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EFFECTS OF 4-ACETAMIDO-4′-ISOTHIOCYANO-2,2′-DISULFONIC STILBENE ON ION TRANSPORT IN TURTLE BLADDERS

GERHARD EHRENSPECK and WILLIAM A. BRODSKY

Department of Physiology and Biophysics, The Mount Sinai School of Medicine of The City University of New York, Fifth Avenue and 100th Street, New York, N.Y. 10029 (U.S.A.)

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Summary

The disulfonic stilbene (4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene) is found to be more potent than acetazolamide as an anion transport inhibitor in the turtle bladder, but less potent than acetazolamide as a carbonic anhydrase inhibitor. The anion-dependent (HCO_3^-, Cl^-) moiety of the short-circuiting current is eliminated by 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene, but only after its addition to the serosal bathing fluid. Whereas 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene has no effect on Na^+ transport across the bladder, it is more potent than ouabain as an inhibitor of microsomal ($Na^+ + K^+$)- ATPase of both turtle bladder and eel electric organ.

Cabantchik and Rothstein [1] found that disulfonic stilbenes (e.g. SITS, DIDS) block the carrier-mediated exchange of anions across erythrocyte membranes.Consequently, it occurred to us that there could be a stilbene-sensitive, anion-selective pathway in the plasma membranes of anion-transporting epithelial cells. If so, the disulfonic stilbenes should inhibit the anion transport across the bladder epithelium of Pseudemys turtles [2,3]; and this was confirmed in the present experiments.

The purpose of the present study was to compare the effects of SITS, acetazolamide, and ouabain on ion transport and on the activities of two transport-related enzymes. Methods used were those previously described for evaluating transepithelial potential (*PD*), dc-resistance (*R*), and short-circuiting current (I_{sc}) in turtle bladders mounted in Rehm-Ussing chambers [2] and for assaying ($Na^+ + K^+$)-ATPase activity in microsomes of this

Abbreviations: SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; DIDS, 4,4′-di-isothiocyano-2,2′-disulfonic stilbene.

tissue [4] and of eel electric organ [5]. Carbonic anhydrase activity was determined from the time-dependent decrease in pH during CO_2 hydration according to the Wilbur-Anderson method [6].

For studies on anion-transport, bladders were bathed on both surfaces by Na^+-free (choline) Ringer solutions, under which conditions the short-circuiting current (I_{sc}) is equal to the net flux of Cl^- and HCO_3^- from mucosa to serosa [2,3]. When the bladders were pretreated with ouabain and amiloride, which inhibit Na^+ transport but not anion transport [7,8], the effect of SITS was the same as that in the absence of these inhibitors.

For studies on Na^+ transport, bladders were bathed in Cl^--free, HCO_3^--free solutions (Na_2SO_4-Ringer); under which conditions I_{sc} equals the net flux of Na^+ from mucosa to serosa [9].

Fig. 1 shows typical plots of the transport parameter, $I_{sc}(t)/I_{sc}(0)$, versus time after exposure of bladders to SITS or acetazolamide. In anion-transporting bladders, the addition of SITS to the serosal fluid (10^{-6} M or 10^{-5} M) was followed by latent periods (15 or 10 min, respectively), after which the I_{sc} (and *PD*) declined rapidly (half-times of 30 or 10 min) to near-zero levels (curves S_1 or S_2), while *R* (not shown) remained unchanged. The same effect was observed with equimolar doses of DIDS, an analogue of SITS. On the other hand, the addition of SITS to the mucosal fluid, even at a higher concentration (10^{-4} M), failed to decrease the I_{sc} or *PD* (line a).

