INHIBITION OF THE PURIFIED AND RECONSTITUTED CALCIUM PUMP OF ERYTHROCYTES BY μM LEVELS OF DIDS AND NAP-TAURINE

Verena NIGGLI, Erwin SIGEL and Ernesto CARAFOLI
Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zurich, Switzerland

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1. Introduction

In the course of investigations on the balance of charges during the operation of the reconstituted Ca^{2+}-pump of the erythrocyte membrane [1] it was found that two inhibitors of the anion transport in erythrocytes, NAP-taurine and DIDS [2], inhibited the ATPase-activity and Ca^{2+}-transport of the enzyme. The observation suggested the possibility that the purified Ca^{2+}-ATPase preparation contained remnants of band III. Preliminary tests suggested that the effect of the two inhibitors mentioned was unrelated to their ability to block the anion channel of plasma membranes (see [1]). More detailed investigations were therefore carried out, and the results are reported here. The finding offers a potentially interesting tool for future studies of the active site of the ATPase.

2. Materials and methods

The Ca^{2+}-ATPase was purified and reconstituted in asolectin or phosphatidylcholine liposomes as in [1] by the cholate dialysis procedure. The lipid–cholate–enzyme mixture was dialysed overnight at 4°C against 500 vol. 120 mM KCl, 10 mM Hepes (pH 7.2), 50 μM $MgCl_2$ and 1 mM dithiothreitol. ATPase activity was measured spectrophotometrically by a coupled enzyme assay, as in [1] in the presence of 10 μM free Ca^{2+} and 0.5 μM A23187. The indicated amounts of DIDS (stock solution: 0.5 mM in dimethylsulfoxide) were added to the medium (containing 4–8 μg reconstituted enzyme), and the activity was followed for ~4 min. The inhibition became maximal after 1 min incubation of the enzyme with the inhibitor. Dimethylsulfoxide alone (10 μl/ml) had no effect on the Ca^{2+}-ATPase, even after 30 min incubation at 37°C. At the concentrations used in the experiments described DIDS had no inhibitory effect on the coupled enzyme system. The inhibition by NAP-taurine was studied in the same way as that by DIDS. A stock solution of NAP-taurine in 10 mM Hepes (pH 7.0) (10 mM) was prepared fresh every day, and protected from light.

Abbreviations: DIDS, 4,4'-di-isothiocyano-2,2'-stilbene disulfonic acid; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonic acid

3. Results and discussion

Fig.1 shows the effect of DIDS on the Ca^{2+}-ATPase reconstituted in asolectin liposomes, where the enzyme is optimally active in the absence of the natural activator calmodulin. In the presence of 0.1 mg phospholipid/ml reaction medium, the ATPase is inhibited 50% by 0.4 μM DIDS, and ~80% by 1.5 μM. The K_i of DIDS depends on the amount of liposomes added: DIDS very probably binds to phospholipids, hence the amount of 'free' inhibitor in the medium decreases as the amount of liposomes increases. This is clearly seen in fig.1, where the concentration of DIDS required to induce 50% inhibition of the ATPase increases from 0.4, to 1.2, to 2.0 μM as the amount of phospholipid in the reaction medium is increased from 0.11 mg/ml to 0.33 mg/ml to 0.55 mg/ml. The phenomenon is analyzed in more detail in fig.2 in which the percent decrease of the ATPase activity has been plotted against the μmol DIDS/mg phospholipid. DIDS inhibits, with about the same

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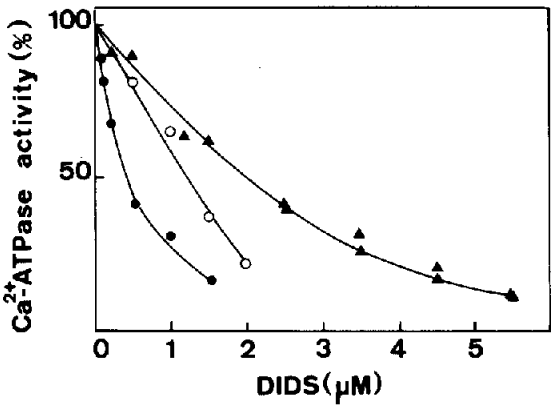

Fig.1. Inhibition of the purified Ca^{2+}-ATPase, reconstituted in asolectin vesicles, by DIDS. The Ca^{2+}-ATPase activity of the reconstituted enzyme was determined in the presence of different amounts of DIDS (as in section 2). Activity of 100% corresponds to the activity in the absence of inhibitor: (•) 10 μl proteoliposomes/ml assay medium; (○) 30 μl proteoliposomes/ml assay medium; (▲) 50 μl proteoliposomes/ml assay medium. The proteoliposomes contained 11 mg lipid/ml and 0.2 mg enzyme/ml (same preparation used in fig.2).

