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REFERENCES

1 A. A. TEIXEIRA-PINTO, L. L. NEJELSKI, J. L. CUTLER AND J. H. HELLER, *Exptl. Cell Research*, 20 (1960) 548.
2 E. J. W. VERWEY AND J. TH. G. OVERBEEK, *Theory of the Stability of Lyophobic Colloids*, Elsevier, Amsterdam, 1948.
3 C. A. COULSON, *Electricity*, Oliver and Boyd, Edinburgh, 1958, p. 42.
4 G. P. HARNWELL, *Principles of Electricity and Electromagnetism*, McGraw-Hill, New York, 1949, p. 75.
5 E. MUTH, *Kolloid Z.*, 41 (1927) 97.
6 E. BRANLY, *Compt. rend.*, 111 (1890) 785; 112 (1891) 90.
7 K. S. COLE, *Cold Spring Harbor Symposia Quant. Biol.*, 1 (1933) 107.
8 T. OGAWA, *J. Appl. Phys.*, 32 (1961) 583.

THE EFFECT OF LOW CONCENTRATIONS OF THIOL-GROUP-BLOCKING AGENTS ON THE OUTER MEMBRANE OF FROG SKIN

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SUMMARY

The effect of some thiol-group-blocking agents in the solution bathing the outside of isolated frog skin was investigated by a simple method permitting of measuring two separate membrane potentials in the skin and of membrane d.c. resistances to individual ions. It was found that, while higher concentrations of these agents bring about a drop in the membrane potentials and permeability changes, similarly to mercuric chloride, lower concentrations, after a short period of incubation of the skin, cause considerable hyperpolarization of the outer membrane. The hyperpolarization is explained as a result of increased permselectivity of the outer membrane in USSING's model of frog skin. The conditions of the reaction involved are described and a crude model of the outer membrane is suggested.

INTRODUCTION

KOEFOED-JOHNSEN AND USSING's[1] model explaining the nature of the potential difference across the frog skin involves two membranes of different properties. It was only the microelectrode technique, which is less suitable for continuously following the effects of external factors, that was used recently with the result of two membrane potentials as proposed by the above model being demonstrated[2,3]. The authors of a

more recent paper[3] supply evidence for a different localization of these two separate potentials than assumed previously, but it seems that this does not invalidate other, well founded properties of the above model. In the author's knowledge the older method of STEINBACH's[4] has not been used since the time of its description.

In the present paper, the author attempted, by a simple combination of several previously applied methods, to measure these two separate membrane potentials and the d.c. resistances of both membranes with respect to individual ions on the basis of the potential–current characteristics in different solutions. The procedure was applied to the investigation of the effect of the thiol-group-blocking agents in the medium bathing the outside of frog skin, where analogies could be expected to exist with respect to the effects of cupric ions[5] and of mercuric chloride[6].

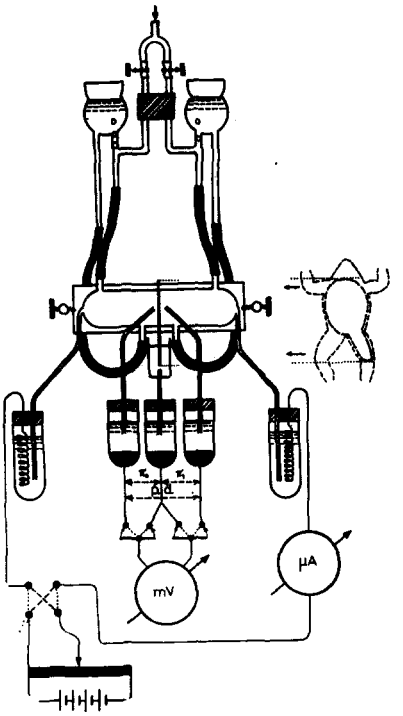

Fig. 1. The apparatus used for measuring two separate membrane potentials and of membrane d.c. resistances in frog skin. For explanation see text.

