Effects of SITS on Sodium Transport, Oxygen Consumption and Na-K-ATPase of the Frog Skin

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=국문초록=

개구리 피부의 Sodium 이동, 산소 소모량 및 Na-K-ATPase에 대한 SITS의 영향

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적출된 개구리 피부에서 Na^+이동, 산소소모량 및 Na-K-ATPase활성도에 대한 SITS(4-acetamido-4′-isothiocyano-2, 2′-disulfonic stilbene)의 영향을 연구하였다. 피부를 통한 능동적 Na^+이동을 추정하기 위하여 short-circuit current(SCC)를 측정하였으며, 산소소모량은 피부조직 및 분리된 표피조직에서 측정하였으며, Na-K-ATPase활성도는 표피조직의 24,000×g 분획에서 측정하였다.

피부를 통한 SCC는 10 mM SITS가 피부외측용액에 첨가될 때 급격히 하강하였으며, 내측용액에 첨가될 때는 20분정도 지난후 하강하기 시작하였으나 그 하강정도는 전자에 비해 약했다. SITS에 의한 SCC억제현상은 용액내에 Cl^-이 없을때도 나타났다. SITS에 의하여 피부 및 표피조직의 산소소모량은 억제되지 않았으나 표피조직분획내 Na-K-ATPase활성도는 심하게 억제되었다.

이상과 같은 성적은 SITS가 개구리 피부에서 능동적 Na^+이동을 강력히 억제함을 나타내는데, 이러한 억제작용은 이 약물이 주로 상피세포의 외측막에 작용하여 나타나는 것으로 사료되지만 Na^+펌프를 억제할 가능성을 전연 배제할 수는 없다.

INTRODUCTION

Disulfonic stilbenes, such as SITS(4-acetamido-4′-isothiocyano-2, 2′-disulfonic stilbene), have been shown to inhibit various anion transport systems. In the red blood cell, SITS inhibits the exchange flows of Cl^- and SO_4^{--}, without affecting cation transport(Cabantchik and Rothstein, 1972). Similarly, in the turtle urinary bladder, SITS suppresses the anion-dependent(HCO_3^-, Cl^-)moiety of the short-circuit current, but has no effect on Na^+ transport(Ehrenspeck and Brodsky, 1976). In Ehrlich ascites tumor cells, SO_4^{--} movement is inhibited by SITS while Cl^- transport is SITS-insensitive(Villereal and Levinson, 1976). In the rabbit kidney slice, SITS induces substantial reduction of organic anion uptake(p-aminohippurate and 2,4,5-trichloro-phenoxyacetate), but has no effect on the transport of organic cation(tetraethylammonium) (Hong et al., 1978).

These effects of SITS have been attributed to its interaction with some component of the cell membrane. Cabantchik and Rothstein(1972) observed that SITS molecules do not penetrate into the red cell membrane, but bind to amino groups at the outer surface that interact electrostatically with sulfonic groups. Ehrenspeck and Brodsky (1976) observed that SITS induces substantial inhibition of microsomal Na-K-ATPase isolated from

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the turtle urinary bladder, although it has no effect on Na^+ transport across the bladder. These authors, therefore, proposed that SITS molecules can not gain access to the Na-K-ATPase in the intact bladder cell.

If indeed SITS alters anion transport in cell membranes, one would expect that cation-anion coupled transport or anion-dependent transport systems may also be affected by SITS. Although the mechanism is not yet precisely elucidated active Na^+ transport in the frog skin has been known to be dependent on Cl^- in the outside bathing medium(Ferreira, 1968; Cuthbert et al., 1969; Ferreira et al., 1973; Ferreira and Bruria, 1978).

We therefore investigated in the present study the effect of SITS on the active Na^+ transport in the frog skin. The results indicate that SITS induces significant inhibition of the short-circuit current across the skin when it is applied to the mucosal surface of the skin.

METHODS

Experimental animal

Frogs, *Rana temporaria*, acquired in Kyungki-do near Seoul, were kept in laboratory at about 25°C for at least 3 days before the experiment.