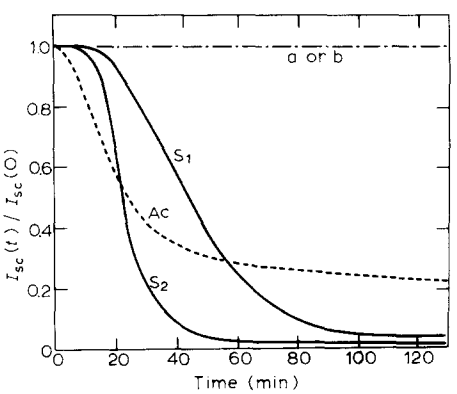

Fig.1. Effect of SITS or acetazolamide on transepithelial ion transport. $I_{sc}(t)/I_{sc}(0)$ is the ratio of short-circuiting current after addition of inhibitor to that just before addition. Curves S_1, S_2, Ac and line (a) show data from anion-transporting bladders (Na^+-free Ringer media). S_1 denotes SITS addition to serosal fluid at 10^{-6} M, and S_2 at 10^{-5} M. Curve Ac denotes acetazolamide addition to serosal fluid at 10^{-4} M. Line (a) denotes SITS addition to the mucosal fluid at 10^{-4} M. Line (b) denotes SITS addition to mucosal or serosal fluid at 10^{-3} M in Na^+-transporting bladders (Na_2SO_4-Ringer media).

After the serosal addition of acetazolamide (10^{-4} M), the degree of inhibition of anion transport (75%) was similar to that reported previously [3], indicating that this carbonic anhydrase inhibitor was much less potent than SITS as an anion-transport inhibitor (compare curves Ac and S_1). This was confirmed by additional data from 4 sets of turtle bladders shown in Table I.

Table I shows steady-state values of I_{sc} before and 120 min after the designated treatments. Even without correction for the time-dependent

TABLE I

EFFECTS OF SITS AND ACETAZOLAMIDE ON ANION-DEPENDENT SHORT-CIRCUITING CURRENT

Mean values ± S.E. of I_{sc} and percentage change in I_{sc} (with statistical parameters) before and after serosal addition of inhibitor at the concentration indicated. Numbers in parentheses denote the number of experiments for each serosal addition. Area of exposed tissue, 1.5 cm^2. Composition of bathing solution (mM): choline chloride, 83.5; choline bicarbonate, 20; KCl, 4; $MgSO_4$, 0.8; K_2HPO_4, 0.61; KH_2PO_4, 0.14; $CaCl_2$, 2; glucose, 11; gassed with O_2/CO_2 (98:2, v/v); final pH, 7.4—7.6.

Serosal additions	I_{sc} (μA)		Mean percentage differences (MPD)* (%)	Probability P(MPD = 0)
	Before	After		
None [5]	14.7 ± 2.1	12.5 ± 1.6	−14 ± 4	< 0.05
SITS, 10^{-6} M [4]	10.3 ± 3.1	1.1 ± 0.1	−87 ± 4	< 0.001
SITS, 10^{-5} M [5]	12.0 ± 3.1	0.6 ± 0.2	−91 ± 4	< 0.001
Acetazolamide, 10^{-4} M [5]	12.9 ± 2.3	4.6 ± 1.3	−65 ± 6	< 0.001

*MPD denotes the mean percentage differences, defined as the mean values ± S.E. of n individual percentage changes in I_{sc} before and after addition of the designated inhibitor in each of the 4 sets of experiments; or

$$\text{MPD}/100 = \left[\sum_{i=1}^{n} (\Delta I_{sc,i}/I_{sc,\text{before},i})\right] \Big/ \left[n\right].$$

decrement of I_{sc} (14%), the percentage decrement of I_{sc} after 10^{-6} M SITS was significantly greater ($P < 0.02$) than that after 10^{-4} M acetazolamide.

The sidedness and potency of the SITS-induced inhibition of anion transport suggest that this inhibitor binds to sites at or near the anion-selective paths in the basal-lateral membranes. The anion-selectivity of the SITS-induced inhibition of transport was underscored by the lack of effect of SITS on Na^+ transport. When added to either the serosal or mucosal bathing fluid at high concentrations (10^{-3} M), SITS evoked no change in I_{sc} (and *PD*) of bladders bathed in Na_2SO_4-Ringer (line b, Fig. 1).

