Biochemical Pharmacology, Vol. 21, pp. 2643–2648. Pergamon Press, 1972. Printed in Great Britain.

IONIC TRANSPORT IN THE ISOLATED FROG SKIN TREATED WITH *STAPHYLOCOCCAL ALPHA TOXIN*—THE EFFECTS OF OUABAIN, ADH AND THEOPHYLLINE

O. KADLEC

Institute of Pharmacology, Czechoslovak Academy of Sciences, Prague 2, Albertov 4, Czechoslovakia

(*Received* 17 *February* 1972; *accepted* 8 *May* 1972)

Abstract—The mechanism of action of *Staphylococcal alpha toxin* (ST) was analysed with the aid of ouabain, ADH and theophylline. These drugs are known to influence the ionic transport of the frog skin in a characteristic way. The addition of ST to the skin causes a fall of its bioelectric parameters (potential difference and short circuit current). The change is reversible and partial recovery in 40 min can be noted. The observed decrease caused by ST was attributed to a block of sodium transport. The administration of ouabain to the preparation pretreated with ST did not produce any further reduction of the bioelectric parameters and the same was true for the administration of these two drugs in the reverse order. The recovery of the bioelectric parameters in the second phase of ST action occurs because there is a transport of chlorides along a concentration gradient from the inside to the outside of the skin. This transport was stimulated by ADH and theophylline and it was blocked by cyanides administration. Theophylline has been shown to induce the same transport of chlorides in the untreated frog skin. The capacity of the frog skin for the transport of chlorides was demonstrated. The two phases of ST action could be well compared with the actions of ouabain and theophylline respectively.

AMPHIBIAN membranes were used by several investigators[1] to analyse the action of *Staphylococcal alpha toxin* (ST). We have described previously[2] that ST induced a block of active transport of sodium ions from the epidermal to the corial side of the isolated frog skin. In the second phase of ST action the transport of chlorides from the corial to the epidermal side was observed. Chlorides were transported down a concentration gradient. Thus, chlorides moved in the opposite direction to the active transport of sodium and evoked a potential difference (PD) and short circuit current (SCC) of the same sign.

In the present paper the effect of ST was compared with the effects of ouabain, antidiuretic hormone (ADH) and theophylline. Such a study allowed the better understanding of mechanism of ST action. The two phases of ST action were similar to the action of ouabain and theophylline respectively. Furthermore, the changes of chloride permeability by ADH and theophylline in ST-pretreated and normal skin were described.

MATERIALS AND METHODS

In the study of ionic transport in isolated frog skin the method of Ussing and Zerahn[3] was used. The skin was clamped between two cylindrical half chambers as a

flat sheet and spontaneous PD and SCC were measured. All values of SCC were recalculated for 1 sq cm. Initially, 20 mM NaCl solution and Ringer solution were bathing the epidermal (outside) border and corial (inside) border of the skin respectively. Frog Ringer of the following composition, bubbled with air and at room temperature, was used (mM): NaCl, 107; KCl, 2; $CaCl_2$, 1; $NaHCO_3$, 7·5 and pyruvic acid 1·5, the pH was 7·0. Ringer sulphate solution of the following composition was used (mM): Na_2SO_4, 50·5; K_2SO_4, 4; $CaSO_4$, 1; $NaHCO_3$, 7·5; and pyruvic acid, 1·5, the pH was 7·1. The slight difference in the osmolarity between the chloride and sulphate Ringer solutions was considered unimportant.[4] The details of the technique were described in the previous paper.[2]

Commercial *Staphylococcal alpha toxin* from the Institute of Sera and Vaccines, Prague, contained 45,000 HU per millilitre (hemolytic units as defined by Bernheimer[5]) and the toxin was added to the solution bathing the corial side of the skin. Ouabain, ADH (vasopressine) and theopylline or aminophylline were also added to the solutions bathing the corial side of the skin whereas cyanides were added to the solution bathing the epidermal side.

The results are given as the means ± standard error. Two sets of experiments were compared by Student's *t*-test. The number of respective experiments is indicated in parentheses.

RESULTS

Ouabain. The addition of 2×10^{-5} M ouabain to the normal frog skin resulted in 20 min in a significant reduction of PD and SCC. PD was decreased from 47·2 to 30·6 mV and SCC was decreased from 42·0 to 15·5 μA ($P < 0{\cdot}01$; 5).

