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INHIBITION OF ANION PERMEABILITY OF SARCOPLASMIC RETICULUM VESICLES BY STILBENE DERIVATIVES AND THE IDENTIFICATION OF AN INHIBITOR-BINDING PROTEIN

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Summary

The permeability of sarcoplasmic reticulum vesicles to sulfate ions was inhibited by diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid (H_2DIDS), which is a potent inhibitor of anion permeability in red blood cell membrane. The amount of H_2DIDS bound to the vesicles was determined by using [^{3}H]-H_2DIDS. Apparent half inhibition of sulfate permeation was observed on the binding of 2.5 μmol/g protein. SDS-polyacrylamide gel electrophoresis of the vesicles treated with [^{3}H]H_2DIDS showed that about 10% of the total bound H_2DIDS corresponds to a 100 000-dalton protein, but the remaining 90% to non-protein components. The content of the H_2DIDS-binding protein was about 0.5 μmol/g protein. These results suggest that the H_2DIDS-binding protein is different from the calcium pump protein and is possibly an anion transport system similar to band 3 in red blood cell membrane.

Introduction

In a previous paper [1], we found that 4-acetoamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS), a potent inhibitor of the anion permeability of red blood cell membrane [2,3], inhibited the permeability of sarcoplasmic

Abbreviations: SITS, 4-acetoamide-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS, diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

reticulum for anions such as Cl^-, P_i and methanesulfonate, whereas it had little effect on cation permeability. Binding of 5 μmol SITS/g protein was necessary for the complete inhibition of anion permeability. These results suggest the existence of the anion transport system in sarcoplasmic reticulum membrane. A similarity between band 3 protein in red blood cell membrane and this anion transport system was suggested. However, since the permeability of sarcoplasmic reticulum vesicles was measured by following the change in the intensity of scattered light due to the osmotic volume change of the vesicles, the quantitative analysis of the permeability was not made sufficiently.

In this paper, much stronger inhibitors of anion permeability, such as DIDS and the dihydro analog of DIDS (H_2DIDS), were used. The permeability for SO_4^{2-} was measured by using radioactive tracers. Radioactive [^{3}H]H_2DIDS was used in an attempt to identify the anion transport system.

As a result, SDS-polyacrylamide gel electrophoresis of the vesicles treated with radioactive H_2DIDS showed that the major H_2DIDS-binding protein has a molecular weight of about 100 000. This 100000-dalton band overlapped that of the calcium pump protein (Ca^{2+}-ATPase), but differs from calcium pump protein.

Materials and Methods

Materials. Sarcoplasmic reticulum vesicles were isolated from rabbit dorsal and hind leg muscle as a microsomal fraction by the method of Weber et al. [4], slightly modified [5]. The vesicles were stored in 5 mM K^+-Hepes (pH 6.5) at 0°C and used within 1 week of isolation. H_2DIDS and [^{3}H]H_2DIDS were kinds gifts from Professor A. Rothstein. DIDS was purchased from Pierce (U.S.A.) and $Na_2^{35}SO_4$ was from New England Nuclear (U.S.A.). Other reagents were commercial products of analytical grade.

Assay of permeability. The permeability of membrane vesicles for SO_4^{2-} was measured according to our earlier method [6]; sarcoplasmic reticulum vesicles were incubated for about 2 h in a solution containing 67 mM K_2SO_4/5 mM K^+-Hepes (pH 6.5) and small amount of $Na_2^{35}SO_4$ (about 10 μCi/ml, 15 μM) at room temperature. The mixture was diluted 100 times with 67 mM K_2SO_4 and 5 mM K^+-Hepes (pH 6.5). Then, at each measurement time, 1.0 ml of the diluted mixture was rapidly filtered through a Millipore filter (HAWP 02500, Millipore Co. U.S.A.). The radioactivity remaining on the filter was measured in a liquid scintillation counter (LS-500, Horiba, Japan) as described earlier [6].

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. [7]. The molecular weight was determined using cross-linked hemoglobin and cross-linked bovine serum albumin (Sigma, U.S.A.).

Assay of protein. Protein concentration was assayed by the Biuret method using bovine serum albumin as a standard [8].

