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Characterization of anion binding sites of sarcoplasmic reticulum vesicles by ^{35}Cl-NMR

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Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle. It could be demonstrated that the anion binding sites on this membrane can be studied by ^{35}Cl-NMR spectroscopy. Titration of sarcoplasmic reticulum vesicles with the sulfate exchange inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) revealed specific binding of this compound to the sarcoplasmic reticulum membrane. A new inhibitor, pyridoxalphosphate-6-azophenyl-2′-sulfonic acid (PPAPS) was introduced and proved to displace chloride equally well from its binding sites. Two binding sites could be distinguished by titration with inorganic phosphate in the presence and absence of the inhibitors. Because of the insensitivity of ^{35}Cl-NMR spectroscopy these anion binding sites have to be located on a protein being present in considerable amount in the sarcoplasmic reticulum membrane.

Introduction

The membranes of sarcoplasmic reticulum from skeletal muscle are permeable to many different anions as has been demonstrated by flux measurements of oxalate [1], succinate [2], sulfate, and chloride [3], the latter exhibiting the shortest permeation time.

Their transport through sarcoplasmic reticulum membranes seems to be closely related to the calcium permeability and might play an essential role in compensating the large net charge being transported into sarcoplasmic reticulum vesicles by the active accumulation of calcium [4].

These transport measurements provided only indirect information about the anion binding sites and their location on the sarcoplasmic reticulum membrane. We used ^{35}Cl-NMR spectroscopy to measure the binding of chloride directly. It was used as a probe to monitor anion binding to protein by the change of its signal width depending on the mode and strength of binding [5]. Because of its insensitivity ^{35}Cl-NMR spectroscopy is only suitable for the exploration of binding to, for example, protein structures which are present in considerable and sufficient concentrations [5,6].

Thus Falke et al. [7] applied this method to characterize chloride-binding sites on band 3 protein on the erythrocyte membrane (28% of the total membrane protein). They could distinguish two sets of binding sites with different affinities.

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; PPAPS, pyridoxalphosphate-6-azophenyl-2′-sulfonic acid; TEA, triethanolamine; SR, sarcoplasmic reticulum; H_0, static magnetic field strength; SDS, sodium dodecylsulfate.

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They identified the high-affinity site as the chloride transport site by studying the effects of the chloride transport inhibitor 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS).

In this paper we tried to characterize the anion binding sites on sarcoplasmic reticulum to get further information about the anion transport across this membrane. For competitive studies we used the sulfate exchange inhibitors DIDS and PPAPS. The former is a well known anion transport inhibitor [8], the latter is a derivatized pyridoxalphosphate which shows half-maximal inhibition of sulfate exchange through the sarcoplasmic reticulum membrane at 55 μM [9].

Materials and Methods

SDS was purchased from Serva (Heidelberg), triethanolamine, inorganic salts and ^2H_2O were from Merck (Darmstadt). PPAPS was prepared according to Ref. 9, DIDS was synthesized according to Ref. 10.

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from hind leg muscles of rabbit according to the procedure of Hasselbach and Makinose [11] as modified by de Meis and Hasselbach [12]. The last wash was performed in 50 mM KCl buffered with 1 mM TEA-HCl (pH 7.4) by centrifugation at $80000 \times g$ (Beckmann 45 Ti, 30500 rpm) for 60 min at 4°C. The resulting pellet was resuspended and homogenized in the same buffer. The final protein concentration of the non-fractionated vesicles was 10 to 20 mg/ml. 0.5 ml portions were shock frozen in liquid nitrogen and kept in the deep freeze at −70°C. The quality of the sarcoplasmic reticulum vesicle preparation was checked by SDS-PAGE [13], Ca^{2+}- and Mg^{2+}-dependent ATPase activity, and ATP-supported Ca^{2+} accumulation. The liberation of inorganic phosphate was determined according to Fiske and SubbaRow [14] resulting in an average ATPase activity between 1 and 2 μmoles phosphate liberated per minute and mg protein. Active calcium uptake was measured using $^{45}Ca^{2+}$ as radioactive tracer by the filter assay procedure of Martonosi and Feretos [15]. The total protein concentration was determined by a modified Biuret method according to Ref. 16.

