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Studies on the anion binding selectivity of sarcoplasmic reticulum membranes by ^{35}Cl-NMR

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Anion binding sites on the membranes of sarcoplasmic reticulum vesicles can be characterized with the aid of ^{35}Cl-NMR. Titration experiments with a series of different anions reveal that multivalent, phosphate-like anions bind much stronger to SR vesicles than monovalent anions like halides whereas oxalate seems to have an intermediate position. The binding strength decreases with decreasing ionic radius according to the following sequence: vanadate > phosphate > sulfate ≫ iodide > oxalate > bromide > chloride ≫ fluoride. This is also reflected by increasing dissociation constants. Although vanadate in absolute terms replaces much more chloride than either, phosphate or sulfate, their dissociation constants are very similar. This implicates a special binding mechanism for vanadate. Phosphate analoguous compounds like pyridoxalphosphate-6-azophenyl-2′-sulfonic acid and its 4′-nitroderivative show the strongest binding.

Introduction

The role of cations and anions and their participation in different processes concerning the functions of the sarcoplasmic reticulum membrane has up to now mainly been elucidated on the level of ion transport. Thus, cation selectivity has been explored in a contrasting study of the lanthanide ion sensitivity of skeletal muscle sarcolemma and sarcoplasmic reticulum [1], and in studies of the alkali metal ion selectivity of the K^+-channel of skeletal [2,3] and cardiac SR [4]. Anion and cation permeabilities have been determined by Haynes [5] and by Kometani and Kasai [6]. They found that the half-permeation times for different anions can vary by a factor of 100. Kasai and Kometani [7] and Yamamoto and Kasai [8] could also show that stilbene derivatives like SITS and DIDS inhibit the anion permeability of sarcoplasmic reticulum. Whereas the inhibition was reported to be competitive with chloride it proved to be non-competitive with gluconate [8]. This indicates the existence of different sets of anion binding/transport sites. Campbell and McLennan used DIDS to inhibit phosphate and oxalate efflux with no effect on Ca^{2+}-efflux [9] but showed that phosphate competes with DIDS when it is used to inhibit active Ca^{2+}-transport [9]. Phosphate has also been shown to compete with oxalate as activator for ATP-supported Ca^{2+}-transport [10] whereas Sr^{2+}-uptake (when Sr^{2+} is substituted for Ca^{2+}) is activated only by oxalate but not by inorganic phosphate [11]. The positive effect of different mono- and dicarboxylates on Ca^{2+} transport has been investigated by several authors [12–14], and The and Hasselbach studied the influence of chaotropic anion binding on SR functions [15]. Jilka et al. [16] indicated that the Ca^{2+}-ATPase may be participating in anion transport by showing increased sulfate permeability of vesicles reconstituted with Ca^{2+}-pump protein which has been estimated to be present at an average density of 400 molecules per vesicle [17].

Tanifuji et al. [18], Rousseau et al. [19], and Rousseau [20] using the vesicle-lipid bilayer fusion

Abbreviations: PPAPS, pyridoxalphosphate-6-azophenyl-2′-sulfonic acid; PPANS, pyridoxalphosphate-6-azophenyl-4′-nitro-2′-sulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; SITS, 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; TEA, triethanolamine; SR, sarcoplasmic reticulum; SDS, sodium dodecylsulfate; m, magnetic quantum number; τ_c, correlation time; χ, quadrupolar coupling constant; n.d., not determined.

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technique and Hals et al. [21] with the 'sarcoball' technique succeeded in incorporating single chloride channels from SR into lipid bilayers. They revealed that the channels do not select among monovalent anions [18], that sulfate [18,20,21], phosphate [19], gluconate and some other larger organic anions (21) hardly or do not pass at all through these anion channels. Moreover they found that DIDS completely inhibits the chloride channel [9,20]. The number of channels in native SR has been calculated [18] and estimated [22] as 0.7 and 1.4 per vesicle, respectively.

The importance of anion transport across the SR membrane as a possible regulator or compensator of active Ca^{2+} transport by dissipation of membrane potentials [23] is very obvious and led us to study anion binding selectivity and anionic inhibitor binding to the SR membrane. In a previous publication [24] we could show that anion binding sites on the SR membrane can be investigated with ^{35}Cl-NMR as a monitor for anion binding.

The aim of the present work was to characterize the anion binding selectivity of this membrane by the same method. This could lead to further understanding of anion transport.