It was therefore decided to determine the effect of SITS on two isolated enzymes: carbonic anhydrase, because of its relation to Cl^- and HCO_3^- transport [3], and ($Na^+ + K^+$)-ATPase, because of its relation to Na^+ transport.

Table II presents data on the concentrations of SITS, acetazolamide, or ouabain that were required for a 50% inhibition of each enzyme. Whereas SITS was much less potent (< 1/1000) than acetazolamide as an inhibitor of

TABLE II

INHIBITION OF CARBONIC ANHYDRASE AND ($Na^+ + K^+$)-ATPase BY SITS, ACETAZOLAMIDE AND OUABAIN

Carbonic anhydrase assay (3 experiments): Crystalline enzyme from beef carbonic anhydrase (Sigma), 0.25 μg/ml; Hepes buffer, 12.5 mM; initial CO_2, 38 mM; initial pH, 8.4; final volume, 5 ml. Hydration reaction at 0°C triggered by the addition of 2.5 ml of 76 mM CO_2. ($Na^+ + K^+$)-ATPase assay (3 experiments): Microsomal ATPase isolated from eel electric organ, 150 μg/ml; NaCl, 60 mM; $MgCl_2$, 3 mM; KCl, 25 mM; Tris-EDTA, 0.1 mM; [γ-^{32}P] ATP, 3 mM; Tris-HCl, 40 mM (pH 7.3); final volume, 100 μl. Reaction triggered at 25°C with 15 μl of 20 mM [γ-^{32}P] ATP (ref. 5).

Inhibitor	Molar conc. of inhibitor required for 50% inhibition of:	
	Carbonic anhydrase	($Na^+ + K^+$)-ATPase
SITS	$1 \cdot 10^{-5}$	$1 \cdot 10^{-5}$
Acetazolamide	$5 \cdot 10^{-9}$	no effect
Ouabain	no effect	$4 \cdot 10^{-5}$

carbonic anhydrase from beef erythrocytes, it was apparently four times more potent than ouabain as an inhibitor of $(Na^+ + K^+)$-ATPase in electric organ microsomes. For a 95% inhibition of $(Na^+ + K^+)$-ATPase, 10^{-4} SITS was as potent as 10^{-3} M ouabain in three experiments on electric organ microsomes, and in two on turtle bladder microsomes.

In a tentative hypothesis on anion transport in the turtle bladder, it is assumed that an active transport mechanism in the apical membrane pumps anions from the mucosal fluid into the cell; and that a discrete carrier mechanism in the basal-lateral membrane mediates the passive flow of anions from the cell to the serosal fluid. Within this framework, present data are consistent with the following claims:

(i) Certain disulfonic stilbenes and sulfonamides (SITS and acetazolamide) bind to sites at or near an anion-selective pathway (or carrier) in the basal-lateral membrane and consequently reduce the net flow of anions across the epithelial cell. The anion pathway might consist in part of a membrane-bound carbonic anhydrase [10] and/or a stilbene-binding protein [11]. Whereas soluble carbonic anhydrase has been found in turtle bladders [12], an anion-transport related, stilbene-binding protein remains to be found.

(ii) Even though SITS inhibits microsomal $(Na^+ + K^+)$-ATPase isolated from the turtle bladder, its lack of effect on Na^+ transport suggests that it cannot gain access to the SITS binding sites on this enzyme in the intact bladder cell. Therefore, whatever the nature of SITS binding in the intact bladder, it does not change the character of the Na^+ transport path in the basal-lateral membrane. On the other hand, ouabain inhibits $(Na^+ + K^+)$-ATPase in isolated bladder cell microsomes and Na^+ transport in the intact turtle bladder [7]. Therefore, ouabain does gain access to its binding sites on $(Na^+ + K^+)$-ATPase in the intact bladder cell, and this binding selectively blocks the Na^+ path in the basal-lateral membrane.

(iii) SITS inhibits the anion transport across turtle bladders about 100 times more effectively than it inhibits anion exchange across erythrocyte membranes [1].

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