For NMR measurements the sarcoplasmic reticulum vesicle solutions were placed in 10 mm sample tubes (2.0 ml). 0.5 ml of ^2H_2O was added for frequency lock resulting in a final chloride concentration of 40 mM.

^{35}Cl-NMR spectroscopy

The NMR spectra were obtained using a Bruker NMR spectrometer AM270. The magnetic field strength of 6.34 T (tesla) corresponds to a ^{35}Cl resonance frequency of 26.467 MHz. The spectral width was 1000 Hz; 600 W data points were accumulated and zero-filled to 2K before Fourier transformation. The line-width was determined using a non-linear least-squares fit routine that varied the parameters of the Lorentzian, i.e. resonance position, maximum, and line-width, so that the sum of squares of deviations was smaller than a given value.

Since the experimental line-width contains the systematic error of the H_0 magnetic field inhomogeneity, optimal shimming under measurement conditions was applied. This procedure, corresponding to the application of several thousand dummy scans, minimized also the error due to deviations in sample temperature (300 K). The H_0 inhomogeneity error was compensated by subtracting the line-width obtained from a spectrum of a pure KCl solution taken under identical conditions. The resulting difference is called in the following ^{35}Cl line broadening.

Results and Discussion

The application of ^{35}Cl-NMR in the analysis of chloride binding to sites in biological systems has been described for erythrocytes and other systems [5,7,17]. We applied this technique for the first time to sarcoplasmic reticulum membranes of muscle cells.

From theoretical considerations of spin relaxation of ^{35}Cl nuclei under conditions of chemical exchange it was deduced that the relaxation may be non-exponential if the correlation time of the molecular reorientations is long or if the exchange rate is slow [5,18]. In order to test the validity of the fast-exchange limit and the assumption that

the fraction of bound chloride is $\ll 1$, we investigated the line-shape of the resonance. In all experiments described below, the resonances showed pure single Lorentzian line-shape suggesting that the exchange rate between all binding sites was not in the slow-exchange limit.

In this case the ^{35}Cl line broadening is related to the chloride concentration by the following equation:

$$\Delta\nu = \sum_i \frac{a_i n_i [P]}{[Cl^-] + K_i} \quad (1)$$

In this expression, $\Delta\nu$ is the line broadening, [P] the protein concentration, a_i is a constant describing the difference of line-width in the totally free and totally bound state, n_i is the number of sites of type i on the protein, $[Cl^-]$ is the chloride concentration and K_i the dissociation constant of chloride in binding site i.

Eqn. 1 predicts that the contribution of binding site i to the line broadening is proportional to the number of binding sites of this type. Therefore the line broadening is proportional to the concentration of protein. We measured the line-width applying protein concentrations of 3 to 11 mg/ml and found a linear dependence of the line broadening as expected.

An attempt to characterize binding sites on the sarcoplasmic reticulum membrane was made by using inhibitors like DIDS and PPAPS which are known to occupy selectively certain types of anion binding sites. In this case the line broadening is given by Eqn. 2, where [I] is the free inhibitor concentration and K_I the dissociation constant of the inhibitor.

$$\Delta\nu = \sum_{i=1}^{2} \frac{a_i n_i [P] \times 1/K_i}{1 + [Cl^-]/K_i + [I]/K_{I,i}} \quad (2)$$

DIDS is known to have two binding sites on the Ca^{2+}-ATPase [19]. Another binding site for H_2DIDS has been found on a different protein of the same molecular mass [20]. DIDS also inhibits Ca^{2+}-stimulated ATP hydrolysis. Half-maximal inhibition occurs at 135 μM [9]. Furthermore, it inhibits sulfate exchange across sarcoplasmic reticulum vesicle membranes [21]. Since half-maximal inhibition is already reached at about 4–5 μM