Experimental procedures

Materials

Sodium orthovanadate was obtained from Sigma Chemical Company (München), SDS and pyridoxal 5-phosphate were from Serva (Heidelberg). Triethanolamine, inorganic salts, and ^2H_2O were purchased from Merck (Darmstadt). ATP (sodium salt) was from Boehringer (Mannheim), $^{45}CaCl_2$ and [^{14}C]oxalate were obtained from NEN Research Products (Dreieich). 2-Aminobenzenesulfonic acid from Janssen Chimica (Brüggen) and 2-amino-4-nitrobenzenesulfonic acid, which was a kind gift from Bayer (Leverkusen), were starting materials for the syntheses of PPAPS and PPANS which were performed according to Ref. 24. Asolectin from soybean was purchased from Fluka BioChemika (Neu-Ulm). All chemicals were of purest grade available from the respective companies.

Preparation of sarcoplasmic reticulum vesicles

The preparation of unfractionated SR vesicles was performed as described in a preceding paper [24] following a modified procedure of Hasselbach et al. [25,26]. They were examined by SDS-PAGE [27] and regularly checked for Ca^{2+}- and Mg^{2+}-dependent ATPase activity [28] as well as their ability of ATP-supported $^{45}Ca^{2+}$ and concomitant [^{14}C]oxalate accumulation [29].

NMR measurements

The frequency of the ^{35}Cl resonance was 26.467 MHz using a Bruker AM 270 NMR-spectrometer. The experimental details of the NMR measurements were as described in a previous paper [24]. The line widths were evaluated from the spectra using two different soft-ware routines supplied by the Bruker soft-ware package. The older version is a non-linear least-squares iteration procedure evaluating frequency and height of the signal as well as the line width. The new version is based on closed mathematical expressions which are known to be much faster than iteration procedures. However, this advantage is partially compensated by the fact that the new version evaluates five parameters of the experimental spectra, i.e. frequency and height of the resonance, line width, and phase and zero order base-line correction [30]. Generally, a greater number of fit parameters increases the instability of the fitting routine against deviations of the experimental data points from the ideal Lorentzian. In order to test the stability of the new version by using experimental ^{35}Cl spectra we measured a series of NMR spectra under identical conditions and evaluated the line width using both, the new and the older version. The statistical analysis of the results showed that the new version is faster and and that the S.D. of the line width was not increased.

Effects of chemical exchange on the ^{35}Cl resonance

The spectrum of chloride bound to macromolecules can be quite complex whereas the spectrum of an aqueous free chloride solution only consists of a single resonance [31–33]. The interaction of the quadrupole moment with an electrical field gradient and the Zeeman splitting results in three NMR transitions between the energy levels $m = 3/2,\ 1/2,\ -1/2$, and $-3/2$ with different frequencies. These transitions can be observed in solid state where the electric field gradient is large and the rate of fluctuations is slow. In free chloride solutions the electric field gradient is relatively small and rapidly changing (small correlation time τ_c) so that the differences in the transition frequency are effectively averaged out (extreme narrowing limit) [31]. The result is an exponential decay of transverse magnetization giving a Lorentzian line shape of the single resonance. The binding of a chloride anion to a macromolecule in solution may cause non-exponential decay of the transverse magnetization. The extent of averaging of the quadrupolar interactions depends on the strength of binding at the macromolecular binding sites and the fluctuation rate of the intramolecular motions.

In the case of chemical exchange between free and bound chloride we have to consider the exchange rate and the relaxation rates in the free and bound state.

Forsén and Lindman [31] have reviewed the influence of chemical exchange on the relaxation of ^{35}Cl. They showed that the theory can also be applied to systems comprising more than one binding site.

On sarcoplasmic reticulum membranes chloride binding sites are located both, inside and outside, of the vesicles. The question was if the exchange rates between chloride bound to these sites and free in solution would be within the fast exchange limit. This should also include the trans-membrane exchange rate through native anion channels which are known from the literature [34]. The calculated half-permeation time is in the range of 1 s [6]. This is slow compared to the requirements necessary for the fast exchange model. Considering the trans-membrane exchange to be slow we expected a superposition of two Lorentzian resonances originating in signals from chloride inside and outside the vesicles, respectively. Therefore we analyzed the line shape of the chloride resonance in dependence on the chloride concentration.

A second way to test the validity of the fast exchange model is to analyze the temperature dependence of the chloride line width. In the limit of extreme narrowing the line width is proportional to the correlation time:

$$1/(\pi T_2) = 2\pi \cdot \chi^2 \cdot \tau_c/5$$

where χ is the quadrupolar coupling constant. The correlation time τ_c obeys an exponential temperature dependence, i.e., increasing the temperature results in a decrease of the correlation time τ_c.