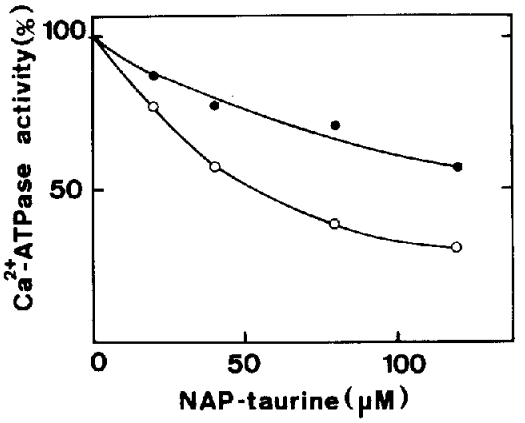

Fig.3. Inhibition of the purified Ca^{2+}-ATPase, reconstituted in asolectin vesicles, by NAP-taurine. The Ca^{2+}-ATPase activity of the reconstituted enzyme was determined in the presence of different amounts of NAP-taurine (as in section 2). Activity of 100% corresponds to the activity in the absence of inhibitor: (○) 10 μl proteoliposomes/ml assay medium; (•) 30 μl proteoliposomes/ml assay medium; proteoliposomes contained 11 mg lipid/ml and 0.2 mg enzyme/ml.

affinity, and to about the same extent, the initial rate of Ca^{2+}-transport by the reconstituted enzyme, as determined by using a Ca^{2+}-sensitive electrode (see [1]).

The other inhibitor of the anion transport in plasma membranes, NAP-taurine, also inhibits the reconstituted ATPase (fig.3), although not as efficiently as DIDS. At 10 μl liposomes/ml reaction medium, 50% inhibition of the ATPase requires ~50 μM NAP-taurine. In agreement with the observations on DIDS, also in the case of NAP-taurine the K_i is shifted towards higher concentrations by increasing concentrations of phospholipids.

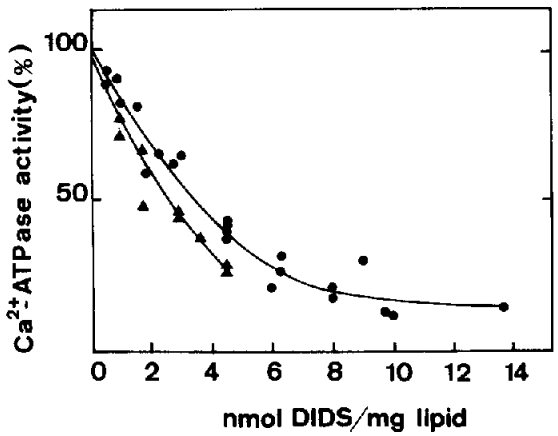

Fig.2. Inhibition of the purified Ca^{2+}-ATPase, reconstituted in asolectin, in the presence and in the absence of A23187, by DIDS: (•) the data of fig.2 were plotted as % activity *vs* nmol DIDS added/mg lipid in the assay medium; (▲) inhibition of Ca^{2+}-ATPase was determined under the conditions in fig.1, with the same enzyme preparation, except that A23187 was not present in the assay medium.

As proposed in [3], the inhibition by DIDS could be due to the blockade of the anion channel, which would normally charge-compensate the electrogenic translocation of Ca^{2+} in red cells. In principle, small contaminating amounts of band III could be present also in the reconstituted ATPase preparation, and function as a charge-compensating pathway for the transport of Ca^{2+}. If this were the case, however, DIDS would be expected to inhibit only the ATPase activity coupled to Ca^{2+}-transport and not the 'uncoupled' ATPase (the uncoupled ATPase is defined in this context as the reconstituted ATPase studied in the presence of A23187, which immediately discharges the transported Ca^{2+} back to the extraliposomal medium). This was found not to be the case. The 'coupled' and the 'uncoupled' ATPases are inhibited to approximately the same extent by the same concentrations of DIDS (fig.2).

The purified Ca^{2+}-ATPase can be activated, in the absence of calmodulin, by acidic phospholipids. When

reconstituted in an ambient medium containing ~20% or more acidic phospholipids (e.g., asolectin) the enzyme is already in the high Ca^{2+} affinity form in the absence of calmodulin. When reconstituted in phosphatidyl choline the enzyme is in the low affinity form and activatable by calmodulin [4]. DIDS inhibits the enzymes reconstituted in asolectin or phosphatidylcholine with about the same efficiency (not shown).

It thus appears evident that DIDS (and NAP-taurine) inhibits the Ca^{2+}-ATPase directly, and not by interfering with the operation of a hypothetical charge-compensating anion channel. The direct inhibition would be difficult to detect in the intact red cell membrane, due to the presence of band III (the anion channel) which has a very high affinity for DIDS (K_i, 0.04 μM [2]) and is ~100-times more abundant than the Ca^{2+} ATPase. It is of interest that SITS, an inhibitor of the anion channel very similar to DIDS, has been found to inhibit the $Na^+ + K^+$-ATPase of microsomal preparations of both turtle bladder and eel electric organ [5] at μM levels. DIDS has also been found to inhibit the transport of Ca^{2+} into liposomes reconstituted with the purified sarcoplasmic reticulum Ca^{2+}-ATPase [6], and to bind the ATPase molecule.

As for the mechanism of the inhibition, the interaction with sulphydryl and/or amino groups essential for enzyme activity seems a possibility. DIDS is known to react covalently with sulfhydryl and amino groups of proteins [2]. In [7] the membrane-bound Ca^{2+} pump was markedly inhibited by low concentrations of sulfhydryl-group reagents.

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