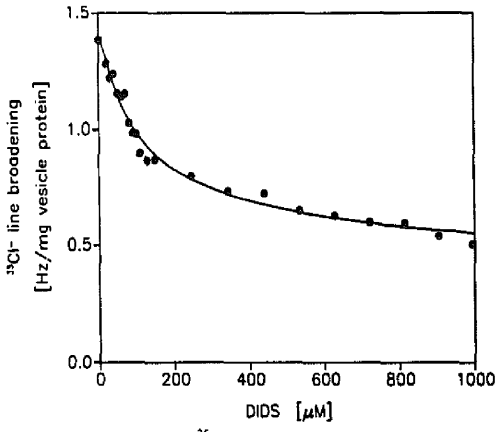

Fig. 1. Dependence of ^{35}Cl line broadening on DIDS concentration. 4 mg/ml sarcoplasmic reticulum vesicle protein, dissolved in 40 mM KCl and buffered with 1 mM TEA-HCl (pH 7.4), were titrated with 10 mM DIDS. The data were fitted according to Eqn. 2 assuming two types of binding sites.

[9,21] there has to be a high-affinity DIDS-binding site.

Therefore we expected that at least two DIDS-binding sites with different dissociation constants could be detectable by NMR investigations.

Fig. 1 shows the dependence of the line broadening on the DIDS concentration. The rapid decrease of the line-width at low inhibitor concentrations is due to the high-affinity sites whereas the smaller slope at high concentration is determined by the low-affinity sites.

In order to evaluate the dissociation constants of the above sites we fitted the experimental results with a mathematical expression according to Eqn. 2, assuming two types of binding sites. Fig. 1 shows that the fit is quite satisfying. It was obtained with dissociation constants of 14 ± 3 and 132 ± 11 μM (S.D.), respectively. Because of the scattering of the data points the existence of further binding sites cannot be ruled out.

So far we have demonstrated that DIDS is a powerful tool for selective inhibition of chloride binding. In order to further characterize anion binding sites we used phosphate, sulfate, oxalate and halides to investigate the effect of these anions on chloride binding. Because of its extremely high charge density we used fluoride in competition with chloride to reveal unspecific chloride binding sites. No chloride displacement could be

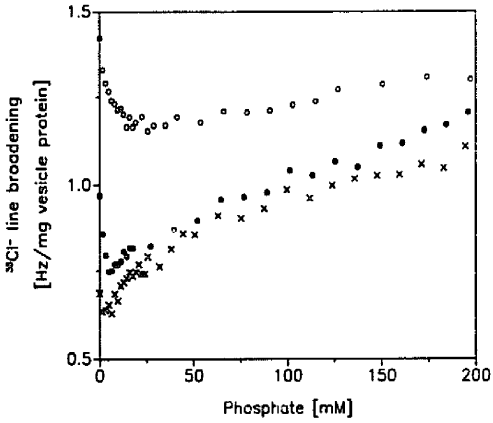

Fig. 2. Dependence of ^{35}Cl line broadening on phosphate concentration in the presence and absence of inhibitors. 4 mg/ml sarcoplasmic reticulum vesicle protein, dissolved in 40 mM KCl and buffered with 1 mM TEA-HCl (pH 7.4), were titrated with phosphate: (●) 100 μM DIDS, (×) 200 μM PPAPS, (○) no inhibitor present.

detected by ^{35}Cl-NMR up to 200 mM fluoride. Phosphate and sulfate proved to be the strongest in replacing chloride. Furthermore, phosphate turns out to increase Ca^{2+} accumulation in sarcoplasmic reticulum in vitro as can be shown by flux measurements [1]. Therefore we describe in the following the investigation of phosphate binding sites.