Results

Inhibition of sulfate permeability by H_2DIDS

Fig. 1 shows the efflux of SO_4^{2-} from sarcoplasmic reticulum vesicles treated

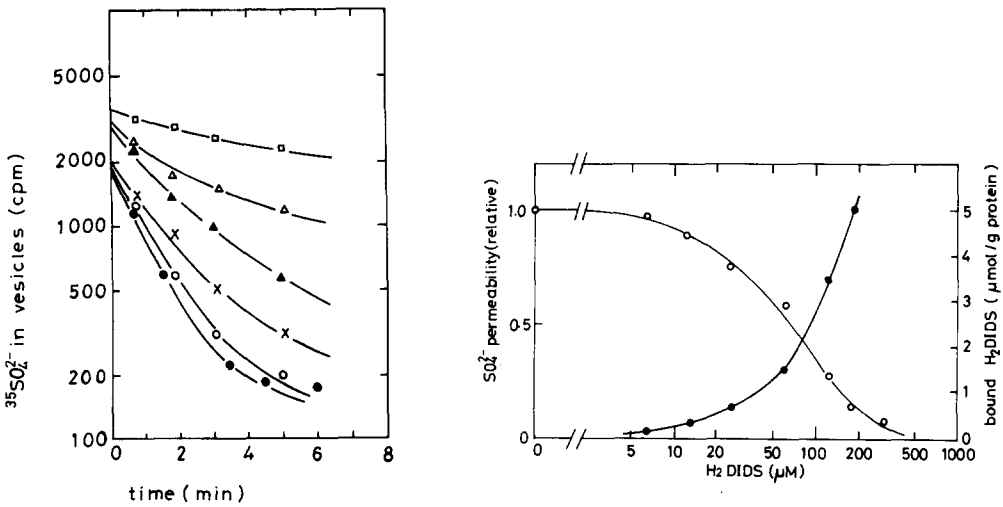

Fig. 1. Inhibition of the sulfate permeability by H_2DIDS. Sarcoplasmic reticulum vesicles were treated with various concentrations of H_2DIDS in 5 mM K^+-Hepes (pH 6.5), and 30 mg protein/ml for 10 min at 37°C. Then the vesicles were incubated with 67 mM K_2SO_4, 5 mM K^+-Hepes (pH 6.5), a small amount of $Na_2^{35}SO_4$ and 20 mg protein/ml for 2 h at room temperature. After the mixture was diluted 100 times with 67 mM K_2SO_4 and 5 mM K^+-Hepes (pH 6.5), the efflux of SO_4^{2-} was followed. H_2DIDS concentration during the treatment was as follows: ●, 0; ○, 12 μM; X, 24 μM; ▲, 60 μM; △, 120 μM; □, 300 μM.

Fig. 2. The relative rate of sulfate efflux and the amount of bound H_2DIDS to the vesicles as a function of H_2DIDS concentration. As a measure of the relative rate of the sulfate efflux τ_0/τ was used, where τ_0 is the permeation time of sulfate not treated with H_2DIDS and τ that treated with H_2DIDS. Experiments similar to that described in Fig. 1 were carried out. The amount of H_2DIDS bound to the vesicles was determined as follows. Vesicles were treated with various concentrations of [^{3}H]H_2DIDS (148 Ci/mol) under the same conditions as in Fig. 1. The treated mixture was diluted 150 times with 5 mM K^+-Hepes (pH 6.5) and centrifuged at 90 000 × g for 30 min. The amount of H_2DIDS bound to the vesicles was determined from the difference between the radioactivity of the solution before centrifugation and that of the supernatant solution after centrifugation. To determine the level of radioactivity, each 200 μl of the solution were mixed with 3 ml Scintisol 500 (Dojin Kagaku, Japan) and counted in a liquid scintillation counter. ○, The relative sulfate permeability; ●, the amount of bound H_2DIDS.

with H_2DIDS. When the concentration of H_2DIDS was increased, the permeability for sulfate decreased. For the quantitative analysis of the permeability, the permeation time, τ, was defined as the time to reach half the initial value, which was estimated by an extrapolation of the curve to zero time. The permeation time is inversely proportional to the permeability coefficient. Without H_2DIDS treatment, the permeation time was about 1 min at room temperature and increased as the concentration of H_2DIDS rose.

In Fig. 2, the relative permeability of SO_4^{2-} ions was plotted against the concentration of H_2DIDS. Half inhibition occurred when the vesicles were treated with about 60 μM H_2DIDS. When DIDS was used instead of H_2DIDS, a similar inhibition curve was observed. In the case of red blood cell membrane, the effect of DIDS is stronger than that of H_2DIDS [9], but DIDS and H_2DIDS both produced similar effects when used on sarcoplasmic reticulum. This concentration is 3-times smaller than that of SITS (0.2 mM) [1]. The concentrations required for the inhibition of sulfate permeability decreased with decreasing concentration. This is explained by the fact that the concentration

