Volume 175, number 2 FEBS 1808 October 1984

The effect of anion channel blockers on enzymatic activity of Na^+/K^+-ATPase and the electrogenic Na^+/K^+ pump

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Received 20 July 1984

Disulfonic stilbenes which block the anion-transport in red blood cells were found to inhibit the brain microsomal Na^+/K^+-ATPase but not the electrogenic Na^+/K^+ pump in intact muscle cells. In contrast to the anion-transport system, the Na^+/K^+-ATPase is inhibited by disulfonic stilbenes, apparently from the cytoplasmic side of the membrane. The pathways for anion and active cation transport are thus different but similar groups of sulfhydryl and/or amino acid residues must play an important role in both systems.

Brain microsome *Skeletal muscle* *Na^+/K^+-ATPase* *Electrogenic Na^+/K^+ pump* *Membrane resistance* *Disulfonic stilbene*

1. INTRODUCTION

The disulfonic stilbenes, SITS, DIDS and DNDS which are covalent binding agents for exposed amino groups on proteins [1] block anion transport in red blood cells [2,3]. SITS also has been found to inhibit the microsomal Na^+/K^+-ATPase of eel electric organ and turtle bladder [4]. We have investigated the effects of disulfonic stilbenes and structurally and/or functionally related compounds on the Na^+/K^+-ATPase activity and the electrogenic Na^+/K^+ pump to determine whether the same population of sites in the membrane may control both anion and active cation transport.

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Abbreviations: DIDS, 4-acetamido-4′-isothiocyano-2,2′-stilbene disulfonic acid; SITS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; DNDS, 4,4′-dinitro-2,2′-stilbene disulfonic acid; DES, diethylstilbestrol; PATAS, 4,4′-bis-4-phenylamino-6-(2-hydroxyethyl)amino-5-triazin-2-yl amino-2,2′-stilbene disulfonic acid

2. METHODS

2.1. *Estimation of Na^+/K^+-ATPase activity*

Enzymatic activity of the Na^+/K^+-ATPase was measured in membrane fractions prepared from the cerebral cortex of white Wistar rats (180 g body weight) according to [5]. The reaction was started by addition of ATP (final concentration 2.5 nM) to the microsomes (0.03–0.05 mg membrane protein per ml) which were preincubated for 5 min at 37°C in 1.25 ml of 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.4.

The precipitated protein was removed by centrifugation and the activity of Na^+/K^+-ATPase was measured as the production of inorganic phosphorus (P_i) [6]. The basal ouabain-insensitive activity of ATPase was measured in a K^+-free solution where 2×10^{-4} M ouabain was present.

2.2. *Measurement of the electrogenic Na^+/K^+ pump*

Electrophysiological experiments were performed on Na^+-loaded hemidiaphragms of female white mice (body weight ~20 g). Muscles were isolated and washed continuous for 5–6 h with an oxygenated (95% O_2, 5% CO_2) solution of the

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00145793/84/$3.00

following composition (in mM): NaCl, 149.8; KCl, 0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 12.8; NaH_2PO_4, 1.0; glucose, 11.0; pH 7.2 [7] at room temperature (20–22°C). The resting membrane potentials (RMP) were recorded from the superficial muscle fibres with an intracellular microelectrode (3 M KCl, 10–20 MΩ) before and 5–10 min after addition of 5 mM K^+ to the bath. The difference between these two values (ΔRMP) was considered to be a measure of the electrogenic Na^+/K^+ pump [8].

2.3. *Measurement of membrane resistance*

The effective input membrane resistance (R_E) of a skeletal muscle fibre was measured using a two-microelectrode intracellular recording technique. One electrode was used for recording membrane potential, the second one (2 M K^+-citrate; 5–10 MΩ), located approx. 20 μm from the first, was used for intracellular injection of an 8–10 ms current pulse of ~0.1 μA. The R_E was calculated from the potential and current changes which were monitored with a Tektronix oscilloscope and photographed.

2.4. *Drugs*

The sources for the compounds used were as follows: ATP (Boehringer); ouabain (Fluka); SITS, DIDS, DNDS and flufenamic acid (Sigma); DES (Serva); PATAS (a gift of Dr S. Fiker of Institute of Hygiene and Epidemiology).