Measurement of short-circuit current(SCC) across the frog skin

As a measure of active sodium transport across the skin the short-circuit current technique of Ussing and Zerahn(1951) was used. The abdominal skin was removed from an animal and mounted as a flat sheet between the two Lucite chambers having a cross-sectional area of 3.14 cm^2. The potential difference(PD) across the skin was measured with a pair of calomel electrodes connected to the chambers by a pair of salt bridges. Current was driven through the skin via Ag-AaCl electrodes connected to the chambers by another pair of salt bridges. The solution bathing both sides of the skin was continuousely stirred and aerated with a stream of air. In most experiments the skin was bathed on both sides with a Ringer solution containing 115 mM NaCl, 2.5 mM $KHCO_3$ and 1mM $CaCl_2$, and pH7.6 at 25°C(Chloride Ringer). In some experiments 115 mM NaCl was replaced by 77 mM Na_2SO_4(Sulphate Ringer). When both PD and SCC were stabilized, SITS(Polysciences, Warrington, Pa) was added to the medium(see below).

Measurement ef Na^+, K^+-activated adenosine triphosphatase(Na-K-ATPase) activity in the frog skin epithelium

Epidermis of the skin was prepared by the method of Rawlins et al.(1970): a piece of ventral skin was mounted between two Lucite chambers containing Ringer solution, and a hydrostatic pressure of 100 cmH_2O was applied to the serosal side for one hour. The skin was then gently blotted on filter paper and separated into dermal and epidermal sheets by scraping with a surgical knife. The epidermal sheet was homogenized in an ice-cold solution of 0.25 M sucrose, 5 mM EDTA and 20 mM Tris-HCl (pH7.4 at 20°C). Homogenates were then centrifuged at 24,000×g for 30 min in a Sorvall refrigerated centrifuge (RC 2-B), and aliquots of the supernatant were taken for ATPase assay(Park and Hong, 1976) and protein determination(Lowry et al., 1951).

The ATPase activity was estimated by measuring inorganic phosphate(Pi) liberated by ATP hydrolysis during incubation of 0.1 ml supernatant with 0.4 ml of an appropriate medium at 37°C. The reaction was initiated by the addition of ATP and terminated by the addition of 0.2 ml perchloric acid(11.67%). The preincubation and incubation period were 15 min each. Concentrations of cations and substrate in the incubation mixture were: Na^+ 100 mM, K^+ 10 mM, Mg^{++} 3 mM and ATP(Na-salt) 3 mM for the total ATPase(i.e., Na-K-+ Mg-ATPase) activity measurement; both Na^+ and K^+ were omitted for the measurement of Mg-ATPase activity. The pH of the medium was adjusted with 40 mM imidazole-HCl to 7.4 at 37°C.

Upon completion of incubation the mixture was centrifuged at 3,000 r.p.m. for 15 min. Inorganic phosphate in the supernatant was measured by the method of Fiske and SubbaRow(1925). The Na-K-ATPase activity was calculated by subtracting the Mg-ATPase activity from the total ATPase activity, and expresses as a specific activity(μ molesPi /mg protein per hr).

Preliminary experiments indicated that the Na^+, K^+-dependent fraction of the ATPase activity was much higher at 37°C than at 25°C, thus the reaction temperature of 37°C was used in order to reduce experimental errors.

Measurements of oxygen consumption in intact skin and separated epidermis

A piece of ventral skin(or epidermis prepared as described above) was cut into several small pieces, which were then divided into two groups: the control and SITS group. In each group approximately 0.15 g tissues were placed in 3ml of air-saturated normal(or 10 mM SITS containing) Chloride Ringer solution at 25°C in temperature controlled, magnetically stirred receptacle, and Po_2 changes in the solution was monitored for 30~60 min with a Clark oxygen electrode and YSI oxygen monitor (model 53). From the value of Po_2 change the rate of oxygen consumption(Qo_2) was calculated and expressed as μl O_2/g wet wt. per hr.

