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THE MECHANISM OF ACTION OF Cu^{2+} ON THE FROG SKIN

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Summary

The mechanism of action of Cu^{2+} when applied to the external side of the frog skin preparation was investigated.

Cu^{2+} acts most probably on the external barrier of this preparation, since it increases the transport pool of Na^+ proportionally to the increase in the short circuit current (I_{sc}).

Cu^{2+} does not open new routes for the Na^+ entry since the stimulated I_{sc} is still completely abolished by amiloride.

The I_{sc} dependence of Na^+ concentration in the external medium is modified by copper, since the K_m value increases in addition to changes in V.

It is suggested that copper acts at the external barrier Na channels in a way similar to that proposed by Zeiske and Lindemann ((1974) Biochim. Biophys. Acta 352, 323—326) for benzoylimidazole-2 guanidine and benzoylthiazole-2 guanidine and by Dick and Lindemann ((1975) Pflügers Arch. ges. Physiol. 355, R72) for *para*-chloromercuribenzenosulfonate and *para*-chloromercuribenzoate.

Introduction

In a previous paper [1] we showed that Cu^{2+} ($1 \cdot 10^{-4}$ M) produced an increase in the short circuit current (I_{sc}) and open circuit voltage of the isolated skin of *Rana ridibunda* and that the increase in I_{sc} was entirely due to an increase in the Na^+ net transport across the preparation.

In this paper we attempt to clarify whether the Cu^{2+} induced stimulation of the I_{sc} is due to an increase in the permeability of the external barrier to Na^+ or to a primary effect on the pumping mechanism for Na^+.

On the other hand, since Cu^{2+} also increases the back-fluxes of ^{22}Na and ^{36}Cl and since the effect of Cu^{2+} is inhibited by external Ca^{2+} [1], we studied also the permeability of the paracellular route by measuring [^{14}C]Mannitol fluxes across the preparation.

Methods

Frogs of the species *Rana ridibunda* Pallas were used. They were kept in a cold room at 4 to 6° C. Experiments were performed from July to December. The skins were mounted in Ussing type chambers of 3.14 cm^2. The short circuit current (I_{sc}) was measured by an automatic device using operational amplifiers and a potentiometric multichannel recorder with calibrated input resistances, in order to read the current values directly. Open circuit voltage (PD) was measured with a high impedance voltmeter (Keithley 610B). Experimental protocols were performed only after these parameters had reached steady values. The Ringer solution has the following composition (mM): Na^+, 114; Cl^-, 121; K^+, 2.4; Ca^{2+}, 2.4. The aerated Ringer solutions were titrated with Tris buffer to a pH of 8 and had an osmolality of 220 mOsm.

Cu^{2+}, as $CuSO_4$, was always used at a concentration of $1 \cdot 10^{-4}$ M on the external side. The effect of amiloride at a concentration of $1 \cdot 10^{-4}$ M was tested on skins previously treated with copper.

Determination of the exchangeable Na^+ from the outside of the frog skin. An equilibration period of 30 min was considered sufficient for the Na^+ involved in the transepithelial transport to reach the same specific activity as the $^{22}Na^+$ from the Ringer, since at the end of this time the transmembrane $^{22}Na^+$ flux reaches a steady value [2].

Paired half skins were mounted in Ussing type chambers. When the I_{sc} was steady, $^{22}Na^+$ was added to the external side of both half skins while Cu^{2+} was added to only one of them. A new steady state was reached after 20 min and after 30 min the skins were removed from the chambers without previous rinsing and were blotted, weighed and placed in 10 ml of 0.1 N HNO_3 for 17 h. Samples were taken to count $^{22}Na^+$. [^{14}C]Mannitol was used as extracellular marker. Intracellular $^{22}Na^+$ was calculated from the total amount measured and conveniently corrected for the extracellular fluid, assuming that in this space the $^{22}Na^+$ is at the same specific activity as in the Ringer.

The effect of Cu^{2+} on transepithelial fluxes of extracellular markers like Mannitol was studied and compared with the effect of hypertonic Ringer solutions on the external side of the frog skin preparation. In the latter case Ringer solution was made up to 400 mOsm with glucose. Paired half skins were used to measure influxes and back-fluxes. After 1 h equilibration with [^{14}C]Mannitol, 30-min periods were defined and the whole fluid of the unlabelled side was collected. Suitable amounts were mixed with scintillation fluid and counted.