In Fig. 2 the upper curve shows the inhibition of chloride binding by phosphate. Low concentrations of phosphate inhibit chloride binding, i.e the line-width of chloride decreases with increasing phosphate concentrations. This effect is dominating up to a concentration of approx. 30 mM. When the concentration is higher than 50 mM, the effect is dominated by a second mechanism which causes an increase in the line-width of the chloride resonance. We studied the relaxation of chloride in the presence and absence of protein and found that the addition of phosphate enhances the relaxation of ^{35}Cl in aqueous solution. Consequently, the increase of the line-width of the chloride resonance in Fig. 2 with increasing phosphate concentration is partially caused by a direct relaxation enhancement in the ternary system KCl-K_2HPO_4-water. This effect is visible in the presence and absence of protein. It is not influenced by application of DIDS and PPAPS (see Fig. 2). Therefore, the relaxation enhancement may be eliminated by subtracting the experimental values obtained in the presence from those in the absence of inhibitors.

The presence of 200 μM PPAPS causes a drastic decrease of the chloride line-width (Fig. 2) whereas the addition of phosphate causes no further decrease. The resulting titration curve is dominated by the chloride-phosphate relaxation enhancement described above. We conclude that small concentrations of PPAPS are able to completely inhibit the access of chloride to all binding sites which can also be occupied by phosphate.

The situation is different if certain binding sites are inhibited for chloride binding using DIDS. Addition of DIDS reduces the line-width of the chloride resonance. However, the reduction of the line-width is approximately two-thirds only compared to that using PPAPS. Consequently, 100 μM DIDS would leave at least one type of binding site unoccupied. This binding site shows inhibition of chloride binding by phosphate which is derived from the data in Fig. 2.

In order to evaluate the data in Fig. 2 in a quantitative way the differences of the line-widths measured with and without PPAPS and DIDS were calculated and depicted in Fig. 3. This results in titration curves in which the chloride-phosphate relaxation enhancement is eliminated. The data

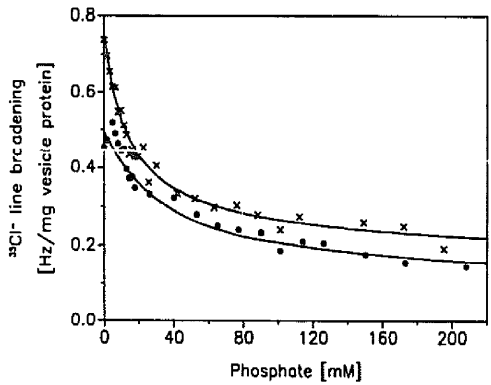

Fig. 3. Phosphate titration of DIDS- and PPAPS-sensitive binding sites. The data points were evaluated from the data in Fig. 2 by subtracting the values obtained in the presence from those obtained in the absence of inhibitor; DIDS (●), PPAPS (×). The data were fitted according to Eqn. 2 assuming one and two types of binding sites, respectively.

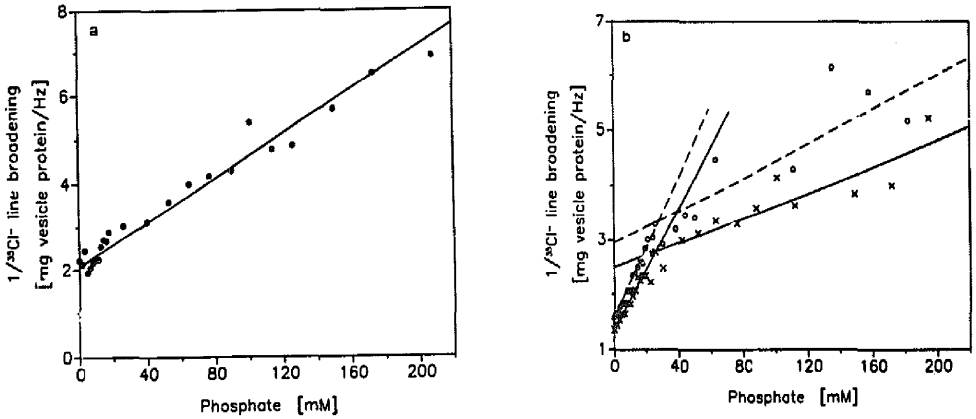

Fig. 4. Phosphate titration in reciprocal presentation. (a) DIDS-sensitive binding sites. (b) PPAPS-sensitive binding sites. For details see Fig. 3.

were fitted to mathematical expressions described above (Eqn. 2). The experimental data for DIDS yielded a good fit when only one phosphate-binding constant was assumed. The experimental data of PPAPS gave a poor fit with systematic deviations; the quality of the fit was greatly enhanced when a mathematical expression for two binding constants was used.