RESULTS

Effect of SITS on SCC across the skin

In order to establish the dose-response relationship, we first examined the SCC response to various concentrations of SITS. Fig. 1 illustrates changes in SCC after the addition of SITS to both mucosal and serosal bathing media(Chloride Ringer) at a concentration of 1, 5, and 10 mM. The SCC was expressed as a percentage of the basal level (before adding the drug). When the concentration of SITS was 1 or 5 mM, the SCC was not apparently different from that of the control preparation

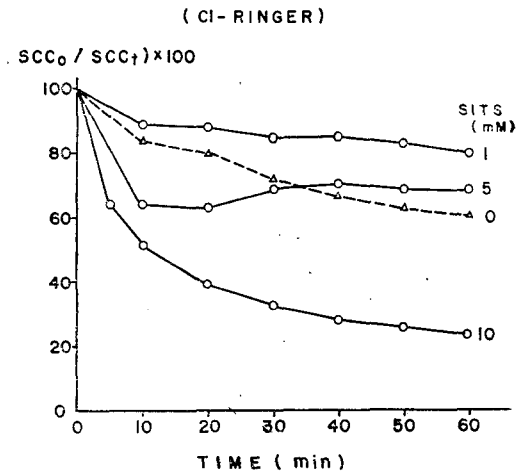

Fig. 1. Changes in SCC of the frog skin after addition of SITS to both mucosal and serosal bathing media(Chloride Ringer) at various concentrations. At time 0, the mean SCC value was 183±10μA/3.14 cm^2 (N=12). Each value represents the mean of 3 skins.

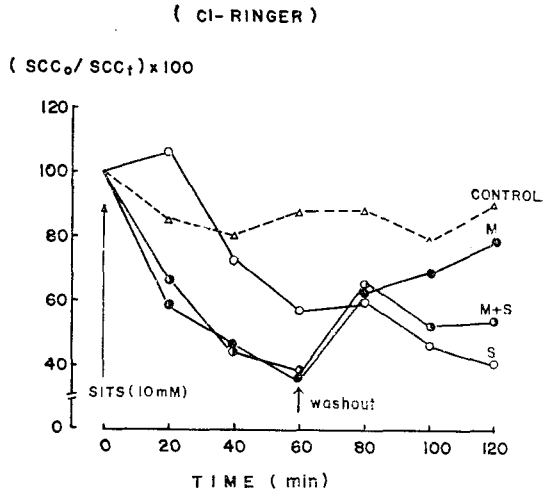

Fig. 2. Changes in SCC of the frog skin after addition of 10 mM SITS to mucosal(M), serosal(S) and both mucosal and serosal (M+S) bathing media(Chloride Ringer). The average SCC at time 0 was 179±15 μA/3.14 cm^2(N=20). Each value represents the mean of 5 skins.

(0 mM SITS). 10 mM SITS, however, evoked a significant inhibition of SCC; the level of SCC at 60 min after the drug addition was corresponded to

approximately 25% of the basal level. We therefore used 10 mM SITS in all subsequent experiments.

Fig. 2 compares the effects of 10mM SITS added to the medium bathing the mucosal, serosal and both sides of the skin on SCC. The addition of SITS to the mucosal surface(M) resulted in immediate reduction of SCC which reached to 40% of the basal level after 60min. In contrast, SITS added to the serosal surface(S) did not alter the SCC during the initial 20 min period after which it gradually inhibited the SCC to a value corresponding to 60% of the basal level at 60min. When SITS was added to both mucosal and serosal media (M+S) the effect was not additive but was identical to that observed with mucosal addition of the drug. These results suggest that the primary site of action of SITS is located on the outer surface of the skin.

In order to study if the SITS-induced reduction of SCC was due to inhibition of Cl^- transport, we examined SITS effect in the skin bathed Cl^--free medium(Sulphate Ringer). The result, however, indicated that 10 mM SITS was equally effective