METHODS

The apparatus used is shown in Fig. 1. The method is a combination of one of STEINBACH[4] and the one used by USSING AND ZERAHN[7]. The ventral skin of the frog (*Rana temporaria*) is cut off from the animal killed by decapitation in the way shown in the above figure, clamped between plexiglas chambers and a piece of the skin from the leg is left to hang out without being damaged by clamping, and connected by means of 0.11 *M* KCl with a calomel electrode. Between this electrode and either of the two electrodes connected by bridges prepared from 3% agar containing 3 *M* KCl with solutions on both sides of the skin, it is possible to measure an injury potential, the

sum of the two equaling the total potential difference across the skin. The unequal changes of both these potentials due to a variety of external factors (*e.g.* the well-known effect of oxytocin, Fig. 2) supplies evidence that this equality is not caused by a division of the total potential difference on the skin surface, but rather that the values measured are approximately identical with the two membrane potentials established in frog skin by using microelectrodes.

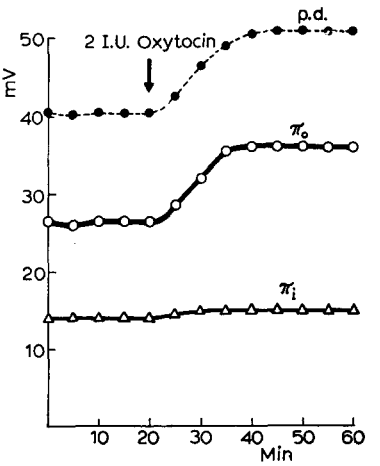

Fig. 2. The effect of 2 I.U. of synthetic oxytocin in the solution bathing the inner surface of frog skin on the individual membrane potentials in steady-state, the frog skin being placed in normal frog Ringer. π_o = potential across the morphologically outer membrane, π_i = potential across the inner membrane, p.d. = potential across the whole skin measured for checking.

It was found by GERSTNER[8], using a technique different from ours, and by CRANE AND DAVIES[9], using a technique similar to the one used here, that in working with whole frog skin the Ohm Law holds at low current densities. The Ohm Law was found here to express the relationship between the current (as applied from a battery by means of two silver chloride electrodes commonly used for short-circuiting experiments) and the two membrane potentials.

The same is true for the skin in modified Ringer solutions (choline Ringer, sulphate Ringer); for this reason the d.c. resistance in choline Ringer (either measured as the slope of the potential–current straight line or, more commonly, derived from the change in the membrane potential caused by a current of 10 μA/cm^2) is taken here as the d.c. resistance of the membranes to chloride ions, as choline does not permeate through frog skin and ions other than sodium and chloride participate only negligibly in the electrically measured d.c. conductance of the whole frog skin in Ringer's solution[6]. Therefore, changes of the d.c. resistance are very likely to reflect the changes in the permeability to chloride ions.

The Ringer solutions used contained 112 mM NaCl (112 mM choline chloride in choline Ringer), 1.88 mM KCl, 0.89 mM $CaCl_2$, 2.38 mM Tris buffer; pH was 8 ± 0.1, the same as in the central vessel with 111 mM KCl, 2.38 mM Tris buffer. The total volume of Ringer's solution in each of the two parts of apparatus was 25 ml; to ensure circulation, atmospheric air without carbon dioxide was bubbled through the system. The exposed skin area was 3.14 cm^2.

RESULTS

Sodium *p*-chloromercuribenzoate and *N*-ethylmaleimide are reported in the literature to be specific agents for blocking –SH groups[10,11]. These compounds, if added to normal Ringer's solution bathing the outside of the frog skin at a final concentration of 0.1 m*M* or somewhat higher, bring about a drop of both frog skin membrane potentials. The outer-membrane resistance increases at first, then also decreases. At still higher concentrations, *e.g.* 1 m*M*, the drop in both membrane potentials is very rapid and the membrane resistance decreases immediately. This action is comparable to the effect of mercuric chloride as described by LINDERHOLM[6].