Different results were observed in the skins pretreated with 1800 HU/ml for 20 min. Sixty min after ST administration and 20 min after the addition of ouabain in the same concentration, PD rose from 17·3 to 19·8 mV and SCC rose from 28·8 to

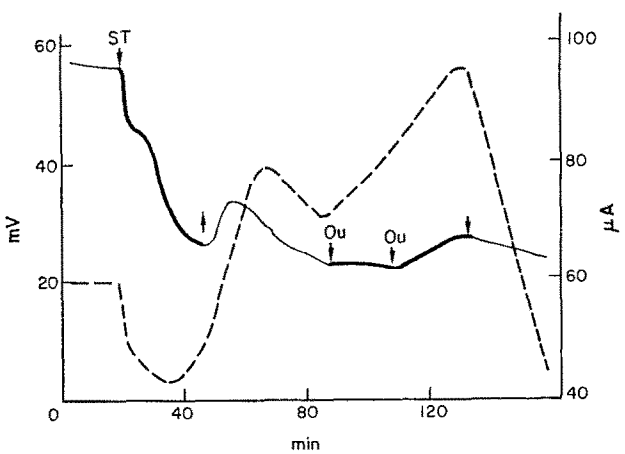

FIG. 1. The action of ouabain in ST-pretreated frog skin. Potential difference (mV; solid line) was recorded continuously and short circuit current (μA; dashed line) was measured intermittently; 1800 HU/ml ST and 3×10^{-5} M ouabain (Ou) were added to the corial side of the frog skin and washed out as indicated by the arrows. In this experiment the concentration of ouabain was doubled by its second addition to enhance the effect.

38·9 μA, but the changes were not significant ($P < 0{\cdot}2$; 7). A typical experiment is shown on Fig. 1. ST and ouabain were also added to the skin in the reverse order. When the administration of ST followed that of ouabain, no further decrease of PD and SCC resulted.

Antidiuretic hormone. Forty min after the addition of 0·1 IU/ml ADH potential difference increased from 49·8 to 52·0 mV and SCC increased from 103 to 137 μA. Both increases were significant ($P < 0{\cdot}05$; $P < 0{\cdot}01$; 7). In normal skin PD and SCC fell to zero as soon as 20 mM KCl solution replaced 20 mM NaCl at the outside facing border. No change of PD and SCC was seen after the addition of ADH to such skin.

As described previously,[2] the frog skin pretreated with 1800 HU/ml ST was insensitive to the replacement of sodium by different cations in the solution bathing the epidermal side. Its PD and SCC did not change significantly when 20 mM KCl or choline chloride, 10 mM hexamethonium bichloride and 13·5 mM $CaCl_2$ replaced 20 mM NaCl. The addition of 0·1 IU/ml ADH to the preparation with any of these solutions bathing the epidermal side of the skin caused significant increases of PD and SCC. On the average PD increased from 24·6 to 26·7 mV and SCC from 45·0 to 59·0 μA ($P < 0{\cdot}01$; 10). The increases were reached usually in 10 min (Fig. 2).

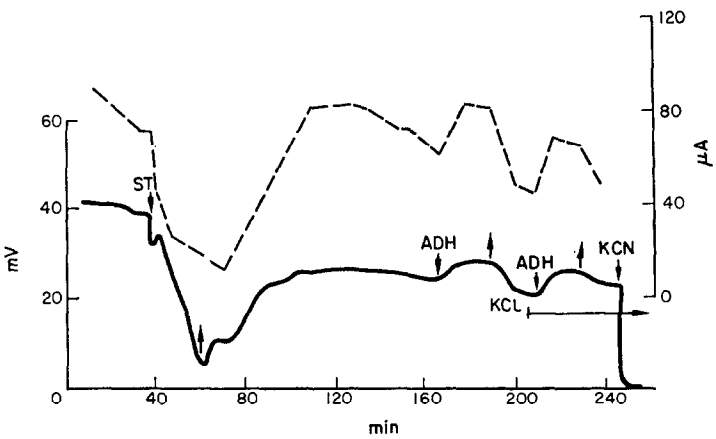

FIG. 2. The action of ADH in ST-pretreated frog skin. Potential difference (mV; solid line) and short circuit current (μA; dashed line) were recorded. To the Ringer solution bathing the corial side of the skin 1800 HU/ml ST and thereafter 0·1 IU/ml ADH were added repeatedly. Twenty mM KCl replaced 20 mM NaCl solution bathing the epidermal side. 4×10^{-3} M KCN was added to the KCl solution. The addition and washout of the drugs and of the KCl solution are indicated by arrows.