In the limit of slow exchange the line width is proportional to the exchange rate. Increasing the temperature will increase the exchange rate and thereby increase the line width.

We carried out both, line shape analysis (which we perform for every experiment) and measurement of the temperature dependence. The temperature dependence of the line broadening of the observable ^{35}Cl signal (Fig. 1) shows that the fast exchange limit is valid.

The question how chloride anions inside and outside of SR vesicles contribute to the ^{35}Cl signals can be answered considering the following arguments. The intensity of the chloride signals is proportional to the amount of nuclei in the respective compartments. Since the chloride concentrations inside and outside of the vesicles are the same because of the presence of chloride channels in the SR membrane [6] the number of nuclei is proportional to the respective volumes. As described by Kasai [35] the average volume of SR vesicles is between 3 and 5 μl per mg protein. The NMR samples contained 12.5 mg protein in 2.5 ml solution. Therefore the percentage of free chloride anions inside the vesicles is between 1.5 and 2.5, respectively. From these data we can conclude that the contribution to the NMR signal intensity of chloride inside the vesicles is negligible. It follows that the results of all experiments carried out under our conditions refer to chloride binding outside the SR vesicles. It also follows from the line shape analysis and temperature dependence studies that the chemical exchange between chloride free and chloride bound at binding sites outside the vesicle membrane is fast on the NMR time scale. The trans-membrane chloride exchange does not interfere with our results.

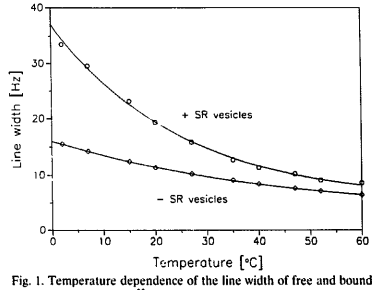

Fig. 1. Temperature dependence of the line width of free and bound chloride. (○) measured ^{35}Cl line width of bound chloride at 4 mg/ml vesicle protein and 40 mM KCl. (◇) line width of free chloride calculated using the Arrhenius equation with $\Delta E = -11.4$ kJ (taken from Ref. 32).

Results and Discussion

Titration of SR vesicles with chloride

In order to be able to evaluate binding data of anions other than chloride properly we first investigated the influence of the chloride concentration on chloride binding. Studying it over a large concentration range, i.e. 5 to 550 mM, we observed a phenomenon which is atypical for chloride titrations. At low concentrations addition of more chloride caused an unexpected increase in line width reaching a maximum at 17 mM. Thereafter, further increase of the chloride concentration brought about the expected behaviour of decreasing line width (Fig. 2) [31].

In a control experiment we titrated chloride into solutions of protein-free vesicles under comparable conditions. The vesicles were reconstituted from SR lipids [36] and used for NMR-measurements at lipid concentrations comparable to those of the SR vesicles under investigation. In the concentration range of 5 to 400 mM KCl the ^{35}Cl line width was constant, i.e., the increase of line width at low concentrations as well as the decrease with increasing KCl concentrations as

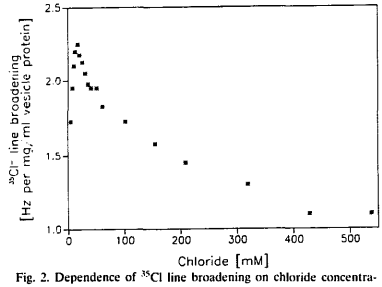

Fig. 2. Dependence of ^{35}Cl line broadening on chloride concentration. 4.3 mg/ml SR vesicle protein were dissolved in 3 mM KCl, buffered with 1 mM TEA-HCl (pH 7.4) and titrated with 2 M KCl.

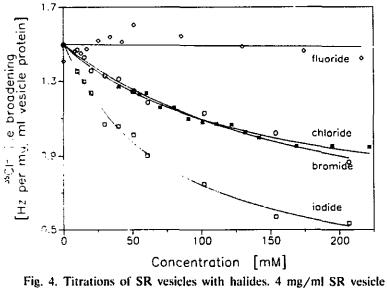

Fig. 4. Titrations of SR vesicles with halides. 4 mg/ml SR vesicle protein were dissolved in 40 mM KCl and titrated with 2 M solutions of fluoride (◇), chloride (•), bromide (○) and iodide (□), respectively. Buffer conditions were as in Fig. 2.

measured with SR vesicles were not detectable (not shown). Therefore we assign the effects seen in chloride titrations of SR vesicles to interactions of chloride with binding sites on SR proteins.