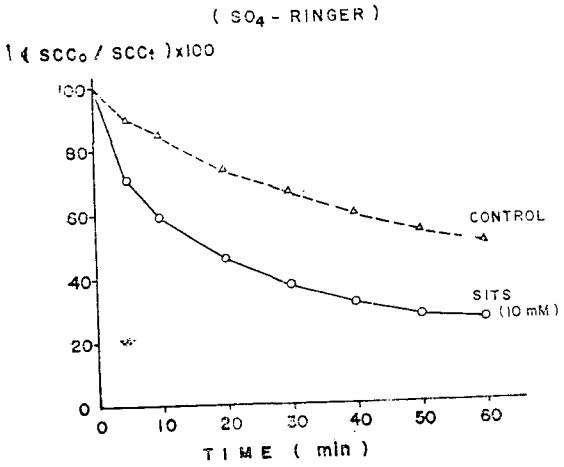

Fig. 3. Changes in SCC of the frog skin after addition of 10 mM SITS to both mucosal and serosal bathing sulphate media. The average SCC at time 0 was 218±38 μA/3.14 cm^2(N=10). Each value represents the mean of 4 skins in the control and 5 skins in the SITS group.

in inhibition of SCC as observed with Cl^- containing medium(Fig. 3). This suggests that SITS inhibits Na^+ transport independent of Cl^- transport.

Effect of SITS on oxygen consumption of the skin

In order to determine whether SITS had an effect on the metabolism of the skin, we next measured oxygen consumption(Q_{O_2}, μl/g wet wt.per hr.) of the skin in the absence and presence of 10 mM SITS. Two series on experiments were conducted: in one series the measurement was made in intact skin and in another series in separated epidermis. As summarized in table 1, the Q_{O_2} of the epidermis was much higher than that of whole skin, probably because the mechanisms for transepithelial Na^+ transport reside in epidermis(Voûte and Ussing, 1958; Voûte and Hänni, 1973). In either case, however, the Q_{O_2} was not reduced by 10 mM SITS, indicating that the SITS-induced inhibition of SCC was not brought about by alterations in tissue metabolism.

Effect of SITS on Na-K-ATPase activity of the skin

In the next series on experiments we have measured the Na-K-ATPase activity of the skin epithelial membrane in the absence and presence of varying concentrations of SITS. Table 2 shows that SITS inhibited the enzyme activity by approximately 50% at 2 and 10 mM and 60% at 10 mM. These indicate that direct application of SITS to the isolated cell membrane resulted in a significant inhibition of the Na-K-ATPase activity.

Table 1. Effect of SITS(10 mM) on Q_{O_2} (μl/g wet wt. per hr) of frog skin

	Control	SITS
Intact skin	140	160
Epidermis	270	285

Each value represents the mean of 2 measurements. The incubation temperature was 25°C.

Table 2. Effect of SITS on Na-K-ATPase activity of frog skin epidermal preparation

SITS (mM)	Specific Activity (μ moles Pi/mg prot. per hr)	%
0	1.21	100
2	0.70	58
10	0.69	58
100	0.61	51

Each value represents the mean of 2 determinations. The assay temperature was 37°C.

DISCUSSION

Transepithelial Na^+ transport in frog skin involves two distinct steps: Na^+ entry across the outer (mucosal) barrier and active extrusion across the inner(serosal) barrier of the epithelial cell. The first step involves a mechanism of carrier mediated transport(facilitated diffusion) (Biber and Curran, 1970; Biber and Cruz, 1973; Cruz and Biber, 1976), and the second step involves the Na^+ pump mechanism(Koefoed-Johnsen and Ussing, 1958). Under normal conditions the overall transport of Na^+ is rate-limited by the mucosal step (Cereijido et al., 1964; Biber and Cruz, 1973; Cruz and Biber, 1976), thus the Na^+ pump mechanism in the serosal membrane is normally operating at submaximal rate. These suggest that agents that inhibit Na^+ entrance across the mucosal barrier can always alter transepithelial Na^+ transport, whereas those affecting active transport mechanism can not induce inhibition of Na^+ transport until the pump activity is suppressed in such an extent that the serosal extrusion becomes the rate-limiting step.

The present investigation reveals that SITS induces definite changes in the Na^+ transport system of the frog skin. Although the exact mode of action of SITS is difficult to assess, the data sheds light on the possible mode of action. It is important to note that SITS is consistently more effective when it is applied to the mucosal surface than to the serosal surface of the skin(Fig. 2). In the presence of 10 mM SITS in the mucosal bathing medium, the SCC decreased rapidly. When SITS was added to the medium bathing the serosal surface, there was about 20 min delay for the inhibition of SCC. Furthermore, the magnitude of SCC inhibition during 60 min exposure to SITS was significantly greater in the case of mucosal application than that of serosal application of the drug. These observation together with the fact that inhibitory effect of SITS on SCC was not additive when the drug was applied to both mucosal and serosal bathing media(Fig. 2) led us to speculate that the primary site of SITS action is on the mucosal facing membrane of the cell.