Theophylline. The administration of 12 mM theophylline to normal skin resulted in changes of PD and SCC of the opposite sign, but both changes were significant. PD was reduced from 49·8 to 42·2 mV and SCC was raised from 31·2 to 60·4 μA ($P < 0{\cdot}02$; 5). In normal skins bathed with 20 mM KCl solution at the epidermal side PD and SCC were at zero levels. Theophylline addition evoked a rise of 17·0 mV PD and of 19·1 μA SCC of the usual sign. The values were significantly different from zero ($P < 0{\cdot}01$; 7). If sulphates replaced chlorides in Ringer solution bathing the corial side or sulphates replaced chlorides on both sides of the skin the charge transport induced by theophylline was abolished (Fig. 3). The dependence of this transport on

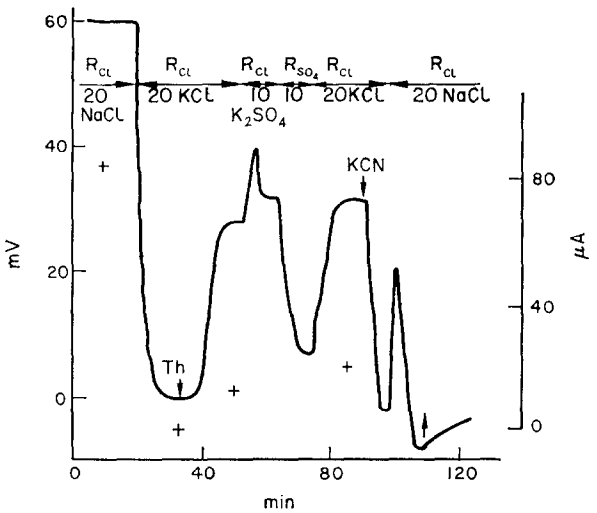

FIG. 3. The influence of 12 mM theophylline (Th) on the potential difference (solid line) and on intermittently measured short circuit current (crosses) of the frog skin. In the above fractions the numerator represents the solution bathing the corial side of the skin and the denominator represents the solution bathing the epidermal side of the skin. R_{Cl}—Ringer chloride solution R_{so_4}—Ringer sulphate solution; 20 mM NaCl; 20 mM KCl and 10 mM K_2SO_4 solutions were present at the respective sides for the time indicated. 4×10^{-3} M KCN was added to the outside solution.

the molarity of the KCl solution bathing the epidermal side, and Ringer chloride solution bathing the corial side at the same time, was measured quantitatively. The average change of PD per 10-fold dilution of KCl solution in the range of concentrations from 20 to 0·2 mM KCl was 12·8 ± 1·5 mV (4) with the highest value of 21·0 mV.

TABLE 1. THE CHANGES OF SHORT CIRCUIT CURRENT (SCC) AND OF POTENTIAL DIFFERENCE (PD) IN NORMAL AND ST-PRETREATED (1800/ml) FROG SKIN. RINGER SOLUTION BATHED THE CORIAL SIDE AND 20 mM NaCl SOLUTION BATHED THE EPIDERMAL SIDE IF NOT INDICATED OTHERWISE.

Drug	Treatment	No of exp.	SCC: μA/cm²	Significance	PD: mV	Significance
Ouabain		5	−26·6 ± 3·16	P < 0·01	−16·6 ± 3·43	P < 0·01
2×10^{-5} M	ST	7	+10·1 ± 5·75	P > 0·2	+2·5 ± 1·76	P > 0·2
ADH		7	+34·0 ± 4·40	P < 0·01	+2·2 ± 0·66	P < 0·05
0·1 IU/ml	ST	7	+14·0 ± 3·58†	P < 0·01	+2·1 ± 0·53†	P < 0·01
Theophylline		5	+29·2 ± 2·10	P < 0·02	−7·6 ± 1·90	P < 0·02
$1{\cdot}2 \times 10^{-2}$ M		7	+19·1 ± 3·46*	P < 0·01	+17·0 ± 2·48*	P < 0·01
	ST	5	+21·4 ± 2·74†	P < 0·01	+13·2 ± 2·40†	P < 0·01

* Measured in experiments when the skins were bathed with 20 mM KCl solution at the epidermal side.

† Measured in a mixed group with either 20 mM NaCl or 20 mM KCl bathing the epidermal side of the skin.

PD and SCC were reduced to zero in the skin bathed with 120 mM KCl at the epidermal side. Theophylline (12 mM) was added to ST-pretreated skin bathed with 20 mM KCl at the epidermal side of the skin. It caused a significant increase of PD from 4·4 to 17·6 mV and of SCC from 6·6 to 28·0 μA ($P < 0{\cdot}01$; 5).

Both NaCN and KCN (4×10^{-3} M) caused a rapid fall of PD and SCC to zero after their addition to the ST-pretreated skin (Fig. 2). In normal skin bathed with Ringer chloride solution at the corial side, the PD and SCC evoked after theophylline addition were immediately inhibited by the addition of cyanides (Fig. 3).

Some of the results are summarized in Table 1.