3. RESULTS

3.1. *Enzymatic activity of microsomal Na^+/K^+-ATPase*

The anion channel blockers (DIDS, SITS, DNDS and flufenamate) and the structurally related compounds (DES, PATAS) inhibited the Na^+/K^+-ATPase (fig.1). DIDS and SITS caused 50% inhibition of Na^+/K^+-ATPase at 7.5 nM and 0.8 μM respectively. The disulfonic stilbene DNDS, and flufenamate, an anion-transport inhibitor [9] but not a disulfonic stilbene, inhibited the Na^+/K^+-ATPase only at millimolar concentrations (inhibition by 20 and 85%, respectively). The inhibition by PATAS and DES was comparable with that of SITS since half-maximal effects were observed at 3.5 and 7.5 μM, respectively.

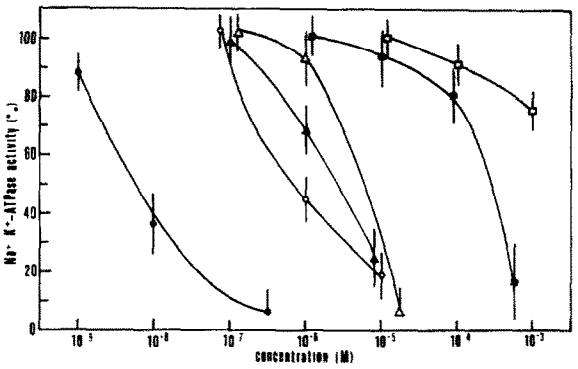

Fig.1. Inhibition of Na^+/K^+-ATPase by disulfonic stilbenes and related compounds. The Na^+/K^+-ATPase activity of brain microsomes was determined in the presence of different amounts of tested drugs: (●) DIDS; (○) SITS; (▲) PATAS; (△) DES; (■) flufenamate; (□) DNDS. Each value represents mean ± SE of 5–6 enzymatic measurements.

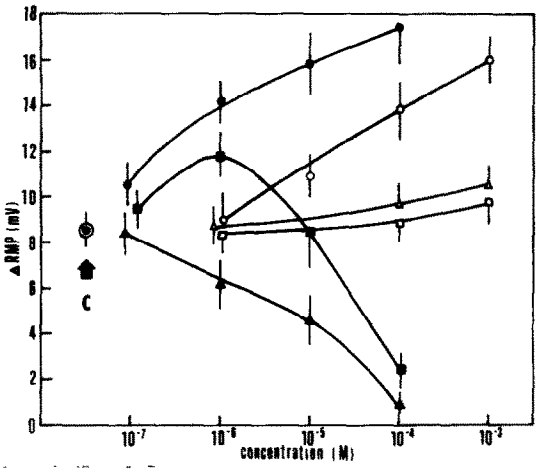

Fig.2. Effect of disulfonic stilbenes and related compounds on the electrogenic effect of Na^+/K^+ pump in Na^+-loaded mouse skeletal muscle. The hyperpolarization (i.e., more negative potential inside the cells) is expressed as ΔRMP measured 5–10 min after adding 5 mM K^+ to the muscle bath with tested drugs: (●) DIDS; (○) SITS; (▲) PATAS; (△) DES; (■) flufenamate; (□) DNDS; C, control. Each value represents mean ± SE from 3–6 muscles where 15–20 fibre potentials were measured in each muscle.

3.2. *Electrogenic effect of the Na^+/K^+ pump*

The average resting membrane potential (RMP) of muscles equilibrated for 5–6 h in a K^+-free solution was 63.7 ± 0.7 mV. After application of 5 mM K^+ the RMP hyperpolarized to 72.2 ±

Table 1

The effect of disulfonic stilbenes and related compounds on the effective input membrane resistance (R_E)

Drug present	R_E (10^5 Ω)	n
None	7.37 ± 0.39	15
DIDS	7.62 ± 0.38	4
SITS	7.35 ± 0.70	3
DNDS	7.31 ± 1.05	3
DES	7.23 ± 1.24	2
PATAS	7.20 ± 0.67	3
Flufenamate	7.78 ± 1.00	4

Measurements were carried out on freshly isolated muscles incubated in a normal K^+-containing solution. The effects of the drugs (all were used at 0.1 mM) were tested between 10–30 min after application. Values are given as a mean ± SE of n muscles where 10–15 fibres were measured in each muscle

0.9 mV. The difference between these two values (ΔRMP = 8.5 mV) represents the electrogenic effect of the Na^+/K^+ pump [8]. Preincubation of Na^+-loaded muscles for 10 min with DIDS and SITS not only failed to inhibit the electrogenic effect but the ΔRMP was enhanced by ~100% at 10 μM and 1 mM, respectively (fig.2). DNDS and DES were without effect over a wide range of concentrations (1 μM–1 mM). PATAS and flufenamate reduced the electrogenic effect of the Na^+/K^+ pump half-maximally at 10 μM and 0.1 mM, respectively. Flufenamate at 1 μM stimulated the electrogenic Na^+/K^+ pump by 30%.