At lower concentrations (about 0.04 m*M*), the above agents, when added to the outside medium in the first hour of incubation of the skin cause a considerable rise in the potential difference across the morphologically outer membrane of frog skin, frequently followed by an increase in its resistance and by a slow drop of the potentials. The effect of 0.04 m*M* *p*-chloromercuribenzoate on the membrane potentials is shown in Fig. 3. A similar effect is exhibited by the mercurial diuretic chloromerodrine [3-(chloromercuri)-2-methoxypropylurea], its effect being always greater and the effect of *N*-ethylmaleimide always smaller than that of *p*-chloromercuribenzoate. No hyperpolarization caused by mercuric chloride was observed under these conditions. In the steady-state (after 2 h of incubation) when the d.c. resistance of the outer

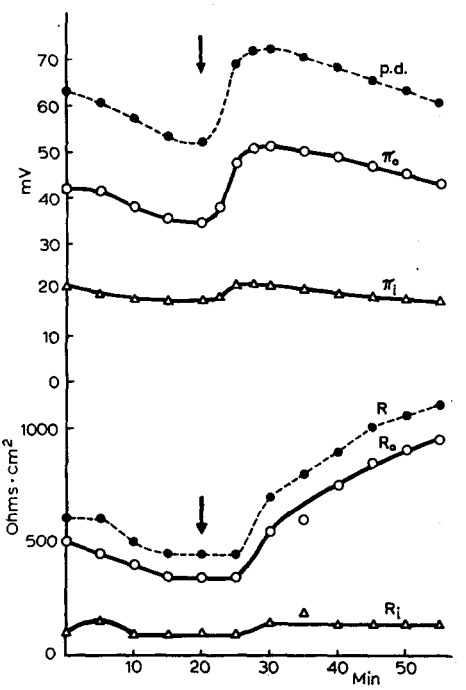

Fig. 3. The effect of *p*-chloromercuribenzoate in the outside solution, the final concentration being 0.04 m*M*, on the individual membrane potentials and d.c. resistances in normal frog Ringer (R_0 = resistance of the outer membrane, R_i = resistance of the inner membrane, R = resistance of the whole skin). The arrow marks the point of addition of the agent. Zero time corresponds to the first measurement, immediately after clamping the skin into the apparatus.

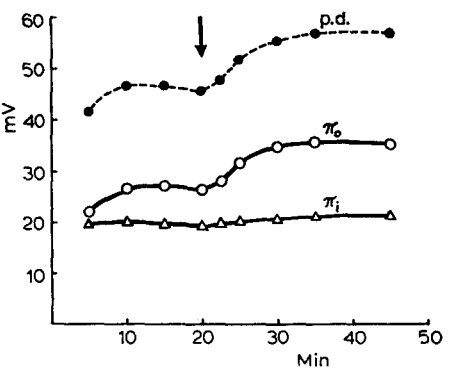

Fig. 4. The effect of *p*-chloromercuribenzoate in the outside solution on the individual membrane potentials induced by the polarizing current of 20 μA/cm² in choline Ringer solution. The effect reflects the drop in permeability to chloride ions. The arrow marks the point of addition of the agent to the outside solution, to a final concentration of 0.04 m*M*.

membrane is high and does not change any more, little or no hyperpolarization is brought about by the above agents.

The hyperpolarization described above was suggestive of the effect of cupric ions as reported by USSING[5]: skins incubated within 2 h of removal from the animal in Ringer's solution containing 0.01 m*M* cupric sulphate, showed a low permeability to chloride ions and a high potential difference, due to this limitation of internal short-circuiting. For this reason, it was investigated whether the decrease in chloride permeability of the outer membrane, *i.e.* the rise in its d.c. resistance when the skin was placed in choline Ringer, took also place in the case examined here.