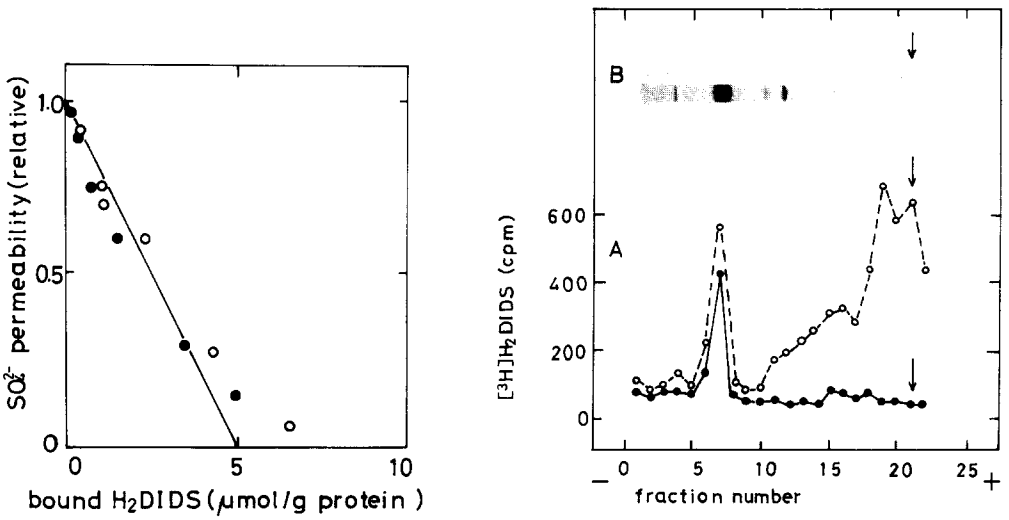

Fig. 3. Relationship between the sulfate permeability and the amount of H_2DIDS bound to the vesicles. •, Data were taken from Fig. 2; ○, data obtained by using a different preparation. The line represents the results expected for linear inhibition of 5 μmol/g protein is required for complete inhibition.

Fig. 4. Identification of H_2DIDS-binding protein. A, SDS-polyacrylamide gel electrophoresis was carried out in the presence of 5% 2-mercaptoethanol using sarcoplasmic reticulum vesicles (66 μg protein) treated in 200 μM [^{3}H]H_2DIDS/5 mM K-Hepes (pH 6.5) for 10 min at 37°C. The total amount of bound H_2DIDS was 5 μmol/g protein from the centrifugation analysis, as in Fig. 2. After the electrophoresis, the gel was stained Coomassie brillant blue and destained in a solution of 5% methanol/7.5% acetic acid for 36 h. • , The destained gel was then sliced in 4-mm sections and each section was incubated in 0.5 ml H_2O_2 for 1 h at 90°C. After addition of 7 ml Scintisol 500, radioactivity was measured. ○, The gel was assayed without staining. B, Electrophoretic pattern. Sarcoplasmic reticulum vesicles (33 μg protein) were electrophoresed. Arrows show the position of tracking dye.

of free inhibitors decreased as a result of the covalent bond formation when concentrated vesicles were used for the reaction. Accordingly, if we plotted the sulfate permeability as a function of free inhibitor, the inhibition curves at different protein agreed with each other and half inhibition occurred at 15 μM for both DIDS and H_2DIDS and at 70 μM for SITS. The H_2DIDS-binding substance must be an anion transport system. In Fig. 2, the amount of H_2DIDS bound to the vesicles was also shown. The result was obtained by using [^{3}H]H_2DIDS.

In Fig. 3, the amount of bound H_2DIDS was plotted against the permeability for sulfate. Apparent half inhibition of sulfate permeation occurred at the binding of 2.5 μmol H_2DIDS/g protein. When this relation is extrapolated linearly, it is expected that the binding of 5 μmol/g protein is required for complete inhibition. This result is consistent with the previous results obtained using SITS [1]. This value agreed with the content of the calcium pump protein, since its molecular weight is 100 000 and its content is about 50% in our preparation [10].

H_2DIDS-binding protein

In order to clarify the relation between the anion transport system and the calcium pump protein, SDS-polyacrylamide gel electrophoresis was carried out

using H_2DIDS-labelled vesicles. As shown in Fig. 4, when the gel was counted before the staining, radioactivity measurement showed a peak at the position of the 100 000-dalton protein and a large broad band in the low molecular weight region. The former peak corresponds to the position of the calcium pump protein, but the latter broad band does not correspond to the staining of protein. When the gel was counted after the staining, the peak at 100 000 daltons decreased slightly. On the contrary, most of the large broad band in the low molecular weight region disappeared, although small peaks remained at the position corresponding to the proteins of about 30 000 and 20 000 daltons which are faintly seen in Fig. 4B. Most of the radioactivity appearing in the low molecular region may originate from phosphatidylethanolamine reacted with H_2DIDS, since there was no staining of protein corresponding to the radioactivity which disappeared during the staining process. This result suggest the possibility that the 100 000-dalton protein is the anion transport system. At present, however, we do not rule out the possibilities that some proteins binding H_2DIDS are removed during the staining and that free H_2DIDS exists which is bound to some proteins tightly but non-covalently during the flux measurement.