In Figs. 4a and 4b the reciprocal values of the ^{35}Cl line broadening were plotted as a function of the phosphate concentration. The linear dependency of the data derived from the experiments using DIDS is clearly demonstrated in Fig. 4a. This is in contrast to the behaviour of the chloride line broadening as a function of phosphate concentration in the presence of PPAPS (Fig. 4b). We can distinguish two linear regions with differing slopes. Two sets of data points, taken from individual sarcoplasmic reticulum preparations, are shown to demonstrate the biological variation and to verify the biphasic behaviour. They may also represent varying amounts of 'light' and 'heavy' sarcoplasmic reticulum vesicles being present.

The plots suggest that within the limits of error the titration curve with phosphate in the presence of DIDS may well be described by one and that in the presence of PPAPS by two binding constants.

Conclusions

The results described in Figs. 2–4 of this paper are in accordance with the assumption that there are at least two different types of anion binding sites on the sarcoplasmic reticulum membrane. Both types are accessible to chloride and phosphate as well as to the sulfate exchange inhibitors PPAPS and DIDS, their affinities being expressed by different dissociation constants. At 100 μM DIDS only one type of sites becomes occupied whereas application of 200 μM PPAPS makes both inaccessible to phosphate.

As mentioned above PPAPS is a derivatized pyridoxalphosphate. The parent compound itself has been shown to inhibit calcium-dependent ATPase activity [22] which can be attributed to the labelling of a single lysine residue on the

TABLE I

INHIBITION OF ION TRANSPORT AND BINDING IN SARCOPLASMIC RETICULUM MEMBRANES BY DIDS

	Half-maximal inhibition [μM]		Method	Ref.
	high affinity	low affinity		
Chloride binding	14	132	^{35}Cl-NMR	[a]
Sulfate efflux	4		tracer	9
Sulfate efflux	5		tracer	22
Phosphate efflux	3		tracer	23
Ca^{2+} uptake [b]	4		tracer	23
ATP hydrolysis		135	P_i determination	9

[a] This work.

[b] Presence of oxalate or phosphate.

ATPase molecule. Loss of ATPase activity can be prevented by protection with Ca-ATP [22]. Therefore PPAPS might also bind to nucleotide binding sites on the Ca^{2+}-ATPase. This is an interesting assumption which we are investigating presently.

The available data concerning the effect of DIDS are summarized in Table I. It contains data from the literature as well as results from our own work and compares the effects of DIDS on sulfate and phosphate flux measurements, active Ca^{2+} uptake, Ca^{2+}-dependent ATP hydrolysis and chloride binding.

Inspection of the data suggests that there are two sets of constants differing by at least one order of magnitude. The K_i of the high-affinity DIDS-binding site corresponds to those inhibition constants which have been found for sulfate and phosphate efflux [9,22,23]. Therefore this binding site could cautiously be interpreted as being connected with anion transport. We do not know if the coincidence of the half-maximal inhibition constant for oxalate and phosphate supported active Ca^{2+} transport with the K_i of high-affinity DIDS binding as measured by ^{35}Cl-NMR is accidental.

Interestingly, the K_i of the low-affinity DIDS-binding site calculated from our ^{35}Cl-NMR data (132 μM) is very close to the value found for half-maximal inhibition of Ca^{2+}-dependent ATP hydrolysis (135 μM).

These results will have to be supported by further biochemical and NMR measurements. We have shown that ^{35}Cl-NMR is a useful method to study chloride binding which is also suitable to analyze phosphate binding. The NMR results combined with additional information through functional studies of kinetics, e.g. transport measurements, determination of phosphorylation rates and phosphate liberation will increase our understanding of ion transport mechanisms across the sarcoplasmic reticulum membrane. It might then be feasable to reveal possible mutual interactions between ion transport sites and phosphorylation sites mediated by conformational changes.

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