In choline Ringer, the potential difference across the skin is nil, the centre of the skin being by some 10 mV more negative than the solutions on either side—probably the result of Donnan equilibrium. When 0.04 m*M* *p*-chloromercuribenzoate was added under these conditions, no rise in the d.c. resistance of the outer membrane (derived from the potential changes caused by a current of 10 μA/cm^2—when the Ohm Law always holds) was ever observed. On the other hand, a rise of the d.c. resistance of the outer membrane in choline Ringer was always observed, when the skin was polarized throughout the experiment by a current of 20 μA/cm^2 in the same sense, as is the case in normal Ringer solution. Fig. 4 shows this increase of the externally induced potential of the outer membrane, which is equal to current multiplied by resistance. The electro-neutral molecules of *N*-ethylmaleimide and chloromerodrine bring about an increase of the d.c. resistance of the outer membrane (the skin being placed in choline Ringer) independently of the polarity of this membrane.

DISCUSSION

Thiol-group-blocking agents were found to bring about a decrease in the permeability of the outer frog skin membrane to chloride ions. The hyperpolarization observed as the result of their action may be readily explained as being due to increased perm-selectivity of this membrane, which thus acts as a more perfect sodium electrode. The peculiarities of action of individual agents tested make it possible to suggest a tentative hypothesis on the mechanism of their action and on the structure of the outer membrane, permitting to predict some further results. Fig. 5 shows a suggested

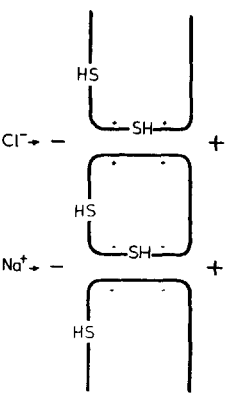

Fig. 5. The crude and simplified model of the outer membrane of frog skin explaining the observed phenomena. For explanation see text.

crude model of the outer membrane. The membrane is porous, as indicated on the differences between the permeability of frog skin derived on the basis of osmotic flow, on the one hand, and of the diffusion of isotopic water, on the other[12]. It is possible to influence the permeability to chloride ions separately; this might suggest the existence of two kinds of pores, cation- and anion-permeable, due to fixed negative and

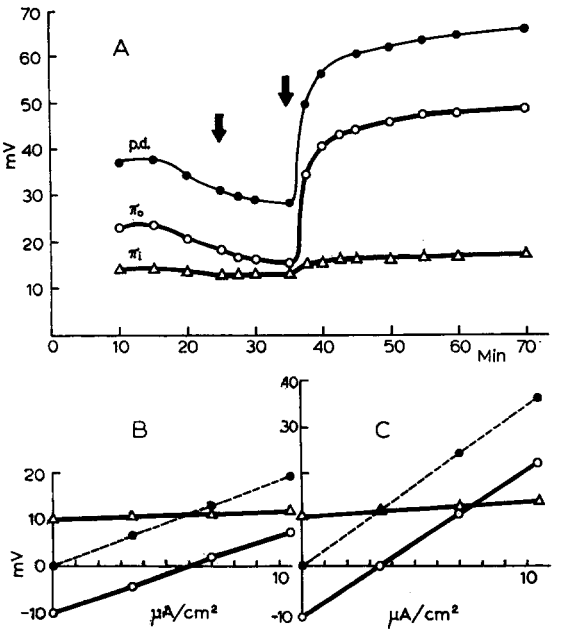

Fig. 6. A, the effect of Pb^{2+} (the left-hand arrow) and of *p*-chloromercuribenzoate (the right-hand arrow) on the potentials induced by a polarizing current of 20 $\mu A/cm^2$. For explanation see text. B and C, straight lines expressing the Ohm Law in the 5th (B) and the 60th (C) min of the above experiment.

positive charges, respectively. The effect of *p*-chloromercuribenzoate anion is dependent on the polarity of the membrane —it appears that it is the direction of electrostatic field in the pores that controls the effect. Fig. 5 shows the polarity at which the above effect is exhibited, irrespective of whether it results from active transport of sodium or from the current passing through from an external source. When the polarity is reversed the electrostatic field repels the negatively charged *p*-chloromercuribenzoate anion from the site of action.