Another important aspect to note is that SITS induces SCC inhibition even in the absense of Cl^- in the medium bathing the skin(Fig. 3). In fact, the magnitude of inhibition was practically identical to that observed with Cl^- containing medium. This strongly suggests that the inhibition of SCC by SITS was not secondary to the alteration of Cl^- movement.

Oxygen consumption of skin tissues was not affected by SITS (Table 1). This results is somewhat surprising, since ion transport is a major energy-requiring process of cells, it's inhibition by SITS would be accompanied by a reduction in tissue metabolism. In fact, ouabain, which inhibits the serosal Na^+ pump, and amiloride, which retards the mucosal entry of Na^+, interfere tissue respiration in various epithelia(Coplan and Maffly, 1972; Willis, 1968; Parisi and Bently, 1970). In any event, the results strongly suggest that SITS inhibition of SCC was not due to reduction of energy supply to the Na^+ pump mechanism.

The present work does not rule out a possibility that SITS may also have an inhibitory effect on the Na^+ pump itself. In the presence of 10 mM SITS in the medium bathing the serosal surface of the skin, the SCC was somewhat inhibited, although the effect appeared slowly and in a relatively small magnitude (Fig. 2). Furthermore, the Na-K-ATPase in a subcellular fraction of the skin was approximately 50% suppressed in the presence of 1-100 mM SITS(Table 2). If SITS molecules do

penetrate into binding sites at the enzyme in the intact skin cell membrane, the later result would indicate that SITS can alter the activity of the Na^+ pump. On the contrary, if the skin cell membrane is not permeable to SITS, as in other membranes (Cabantchik and Rothstein, 1972; Ehrenspeck and Brodsky, 1976), the result may not have any relevance to the SITS effect on *in situ* Na^+ transport. In such case, the observed inhibition of SCC by SITS at the serosal side would indicate that SITS molecules diffuse into mucosal membrane (perhaps through lateral intercellular spaces) before they affect Na^+ transport. Certainly, a better understanding of the membrane permeability of SITS is required to elucidate it's mode of action in the frog skin.

In conclusion, the present study clearly demonstrated that SITS interferes cation transport system in the frog skin epithelium, independent of anion transport. The precise mechanism for this inhibition is yet to be elucidated.

Summary

Effects of SITS (4-acetamido-4′-isothiocyano-2, 2′-disulfonic stilbene) on a Na^+ transport, tissue oxygen consumption and Na-K-ATPase activity were studied in isolated frog skin preparations. Na^+ transport was estimated by measuring the short-circuit current(SCC) across the skin; oxygen consumption was measured in separated epidermis as well as in intact skin; and Na-K-ATPase was assayed in 24,000×g fraction of epidermal homogenates.

The SCC across the skin was rapidly and substantially reduced in the presence of 10 mM SITS in the medium bathing the outside(mucosal) surface of the skin. When the drug was added to the inside(serosal) bathing medium, there was about 20 min delay for inhibition of SCC and the effect was less pronounced. The above effect of SITS was independent of the presence of Cl^- in the bathing medium. The oxygen consumption of the skin tissue was not affected by SITS, but the Na-K-ATPase activity of a subcellular fraction of the skin was significantly inhibited.

These results suggest that SITS retards Na^+ transport across the frog skin primarily by interfering Na^+ entry across the mucosal membrane of the epithelial cell, although an effect on Na^+ pump can not be ruled out completely.