The Na^+ concentration dependence of the I_{sc} was studied in the presence and absence of Cu^{2+}. Six pieces of skin from the same frog (60 g weight) were mounted to test six Na^+ concentrations. A control period with a 114 mequiv./l Na^+ Ringer was made before and after each experimental period. For each piece of skin the I_{sc} under control conditions was considered 100%. All the other values are given as percentages of these control values. The method and assumption used were already described in a previous paper [3]. Na^+ was substituted by magnesium on both sides of the skin in order to avoid any electrochemical gradients across the preparation. All Ringer solutions were made up to the same osmolality with glucose.

Results

As a measure of the permeability of the paracellular route we used fluxes of [^{14}C]Mannitol. In Table I, lower panel, we can see that when we increase the osmolality of the external solution to 400 mOsm with glucose, the inward and outward fluxes of [^{14}C]Mannitol increase, in agreement with the results reported by Ussing [4,5] and Franz and van Bruggen [6], corresponding probably to an opening of the tight junctions [7]. Yet, as shown in the upper panel, no such effect is observed when Cu^{2+} is added to the external solution. It can be seen as well that while in the experiments where the hypertonic solutions were used at the outer face of the frog skin, the total conductance increases, in the Cu^{2+} experiments the total conductance does not change. This could be explained by an increase in the Na^+ conductance with a simultaneous decrease in the shunt conductance. Therefore, the increase in Na^+ and Cl^- back-fluxes as described in a previous paper remain to be explained.

The transport pool of Na^+. If Cu^{2+} increases primarily the permeability of the mucosal barrier one should expect the transport pool to increase. In fact, the amount of intraepithelial Na^+ that exchanged with Na^+ from the external medium over a period of 30 min increased by 100% in the presence of external Cu^{2+} (3.35 ± 0.41 vs. 6.73 ± 0.53 mequiv./kg wet wt.). This increase agrees with the observed increase in I_{sc} (15.9 ± 1.64 vs. 30.0 ± 2.0 μA/cm^2). (See also Fig. 1).

Effect of copper on the external barrier. If Cu^{2+} increases the permeability of the external barrier to Na^+ one should ask whether this increase is due to the opening of new channels for Na^+, or to an increase in a nonspecific cation permeability, or if it is due to a modification of the physiological Na^+ entry mechanism: increase in affinity and/or capacity of this mechanism. The fact that amiloride at $1 \cdot 10^{-4}$ M inhibits 100% of the Cu^{2+}-stimulated I_{sc} (Fig. 2) supports the latter hypothesis, since Nielsen [8] showed recently that when the I_{sc} is increased by opening an alternative route for Na^+ entry (with Filipin) the increased current is amiloride-insensitive.

TABLE I

[^{14}C]Mannitol fluxes in the presence of $1 \cdot 10^{-4}$ M $CuSO_4$ outside, or in the presence of hypertonic solution outside (400 mOsm), compared with fluxes under control conditions. Each value is the result of six experiments with two periods of 30 min performed for each condition. Conductances were measured in the middle of each period (obtained from I_{sc}/PD). The results are expressed as means and standard errors of the mean. *P*, (permeability) expressed in cm · s^{-1} × 10^8; *G*, (conductance) (glucose) expressed in mmho/cm^2.

	Influx		Back-flux	
	Control	Cu^{2+}	Control	Cu^{2+}
P	10.86 ± 1.67	9.63 ± 1.47	9.23 ± 1.8	9.44 ± 1.86
G	0.32 ± 0.04	0.33 ± 0.04	0.34 ± 0.03	0.4 ± 0.02
	Control	Hypertonic	Control	Hypertonic
P	10.65 ± 3.0	26.75 ± 4.5	6.89 ± 1.8	11.92 ± 1.9
G	0.23 ± 0.02	0.48 ± 0.05	0.22 ± 0.02	0.40 ± 0.03

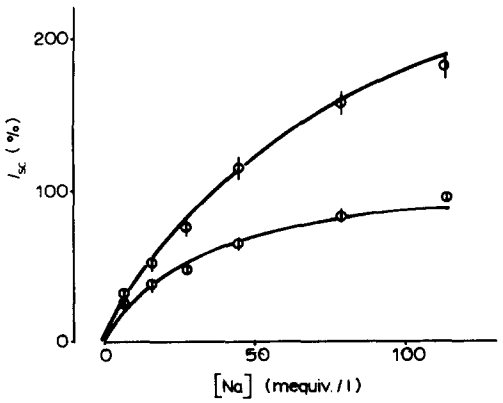

Fig. 1. Direct plot of the percentual I_{sc} as a function of the Na^+ concentration of the Ringer solution. The I_{sc} of the experimental periods is expressed as percentage of the I_{sc} measured when full NaCl Ringer is used, in order to normalize the results.

