1	0.186 ± 0.043	0.121 ± 0.024
2	0.168 ± 0.032	0.123 ± 0.041
3	0.161 ± 0.027	0.122 ± 0.042
4	0.163 ± 0.029	0.128 ± 0.049

(b) Cu^{2+} 10^{-4} M outside

Period	Influx	Backflux
5	0.191 ± 0.033	0.109 ± 0.055
6	0.175 ± 0.029	0.270 ± 0.099
7	0.184 ± 0.026	0.906 ± 0.602
8	0.230 ± 0.033	1.848 ± 1.12

TABLE V

^{42}K BACKFLUXES IN TWO PAIRS OF PAIRED HALF-SKINS, TO TEST THE EFFECT OF OUABAIN ON THE INCREASED ^{42}K BACKFLUXES PRODUCED BY Cu^{2+}

Each period is 30 min. After 4 control periods Cu^{2+} is added to one half and Cu^{2+} and ouabain to the other half of the same skin. 20 min elapsed before the other 4 steps were performed. By the 2 last periods the I_{sc} was already 0. K fluxes in (μequiv./cm^2 · h) × 10. Between periods 4 and 5 Cu^{2+} was added to the 1st half and Cu^{2+} + ouabain to the 2nd half of each skin.

Period (30 min)	Skin I		Skin II	
	1st half	2nd half	1st half	2nd half
1	0.124	0.148	0.112	0.113
2	0.136	0.146	0.110	0.111
3	0.119	0.130	0.101	0.108
4	0.113	0.114	0.104	0.106
5	0.335	0.272	0.110	0.145
6	0.982	0.537	0.368	0.384
7	0.890	0.636	0.755	0.392
8	0.487	0.306	0.761	0.179

the Cu^{2+}-induced increase in the permeability to K of the external barrier unmasks an active K transport. This K pump can either be an independent one as described by Finn [16] in toad bladder, or Valenzeno and Hoshito [17] in *R. pipiens*, or the Na-K exchange pump described by Huf et al. [18] in *R. pipiens*, Koefoed-Johnsen [19] and Koefoed-Johnsen and Ussing [3] in *R. temporaria*.

If this pump is pumping K into the cells across the internal barrier it can only produce a net outward flux if the external barrier gets permeable to potassium. Otherwise as it happens under normal conditions the K recirculates across the inner barrier.

Ouabain was tested on the increased $^{42}K^+$ outfluxes induced by Cu^{2+}, to see if the Na-K ouabain-sensitive exchange pump can be responsible for the net outward K flux. The results of these experiments can be seen in Table V. Following 4 control periods ouabain (10^{-3} M) was added to one half-skin while Cu^{2+} was added to both. After 20 min 4 periods of 30-min fluxes were measured. The reason for this is that ouabain takes almost 1 h to abolish the I_{sc}. It can be seen that ouabain reduces the Cu^{2+}-stimulated $^{42}K^+$ backfluxes substantially.

Discussion

The possibility that the effect of copper on the I_{sc} could be related to a previous adaptation to cold of the frogs [7,8] was not confirmed in our experiments. It seems rather that it is the functional state of the skin that conditions the effect of copper. High I_{sc} and low conductance preparations respond poorly if at all to copper. This was clearly seen in *R. temporaria*. The batch of *R. pipiens* studied had a very low average control I_{sc} and exhibit a large response to copper. A species difference cannot be ruled out since *R. ridibunda* seems to respond to copper even when their basal I_{sc} are not low.

Our results show that Cu^{2+} stimulates the frog skin I_{sc} by a mechanism similar to that of DTNB a well known sulphydryl oxidizing reagent and is antagonized by dithiothreitol a sulphydryl reducing agent. We therefore suggest that the effect of Cu^{2+} on the I_{sc} can be explained by its oxidizing effect on the SH groups of the skin and furthermore that this state of the SH groups favours Na^+ transport. Also like sulphydryl oxidising reagents Cu^{2+} increases the transepithelial K fluxes, the outflux increasing much more than the influx. This corresponds to an "active" K transport probably resulting from an active uptake across the internal barrier and a passive diffusion across the external barrier. In the absence of Cu^{2+} and in steady state the potassium pumped in the cells across the internal barrier leaks out across the same barrier. If now the permeability of the external barrier is increased by Cu^{2+} one should expect that a much larger fraction of the pumped K, leaks out through the external barrier and consequently that the net flux of K across the preparation should increase. This was shown by the K unidirectional fluxes. From a mechanism like the one described above one should predict the flux ratio to be constant but the difference between the fluxes to increase. Yet the results show that the flux ratio (out/in) increased from 0.7 (control) to a final value of 8 (after adding Cu^{2+}). This may be explained if one considers that under normal conditions the transepithelial $^{42}K^+$ fluxes are mainly paracellular. If the effect of copper were on the

paracellular route both fluxes would increase and the flux ratio would be the same. The results above can only be due to an effect on the cell membrane so that the potassium that is pumped in across the basolateral membranes diffuses out across the external barrier. Similar results were obtained by Nielsen [20] with Amphotericin B.

The effect of ouabain suggests that the Na-K pump is responsible for the net outflux observed in the presence of Cu^{2+}. The fall in the outward $^{42}K^{+}$ fluxes produced by the glycoside is probably due to the inhibition of the Na-K exchange pump and consequently to a fall in the intracellular concentration of K [21]. However the potassium fluxes will remain higher than in the control periods not only because the permeability of the mucosal barrier is increased but also because the decrease in cell potential induced by ouabain [22] will favour an outflux of K.

Acknowledgement

I wish to thank Professor R.D. Keynes for helpful discussions and Dr. H.G. Ferreira for his critical reading of the manuscript.

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