REFERENCES

1) Biber, T.L.L. and L.J. Cruz: *Effect of antidiuretic hormone on sodium uptake across outer surface of frog skin. Am. J. Physiol. 225:912-917, 1973.*

2) Biber, T.U.L. and P.F. Curran: *Direct measurement of uptake of sodium at the outer surface of the frog skin. J. Gen. Physiol. 56:83-89, 1970.*

3) Cabantchik, Z.I. and I. Rothstein: *The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. J. Membrane Biol. 10:311-330, 1972.*

4) Cereijido, M., F.C. Herrera, W.J. Flanigan and P.F. Curran: *The influence of Na concentration on Na transport across frog skin. J. Gen. Physiol. 47:879-893, 1964.*

5) Coplon, N.S. and R.H. Maffly: *The effect of ouabain on sodium transport and metabolism of the toad bladder. Biochim. Biophys. Acta 282: 250-254, 1972.*

6) Cruz, L.J. and T.U.L. Biber: *Transepithelial transport kinetics and Na entry in frog skin: effects of novobiocin. Am. J. Physiol. 231: 1866-1874, 1976.*

7) Cuthbert, A.W., E. Painter and W.T. Prince: *The effect of anions on sodium transport. Br. J. Pharmac. Chemother. 36:97-106, 1969.*

8) Ehrenspeck, G. and W.A. Brodsky: *Effect of 4-acetamido-4′-isothiocyano-2, 2′disulfonic stil bene on ion transports in turtle bladders. Biochim. Biophys. Acta 419:555-558, 1976.*

9) Ferreira, K.T.G.: *Anionic dependence of sodium transport in the frog skin. Biochim. Biophys.*

Acta 150:587-598, 1968.

10) Ferreira, K.T.G. and H. Bruria: *Chloride dependence of active sodium transport in frog skin the role of intercellular spaces. J. Physiol. 283:283-305, 1978.*

11) Ferreira, K.T.G., M.M. Guerreiro and H.G. Ferreira: *Kinetic characterization of the chloride dependence of sodium transport in the frog skin. Biochim. Biophys. Acta 291:269-273, 1973.*

12) Fiske, C.H. and Y. SubbaRaw: *The colorimetric determination of phosphorous. J. Biol. Chem. 66:375-400, 1925.*

13) Hong, S.K., J.M. Goldinger, Y.K. Song, F.J. Koschier and S.H. Lee: *Effect of SITS on organic anion transport in the rabbit kidney cortical slice. Am. J. Physiol. 234:F302-F307, 1978.*

14) Koefoed-Johnsen, V. and H.H. Ussing: *The nature of frog skin potential. Acta Physiol. Scand. 42:298-308, 1958.*

15) Lowry, O.H., N.D. Rosebrough, A.L. Farr and R.J. Randall: *Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.*

16) Parisi, M. and P.J. Bentley:*Effects of amiloride on respiration of the urinary bladder, intestine and sartorius muscle of toads. Biochim. Biophys. Acta 219:234-237, 1970.*

17) Park, Y.S. and S.K. Hong: *Properties of toad skin Na-K-ATPase with special reference to the effect of temperature. Am. J. Physiol. 231:1356-1363, 1976.*

18) Rawlins, F., L. Mateu, F. Fragachan and G. Whittembury: *Isolated toad akin epithelium: transport characteristics. Pflügers Arch. 316: 64-80, 1970.*

19) Ussing, H.H. and K. Zerahn: *Active transport of sodium as a source of electrical current in the short-circuited frog skin. Acta Physiol. Scand. 23:110-127, 1951.*

20) Villereal, M.L. and C. Levinson: *Inhibition of sulfate transport in Ehrlich ascites tumor cells by 4-acetamido-4'-isothiocyanostilbene-2, 2'-disulfonic acid(SITS). J. Cell Physiol. 89:303-311, 1976.*

21) Voûte, C.L. and S. Hänni: *Relation between structure and function in frog skin. In: Transport Mechanisms in Epithelia, edited by H.H. Ussing and N.A. Thorn, pp 86-93, Academic press, New York, 1973.*

22) Voute, C.L. and H.H. Ussing: *Some morpholological aspects of active sodium transport-The epithelium of the frog skin. J. Cell Biol. 36: 625-638, 1958.*

23) Willis, J.S.: *Ouabain inhibition of ion transport and respiration in renal cortical slices of ground squirrels and hamsters. Biochim. Biophys. Acta 163:506-515, 1968.*