DISCUSSION

In our previous study[2] the block of active transport of sodium by ST in the frog skin was proved by means of radioisotopes. PD and SCC were both declining at the same time. The active transport of sodium in the frog skin is also blocked by ouabain.[6,7] The mechanism of action of this compound is the inhibition of Na-K ATPase which is manifested by the decline of both PD and SCC. Described above results clearly demonstrated that ST and ouabain mutually prevented the effect one to the other when administered successively. Thus, one can make the conclusion that ST blocks the active transport of sodium by a mechanism similar to the action of ouabain—through the inhibition of transport Na-K ATPase. The ST-induced inhibition of the mitochondrial Na-K ATPase was described recently.[8]

ADH augments the transport of sodium and water across several amphibian membranes.[9] In our experiments ADH not only increased the transport of sodium in normal skin (NaCl solution bathing the epidermal side) but also the transport of charge was increased in ST-pretreated skin. In the latter case the transport of charge could have been also increased in spite of the fact that potassium or another cation were bathing the epidermal side of the skin. So, the increased transport of charge observed before and after the toxin action is different in nature. A new source of PD and SCC in the second phase of ST action was attributed to the transport of chlorides from the corial to the epidermal side.[2,10] Chlorides could be added to the molecules which movement might be facilitated by ADH in the frog skin.

Baba *et al.*[11] proposed the same mechanism of action of ADH and theophylline in the frog skin. The nucleotide cyclic 3′,5′-AMP is the putative intracellular mediator of their action and its level rises after an exposure to ADH and theophylline. In our experiments the transport of chlorides in ST-pretreated skin was increased by theophylline as well as by ADH. After their addition PD and SCC rose even in the case when 20 mM KCl solution was bathing the epidermal side.

However, the action of theophylline in the normal skin seems to be different to that of ADH. The SCC increased but PD decreased after the addition of theophylline. Cuthbert and Painter[12] suggested that theophylline reduced the anion drag on sodium ions by increasing chloride permeability rather than that it increased the concentration of cyclic 3′,5′-AMP like ADH. Such opinions on the different mechanisms of action of ADH and of theophylline is in agreement with our finding of theophylline action in the normal skin bathed with 20 mM KCl at the epidermal side. The transport of charge evoked after its addition was of usual sign. The critical role of chlorides was manifested when they were replaced by sulphates. The transport of charge was

immediately abolished. Further, PD induced by this transport was clearly dependent on the concentration gradient of chlorides across the skin. Thus, theophylline might not only increase the transport of chlorides after the action of ST, but it would be able to induce similar process in a normal skin.

Cyanides inhibit oxidation processes providing energy for the active transport. The active transport of sodium was rapidly reduced after the addition of cyanides and PD and SCC declined to zero.[13] Cyanides also inhibited the transport of chlorides induced either by the treatment with ST or by addition of theophylline. Though, this transport was observed only under a favourable concentration gradient, its stimulation by theophylline and ADH and its inhibition by cyanides suggested a link to the metabolism of the tissue.

In conclusion, these experiments have shown that *Staphylococcus alpha toxin* exerts rather a specific membrane effect that could be well compared with the effects of drugs established as tools in membrane biology, namely ouabain and theophylline. Ouabain type of block of membrane Na-K ATPase corresponds to the block of ionic transport caused by ST; and subsequent rise of ionic transport attributed to the transport of chlorides corresponds to the action of theophylline. Moreover, the toxin disclosed the capacity of the frog skin to transport chlorides.

REFERENCES

1. J. RAHAL, M. PLAUT and L. WEINSTEIN, *J. Clin. Invest.* **47,** 1603 (1968).
2. O. KADLEC and R. ČAPEK, *Naunyn-Schmiedebergs Arch. Pharmac.* **270,** 262 (1971).
3. H. USSING and K. ZERAHN, *Acta physiol. Scand.* **23,** 110 (1951).
4. C. R. HOUSE, *J. Physiol.* **202,** 631 (1969).
5. A. W. BERNHEIMER, *Ann. N.Y. Acad. Sci.* **128,** 112 (1965).
6. J. C. SKOU, *Physiol. Rev.* **45,** 596 (1965).
7. J. KAWADA, R. E. TAYLOR and S. B. BARKER, *Comp. Biochem. Physiol.* **30,** 965 (1969).
8. E. NOVÁK, J. SEIFERT and H. RAŠKOVÁ, *Toxicon* **8,** 261 (1970).
9. A. LEAF, *Rev. Physiol.* **56,** 216 (1965).
10. O. KADLEC and R. ČAPEK, *Biochem. Pharmac.* **18,** 1775 (1969).
11. W. I. BABA, A. J. SMITH and M. M. TOWNSHEND, *Quart. J. Exp. Physiol.* **52,** 416 (1967).
12. A. W. CUTHBERT and E. PAINTER, *J. Pharm. Pharmac.* **20,** 492 (1968).
13. E. G. HUF, N. S. DOSS and J. P. WILLS, *J. Gen. Physiol.* **41,** 397 (1957).