In order to establish whether the increase in chloride binding at low concentrations was due to a chloride specific effect or a totally unspecific influence we repeated the titration with increasing concentrations of potassium fluoride at a constant low chloride concentration of 5 mM (Fig. 3). A distinct increase of the line width is observable up to 30 mM KF/KCl (total) whereas it remains practically constant after further addition of KF.

Contrary to all other anions used in titration experiments (see below) potassium fluoride even at ionic strengths corresponding to concentrations of 25 mM or more does not show any significant positive or negative influence on chloride binding, i.e., there seems to be no competition of binding between chloride and fluoride ions detectable by ^{35}Cl-NMR within the limits of experimental error.

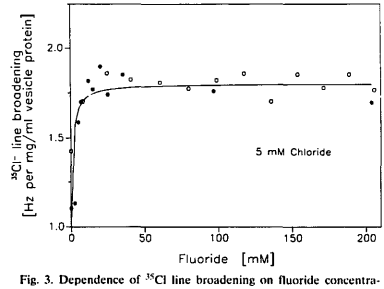

Fig. 3. Dependence of ^{35}Cl line broadening on fluoride concentration in the presence of 5 mM chloride. 4 mg/ml SR vesicle protein were dissolved in 5 mM KCl. Buffer conditions were as in Fig. 2. Two different samples from two SR preparations were titrated with a 2 M KF solution.

We conclude that the increase in chloride binding at low concentrations (Figs. 2 and 3) is due either to unspecific effects which are caused by the change of ionic strength or to an influence of K^+ on chloride binding which cannot be excluded. Further experiments including the analysis of this effect will be published in a seperate communication. Because of the absence of any change of chloride binding above 25 mM KF all subsequent titrations were performed at a constant KCl concentration of 40 mM.

Titration of anion binding sites with halides

The results above suggest that the binding sites investigated might be selective for chloride ions. In order to examine the selectivity of the binding sites we measured chloride binding in the presence of varying concentrations of halides. The results shown in Fig. 4 reveal that the binding of iodide is stronger than that of bromide, chloride, and fluoride. The dissociation constants for iodide, bromide and chloride were calculated according to Ref. 24 as 31 ± 7 mM, 56 ± 13 mM and 132 ± 26 mM (S.E.), respectively. Hence, there is no exclusive specificity for the binding of chloride. On the contrary, the dissociation constants decrease with increasing ionic radii. We conclude that the charge density is not the dominating factor for the binding behaviour of the spherically symmetrical halide ions. We are led to the assumption that the geometry of the binding site, i.e., the three-dimensional distribution of positive charges and the influence of van-der-Waals repulsive forces might dominate the binding of anions.

Titration with multivalent anions

The described titrations with halides show that iodide is the best inhibitor of chloride binding. Because of the very low concentration of iodide in muscle cells this is not likely to be physiologically meaningful. Nevertheless should the physiological anion(s) resemble iodide in ionic diameter and distribution of electron density more than any other anion used.

Since bromide and iodide bound to the SR membrane with an affinity high enough to be detected by ^{35}Cl-NMR measurement the existence of an anion binding/transporting protein seems to be likely. The functional relevance of such a protein could be speculated to be a compensator for large amounts of positive charge being transferred across the membrane during active Ca^{2+}-transport.

Since there was a preference of anion binding for anions with larger diameters we describe in the following titrations with anions still larger than iodide but carrying different charge. The influence of a number of chaotropic anions on the functions of the SR membrane has been shown by The and Hasselbach [15]. A good physiological candidate is phosphate which has an ionic diameter of 4.8 Å compared to that of iodide of approx. 4.2 Å (see Table I).

To test the influence of the ionic diameter, we investigated phosphate and similar ions, which are known to permeate the SR membrane in vitro or affect other functions of sarcoplasmic reticulum. These ions are sulfate, oxalate and vanadate.