On the basis of this hypothesis the small Pb^{2+} cations possessing an affinity for thiol groups will be repelled by the electrostatic field, caused by current polarization of the same sense as in sodium solutions, and will exhibit no effect on the resistance in choline Ringer, nor hinder the subsequent effect of negatively charged *p*-chloromercuribenzoate. Fig. 6A shows this to be really the case. Fig. 6B and 6C express the Ohm Law at the 5th and 60th min of the same experiment; they confirm that the increase in the externally induced potential (current multiplied by resistance) is not due to nonlinearity at higher current densities.

The action of low concentrations of thiol-group blocking agents (the decrease of permeability to chloride ions) is understood in this model as a steric hindrance in the

anion-permeable pores, as the effect on the membrane potentials seems to be more pronounced the larger the molecule of the inhibitory agent (in the series: N-ethylmaleimide < *p*-chloromercuribenzoate < chloromerodrine). It appears that in heteroporous membrane with an all-or-none response of the individual pores (their size being commensurate with that of the blocking molecules) the fraction of blocked pores (*i.e.* the magnitude of the effect observed) would be greater with larger molecules of the agent. After a short incubation of the skin the binding of thiol-groups in anion-permeable pores is more readily accomplished than in the cation-permeable pores, probably for steric reasons—this is suggested by the fact that the small molecule of mercuric chloride does not prefer the anion-permeable pores. Agents with larger molecules block the thiol groups in the cation-permeable pores only after a lag or at higher concentrations and only then does the permeability to sodium ions decrease, as indicated by the marked increase in the d.c. resistance found in normal Ringer solution. In the steady-state, the conductance of the frog skin (or the diameter of its pores in our model) is lowered corresponding to the "Abdichtungsprozess" described by GERSTNER[13]; the permeability to chloride ions is lowered more markedly than that to sodium ions, resulting in an increased potential difference. For this reason, no hyperpolarization but rather a drop in both membrane potentials, due to lowering of the permeability to sodium ions, is observed.

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REFERENCES

1 V. KOEFOED-JOHNSEN AND H. H. USSING, *Acta Physiol. Scand.*, 42 (1958) 298.
2 L. ENGBAEK AND T. HOSHIKO, *Acta Physiol. Scand.*, 39 (1957) 348.
3 B. T. SCHEER AND M. W. MUMBACH, *J. Cellular Comp. Physiol.*, 55 (1960) 259.
4 H. B. STEINBACH, *J. Cellular Comp. Physiol.*, 3 (1933) 1.
5 H. H. USSING, *Acta Physiol. Scand.*, 19 (1949) 43.
6 H. LINDERHOLM, *Acta Physiol. Scand.*, 27, *Suppl.* 97 (1952) 1.
7 H. H. USSING AND K. ZERAHN, *Acta Physiol. Scand.*, 23 (1951) 110.
8 H. GERSTNER, *Pflüger's Arch. ges. Physiol.*, 242 (1939) 113.
9 E. E. CRANE AND R. E. DAVIES, *Trans. Faraday Soc.*, 46 (1950) 598.
10 F. P. CHINARD AND L. HELLERMAN, in D. GLICK, *Methods of Biochemical Analysis*, Vol. I Academic Press, Inc., New York, 1954, p. 1.
11 E. ROBERTS AND G. ROUSER, *Anal. Chem.*, 30 (1958) 1291.
12 V. KOEFOED-JOHNSEN AND H. H. USSING, *Acta Physiol. Scand.*, 28 (1953) 60.
13 H. GERSTNER, *Pflüger's Arch. ges. Physiol.*, 242 (1939) 587.