In the case of Fig. 4, the radioactivity measured in the 100 000-dalton band was about 10% of the total bound H_2DIDS, and the amount of H_2DIDS was about 10% of the calcium pump protein, although the permeability of sulfate was completely inhibited. As shown in Table I, when the concentration of H_2DIDS and the treatment time were increased, the amount of bound H_2DIDS in the 100 000-dalton band did not increase above about 0.5 μmol/g protein. This value is about 10% of the calcium pump protein. This result suggests that the H_2DIDS-binding protein is a different molecule from the calcium pump protein, although their molecular weights are close. A preliminary experiment

TABLE I

THE AMOUNT OF H_2DIDS BOUND TO THE VESICLES UNDER VARIOUS CONDITIONS

Sarcoplasmic reticulum vesicles were treated at 37°C in a solution containing [^{3}H]H_2DIDS and the amount of total bound H_2DIDS was determined by centrifugation as described in Fig. 2. The amount of H_2DIDS bound to the 100 000-dalton protein was determined from the radioactivity of the three fractions around the peak. The radioactivity was determined using gels before the staining as described in Fig. 4. The amount of sarcoplasmic reticulum vesicles is given as that of protein.

Condition at treatment			Total bound H_2DIDS		H_2DIDS bound to 100 000-dalton protein (μmol/g)
Added H_2DIDS (μM)	Sarcoplasmic reticulum vesicles (mg/ml)	Incubation time (min)	% of added H_2DIDS	μmol/g	
110	5	1	28.2	6.2	0.26
110	5	3	30.0	6.6	0.31
110	5	10	36.3	8.1	0.39
110	5	60	47.7	10.5	0.50
20	2	10	25.0	2.5	0.23
50	8	10	89.6	5.6	0.60
190	10.5	10	40.6	8.5	0.45
220	30	10	79.0	5.0	0.40

supporting this suggestion is obtained as follows. When the vesicles labelled with H_2DIDS were treated at pH 11.3, about 40% of the total protein was removed and the absorbance of Coomassie brillant blue at 100 000 daltons decreased to about 1/3 of the control, but the radioactivity of H_2DIDS at this position did not decrease. When the electrophoresis was carried out without 2-mercaptoethanol, a similar distribution of radioactivity was obtained.

Discussion

In this paper, we confirmed the existence of anion transport system in sarcoplasmic reticulum vesicles, and that anion permeability was inhibited by H_2DIDS. This results was obtained by using radioactive SO_4^{2-}. In the previous paper [1], ionic permeability was measured by osmotic volume change and the specific effect of SITS was reported. These results suggest that a specific anion transport system exists in sarcoplasmic reticulum similar to that in red blood cell membrane [2,3]. With the scattering method the net movement of ions can be followed while the tracer method permits the observation of exchange diffusion [3,11]. When the net movement of SO_4^{2-} was followed by the scattering method, it was found that the net movement of sulfate was slightly slower than that of the exchange diffusion (unpublished data). In the case of red blood cells, the rate of the exchange diffusion of sulfate is several times faster than that of the net movement [3]. There must be some difference between the anion transport system in sarcoplasmic reticulum and that in red blood cell membrane.

One of the H_2DIDS-binding substances was identified as proteins of about 100 000 daltons. Although, this value is close to that of the calcium pump, this protein is suggested to be a different substance from the calcium pump protein. Separation of the H_2DIDS-binding protein from the calcium pump is now in progress.

The H_2DIDS-binding protein has a molecular weight similar to band 3 which is known as the anion transport system in red blood cell membrane [2]. It is possible that the H_2DIDS-binding protein is the anion transport system in sarcoplasmic reticulum. However, since only 10% of the total bound H_2DIDS was found on this protein, the remaining 90% of binding sites could be involved in the inhibition of sulfate transport. The following substances remain as candidates of the anion transport system other than the 100 000-dalton protein: small peaks of radioactivity appeared in the low molecular weight region, components which are removed during the staining, and components which can bind H_2DIDS tightly but reversibly. The isolation of the anion transport system and the reconstitution of the vesicles are required.

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