3.3. *Membrane resistance*

To discover if the anion permeability of skeletal muscle was inhibited by the drugs used we measured the effective input membrane resistance (R_E) of muscle fibre membrane. None of tested drugs had any significant effect on membrane resistance 30 min after application (table 1).

4. DISCUSSION

Disulfonic stilbenes and compounds with similar structure and/or function were tested for their effect on the Na^+/K^+-ATPase as well as on the electrogenic Na^+/K^+ pump in intact cells. The Na^+/K^+-ATPase was inhibited by the tested drugs, with the following order of potency: DIDS ➤ SITS > PATAS > DES ➤ flufenamate > DNDS. The effectiveness of disulfonic stilbenes seems to correlate closely with the number of NCS^- groups they contain: DIDS (2 NCS^-) is 100 times more effective than SITS (1 NCS^-) and DNDS (0 NCS^-) had only a minor inhibitory effect at millimolar concentrations and so is 8000 times less effective than SITS.

Isothiocyanates are well known to be potent modifiers of sulfhydryl groups [10] and Na^+/K^+-ATPase inhibitors [11–13]. Nevertheless, some compounds without NCS^-, but possessing disulfonic stilbene or similar structure (PATAS and DES), inhibited the Na^+/K^+-ATPase at micromolar concentrations. In the yeasts, many ATPases were found to be inhibited by DES [14]. Flufenamate, which inhibits anion transport by binding to the stilbenedisulfonate-binding site in band III protein [15], inhibited Na^+/K^+-ATPase at mM concentrations. It indicates that not only NCS^- but also the disulfonic structure is responsible for Na^+/K^+-ATPase inhibition.

When applied extracellularly to intact cells, DIDS, SITS, DNDS and DES were without effect on the electrogenic Na^+/K^+ pump over a wide range of concentrations.

DIDS and SITS enhanced the electrogenic effect. The reason for this stimulation is unknown but again it correlates with the number of NCS^- groups in the disulfonic stilbene molecule: DIDS stimulates the electrogenic effect at 100 times lower concentrations than SITS; DNDS is without any stimulatory effect. Flufenamate and PATAS inhibited the Na^+/K^+ pump in the intact cells. The enhancement of the electrogenic effect in the presence of DIDS and SITS could be due to the increased membrane resistance after inhibition of Cl^- permeability [16]. In the skeletal muscle Cl^- permeability is responsible for 85% of total membrane permeability [17]. However, we did not see any increase in input membrane resistance during 30 min application of tested drugs, which raises the question of whether these compounds have an effect on the Cl^- channels in skeletal muscle.

The inhibition of Na^+/K^+-ATPase by anion channel blockers does not require that active cation and anion-transport be physically coupled. Firstly, anion transport is inhibited by disulfonic stilbenes from the external surface of the membrane [2] whereas Na^+/K^+-ATPase is apparently

accessible for inhibition only from the cytoplasmic side of the membrane since impermeable disulfonic stilbenes were ineffective when applied extracellularly to the intact cells (see also [4]). Secondly, our results are based on the action of anion channel blockers specific for erythrocyte band III protein [3]. Net movement of anions through band III protein is apparently distinct from anion transport in skeletal muscle plasma membrane since the stilbenes were apparently without effect on this system. In the red blood cell the membrane permeability for Cl^- $p_{Cl^-} = 1.49 \times 10^{-3}$ cm/s [3] is much greater than in skeletal muscle where $p_{Cl^-} = 4 \times 10^{-6}$ cm/s [18]. Our study showed that some of the features of the inhibition of Na^+/K^+-ATPase are similar to those observed for inhibition of anion transport in red blood cells. This suggests that a similar population of sulfhydryl and/or amino acid residues must be involved in both processes.

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