To quantify the results of the different titrations shown in Fig. 5 we fitted hyperbolae to the different sets of data points. The calculated constants indicate a similar binding behaviour of phosphate and sulfate whereas vanadate binds stronger and oxalate is the ion with the weakest binding. The titration behaviour of the anions in Figs. 4 and 5 is characterized by the K_i value and the asymptotic behaviour at high anion concentrations. Comparing the latter we can classify the ions into two groups: The first group, comprising the halides, achieve a steady decrease in line width reaching a relatively low level at high anion concentrations. The titration curves of the second group, i.e., phosphate and sulfate, show a rapidly decreasing line width at small concentrations and remain constant at higher concentrations.

TABLE I

Diameters and K_i values of investigated anions

Ion	Ionic diameter (Å)	Ref.	K_i (mM) [a]
Fluoride	2.32–2.72	37–39	> 500
Chloride	3.28–3.62	37–39	132 ±26
Bromide	3.6 –3.92	37–39	56 ±13
Iodide	4.08–4.38	37–39	31 ± 7
Oxalate	≈ 6.4 [b]	39,40	38 ±35
	≈ 5.3 [c]	39,40	
Sulfate	≈ 4.7	39,40	2.1± 0.9
Phosphate	≈ 4.8	39,40	1.6± 0.2
Vanadate	≈ 5.5	39,40	0.5± 0.1
Arsenate	≈ 5.2	39,40	n.d.

[a] Mean ± S.E.
[b] Diagonal dimension.
[c] Lateral dimension.

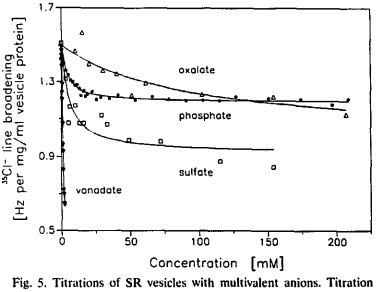

Fig. 5. Titrations of SR vesicles with multivalent anions. Titration conditions were as in Fig. 4 except for vanadate which was used in 10 and 100 mM solutions, respectively. Oxalate (△), phosphate (●), sulfate (□), vanadate (▼).

This indicates that only a limited amount of chloride is displaced by phosphate and sulfate whereas the remaining binding sites are not accessible to these ions. This suggests the binding sites to be specific for phosphate (sulfate).

The impressive absolute amount of chloride replaced by vanadate (indicated by the large reduction of line width) suggests a special binding mechanism for vanadate. To exclude a direct vanadate–phospholipid interaction we also titrated vanadate to vesicles prepared from soybean asolectin. This experiment did not produce any change of the chloride line width up to 10 mM vanadate (not shown).

The binding mechanism of vanadate might involve a conformational change [41] of the protein causing a reduction of the number of chloride binding sites per protein molecule. Therefore the calculated inhibitor constant of vanadate and the K_i values of the other ions have to be compared cautiously.

Vanadate is a very particular example of an anion because it has been found to inhibit the functions of the Ca^{2+}-ATPase of SR at micromolar concentrations [42–44]. It is a stable transition state analogue of phosphate [45–47] which emphasizes the fact that we are investigating phosphate binding sites. Therefore we investigated inhibitors which are known to occupy phosphate binding sites [24].

Titration of anion binding sites with PPAPS and PPANS

So far we have seen that monovalent anions like bromide and iodide as well as multivalent anions like oxalate, sulfate, phosphate, and vanadate can displace chloride from anion binding sites of the SR membrane with varying efficiency. Fluoride with a charge density much higher than that of chloride fails to displace chloride whereas other non-physiological ions like bromide and oxalate do. This leaves two important questions open: (1) Is there a totally unspecific anion binding/transport protein or is it specific for one of the more strongly binding anions? (2) What is the physiological relevance of anion binding and its correlation to anion transport?

From the literature we know that there are chloride channels in the SR membrane with very high efficiency [6] but very low density. The concentration of these channels is such that their exploration with a technique of little sensitivity like ^{35}Cl-NMR would not be possible. Thus, the anion binding sites we are characterizing must be located on anion binding or carrier proteins which are present in considerable concentration in the SR membrane. They must be able to bind chloride (see Fig. 2) but from titration experiments with phosphate we know that the affinity of this anion to a part of the binding sites is much higher than that of chloride itself (see Fig. 5).

The permeability of the SR membrane to phosphate and oxalate has been reported by Beil et al. [10]. During in vitro Ca^{2+}-accumulation in the presence of ATP, oxalate or phosphate can be taken up concomitantly in a 1:1 stoichiometry [29,48,49] with Ca^{2+}. Thus, there have to be binding/transport proteins with binding sites for phosphate, oxalate and similar ions like sulfate and vanadate which can also bind chloride.