That hypothesis was further tested by studying the Na^+ dependence of the I_{sc} in the presence and in the absence of external Cu^{2+}. The results are reported in Fig. 1. The continuous curves were drawn by least-square fitting, assuming simple Michaelis-Menten kinetics, and show that both the maximum I_{sc} and its K_{Na} increased (V 115 vs. 337% and K_m 33 vs. 91 mM).

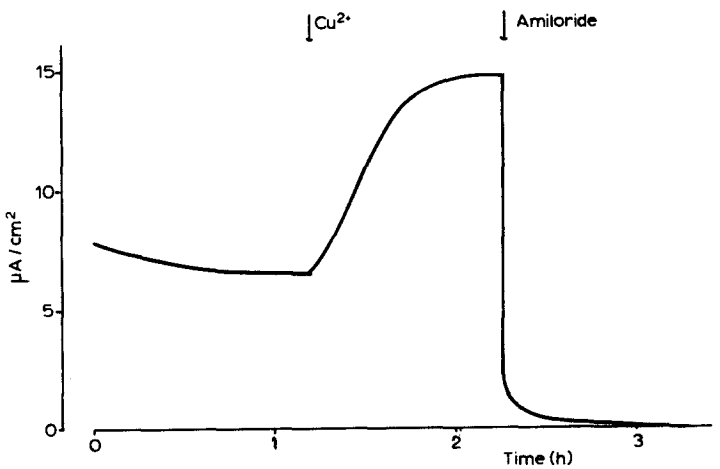

Fig. 2. The effect of Amiloride at $1 \cdot 10^{-4}$ M on the I_{sc} of the frog skin, after treatment with Cu^{2+} on the external side of the frog skin preparation.

Discussion

The results reported in this paper confirm our earlier observations [1] that Cu^{2+} ($1 \cdot 10^{-4}$ M) when applied to the external surface of the frog skin, increases the I_{sc}.

Cu^{2+} does not seem to affect the permeability of the tight junctions to non-polar molecules like [^{14}C]Mannitol. The Cu^{2+} stimulation of the I_{sc} is not due to opening of new pathways for the Na^+ translocation, since the I_{sc} can still be completely abolished by amiloride. The effect of Cu^{2+} on the I_{sc} occurs together with an increase of the fraction of the intracellular Na^+, which can be labelled in 30 min from the external solution. Furthermore, the I_{sc} dependence on Na^+ is clearly modified by copper, since both the K_m and the maximum transport rate are increased.

Similar effects were described by Dick and Lindemann [9] with *para*-chloromercuribenzoate (PCMB) and *para*-chloromercuribenzenosulphonate (PCMBS) and by Zeiske and Lindemann [10] with benzoylimidazole-2 guanidine and benzoylthiazole-2 guanidine and more recently by Zeiske with uranyl ions [11]. It was shown by us [1] that Cu^{2+} on the external side does not interfere with the effect of the antidiuretic hormone (ADH), and it was shown above that it does not open new channels like polyene antibiotics do.

We suggest, therefore, that Cu^{2+} may act as the external barrier of the frog skin, on the sodium channels interfering with the 'substrate inhibitor' effect of the high Na^+ concentration (described by Fuchs et al. [12]), in such a way that it changes the saturation properties of these channels as happens with the other substances mentioned above.

Nevertheless, an additional effect on the sodium pump cannot be ruled out completely.

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References

1 Ferreira, K.T.G. (1970) Biochim. Biophys. Acta 203, 555—567
2 Cereijido, M. and Rotunno, C.A. (1967) J. Physiol. 190, 481—497
3 Ferreira, K.T.G., Guerreiro, M.M. and Ferreira, H.G. (1973) Biochim. Biophys. Acta 291, 269—273
4 Ussing, H.H. (1963) Acta Physiol. Scand. 59, (suppl. 213), 155—156
5 Ussing, H.H. (1966) Ann. N.Y. Acad. Sci. 137, 543—555
6 Franz, T.J. and van Bruggen, J.T. (1967) J. Gen. Physiol. 50, 933—949
7 Dibona, D.R. and Civan, M.M. (1973) J. Membrane Biol. 12, 101—128
8 Nielsen, R. (1977) Acta Physiol. Scand. 99, 339—411
9 Dick, H.J. and Lindemann, B. (1975) Pflügers Arch. ges. Physiol. 355, R72
10 Zeiske, W. and Lindemann, B. (1974) Biochim. Biophys. Acta 352, 323—326
11 Zeiske, W. (1978) Biochim. Biophys. Acta 509, 218—229
12 Fuchs, W., Larsen, E.H. and Lindemann, B. (1977) J. Physiol. 267, 137—166