In previous experiments we have shown that the binding of chloride to the SR membrane can be partially inhibited by the sulfate transport inhibitor PPAPS [24]. Therefore we decided to titrate the chloride/phosphate binding sites with PPAPS and PPANS to further characterize this connecting link between anion binding and anion transport. PPANS in part of its structural features follows the example of DNDS, a reversible inhibitor of anion exchange transport through the erythrocyte membrane [50], while PPAPS follows that of DIDS. The result of these experiments in comparison to a titration with vanadate is shown in Fig. 6. The titration curves of the inhibitors were fitted by hyperbolae. Even if this function may be only an approximation of the actual function describing the experimental data it is satisfactory to characterize the relative abilities of the anions to replace chloride. The inhibition constants calculated from the mathematical fits are listed in Table II. From the literature we know that vanadate inhibits the Ca^{2+}-ATPase half-maximally between 10 and 50 μM [42,43]. However, we could not show the existence of a high affinity vanadate binding site. This discrepancy could be caused by different conformational states of the enzyme or by the existence of a vanadate binding site which is not accessible to chloride.

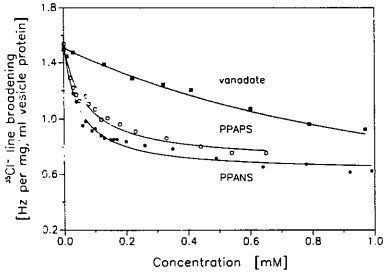

Fig. 6. Titrations of SR vesicles with vanadate, PPAPS and PPANS. 4 mg/ml SR vesicle suspensions (40 mM KCl, pH 7.4) were titrated with solutions of 10 or 100 mM vanadate (•), 11 mM PPAPS (○) and 36.5 mM PPANS (●).

TABLE II

Comparison of inhibitor constants of vanadate and phosphate-analogous compounds

The values have been estimated by non-linear least-squares fits assuming one binding site only.

Inhibitor	K_i (μM) [a]
PPAPS	46 ± 2.0
PPANS	20 ± 2.0
DIDS [b]	14 ± 3
	132 ± 11
Vanadate	500 ± 100

[a] Mean ± S.E.
[b] Taken from Ref. 24.

Conclusions

Sarcoplasmic reticulum membranes do not show a very distinct anion binding selectivity. This suggests that we are investigating an inhomogeneous set of binding sites. We want to stress the fact that the SR anion binding sites so far evaluated by ^{35}Cl-NMR are exclusively located on the outside of the vesicles. The reason is that the contribution of chloride inside the vesicles amounts to maximally 2.5% of the experimental signal intensity. Therefore its influence on the line width is negligible. Moreover, line shape analysis and the dependence of the line width on temperature revealed that the exchange of chloride between various

TABLE III

Proposed binding specificity of anion binding sites on the SR membrane as determined by ^{35}Cl-NMR

	Binding site	
	I	II
Chloride	+	+
PPAPS	+	+
Phosphate	+	–
Fluoride	–	–

binding sites outside of the vesicles and free chloride is within the fast exchange limit.

Our titration experiments with a number of different anions show that there is a tendency of stronger binding affinity for larger anions with a charge greater than one. This is shown by the inhibition constants which decrease with increasing ionic diameter of the halides. This series continues with phosphate and analoguous anions. Oxalate takes an exceptional position concerning both, ionic dimensions and inhibition constant. The binding of PPAPS and PPANS is nearly three orders of magnitude stronger than that of the above anions. Because of the unphysiological character of these derivatized anionic inhibitors the hydrophobic part of the molecules certainly do not contribute to anion binding selectivity but may well increase the binding strength.

The absolute amount of chloride replaced by the anions has shown that there are two types of binding behaviour. Although the halides show relatively weak binding they replace larger amounts of chloride than phosphate and sulfate. This indicates that there is at least one set of chloride binding sites not accessible to phosphate and sulfate. Additionally, PPAPS is still able to displace chloride even when phosphate binding sites are occupied by phosphate (see Figs. 2 and 3 in Ref. 24). The experiments described in our previous paper [24] and the present results revealed the existence of at least two types of binding sites as shown in Table III. The two sets of binding sites named I and II are accessible to chloride, bromide and iodide and can be affected by PPAPS and PPANS. The second set of binding sites (II) is not accessible to phosphate. Although these results do not give final information concerning the physiological role of the anions investigated, they may help to classify the binding sites according to their binding affinity with regard to their biological importance.

Acknowledgements

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