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Equilibrium Binding of Calcium to Fragmented Human Red Cell Membranes and its Relation to Calcium-Mediated Effects on Cation Permeability

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Summary. We have measured Ca binding to fragmented human red cell membranes under equilibrium conditions in the presence of low concentrations of EGTA-buffered, ionized Ca. The ionic strength of the assay medium was maintained at 0.16. Two high affinity Ca binding sites were identified: Site I was pH-sensitive. Its apparent dissociation constant (K') increased from 2×10^{-7} to 6×10^{-7} M as the pH was shifted from 6.8 to 6.0. Between pH 8.5 and 6.8 K' remained constant. The capacity of the same site decreased between pH 8.5 and 6.8 from 0.16 to 0.04 nmoles/mg protein. Site II was insensitive to pH changes between 8.5 and 6.0. It had a K' value of $\sim 3 \times 10^{-6}$ M and a capacity of ~ 0.2 nmoles/mg protein. Mg and the local anesthetic propranolol (but not tetracaine) inhibited Ca binding to site I competitively and to site II noncompetitively. The properties of the high affinity Ca membrane binding sites are consistent with the assumption that site I corresponds to the site at which Ca initiates an increase in K permeability in resealed red cell ghosts. Site II is possibly involved in the Ca-mediated resealing of red cell ghosts after osmotic hemolysis. In the presence of MgATP, only a single saturable high affinity Ca binding site was observed ($K' \sim 6 \times 10^{-7}$ at pH 6.8). The capacity of this site (~ 1.8 nmoles/mg protein) was almost 10 times higher than the combined capacities of sites I and II under control conditions. The results are discussed in the light of inevitable but severe shortcomings due to the evaluation of binding constants from nonlinear Scatchard plots by a curve-fitting procedure.

Good evidence supports the view that membrane-bound Ca – at least in red cells – plays an important role in controlling membrane permeability: (i) Ca can reverse the cation leak developing in red cells which are exposed to media of low ionic strength [3]; (ii) Ca (or Mg) is required for the restitution of a low cation permeability in erythrocyte ghosts ("resealing") after hypotonic hemolysis of red cells [2, 12, 25]; (iii) Ca can induce a large selective increase in passive K permeability if it has access to the inside of the cell membrane [1, 18, 19, 24, 25, 27, 37]. A better characterization of the membrane sites which are involved in these Ca-mediated changes of membrane function requires the direct de-

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termination of their binding affinities and capacities. The binding of Ca to human erythrocyte membranes has been measured repeatedly [5, 9, 10, 20, 26, 28, 40]. However, the binding parameters reported in these studies were determined either under nonequilibrium conditions and/or in media of low ionic strength. Hence, the Ca binding sites which were described cannot be related easily to specific effects of Ca on the cation permeability of intact cells. Therefore, in the present investigation we used an equilibrium binding assay and an ionic strength close to 0.16. The method was sensitive enough to detect high affinity Ca binding sites with capacities below 0.1 nmole/mg membrane protein. Our results suggest that, in fact, two high affinity Ca binding sites with low capacities exist in the red cell membrane. Moreover, evidence is presented that those sites may be related to the receptors which mediate the biological effects of Ca on membrane permeability.

Materials and Methods

Preparation of Fragmented Red Cell Membranes

Fresh erythrocytes were supplied by the Swiss Red Cross Blood Transfusion Service in Bern and were used within 5 days after withdrawel of the blood. The cells were washed 3–4 times in isotonic NaCl solution. The white buffy layer was carefully removed by suction. One volume of cells was then hemolyzed at 22 °C in 10 vol of a hypotonic solution which contained 10 mM HEPES[1] and 1 mM EGTA and was buffered to pH 7.6. After 2–4 min the hemolysate was transferred to a refrigerated centrifuge (Sorvall RC2B) and spun down at 19,000 × g. For all subsequent steps of the preparation the temperature of the membrane suspension was kept at 0–2 °C. The membrane sediment from the first centrifugation was subject to two hypotonic washes (*soln. I* in Table 1) followed by two isotonic washes (*soln. II*). Subsequently, the membranes were frozen in liquid nitrogen, thawed in a water bath at 25–30 °C, and again washed in isotonic saline (*soln. II*). The freezing-thawing cycle was repeated at least 5 times. We found that the capacity of the membrane preparation to form sealed vesicles capable of pumping Ca out of the intravesicular space decreased with increasing numbers of freeze-thawing cycles. No such pumping was observed in membrane preparations subject to 5 consecutive freeze-thawing cycles. Thereafter, the membranes were freed of EGTA by 3 more washes in an isotonic KCl solution (*soln. III* or *IV*). The pH of the final wash solution was adjusted to the pH of the binding assay medium. A stock suspension of the membrane sediment in the same medium was prepared to contain 6–10 mg protein/ml. The method yielded about 150 mg membrane protein per 100 ml of sedimented

1 *Abbreviations used:* Me^{++}-buffers: EGTA = ethyleneglycol-bis(-aminoethylether)-N, N′-tetraacetic acid; HEDTA = N′-(2-hydroxyethyl)-ethylenediamine-N, N′-triacetic acid. – H^{+}-buffers: HEPES = N′-(2-hydroxyethyl) piperazinyl-(1)-ethane-sulphonic acid; PIPES = Piperazine-N, N′-bis (2-ethanesulfonic acid) Tris = Tris (hydroxymethyl)-aminomethane. – $[Ca^{++}]$ denotes the free ionized concentration of that ion. The symbols Na and K are used for sodium and potassium without specification of the respective free ion concentrations. – The symbol K' is used to denote the apparent Ca dissociation constant of a binding site.

red cells. Two major modifications of this general procedure were tried: (i) Membranes were prepared in the absence of the Ca-complexing agent EGTA. Such preparations did not show significantly different Ca binding characteristics, but the experimental scatter was increased considerably. (ii) Membranes were prepared exclusively by multiple freezing-thawing cycles, thus maintaining isotonic conditions throughout the whole procedure. This preparation retained a faintly pink color due to some residual hemoglobin which could not be removed. The Ca binding characteristics differed somewhat from those of the standard preparation and will be discussed separately in *Results*.

Ca Binding Assay

Equilibrium binding of Ca was estimated according to a method of Madeira and Carvalho [21] adapted to our special requirements. 5 ml of an isotonic KCl solution buffered to the desired pH value (*soln. IV* or *V*) were pipetted into preweighed conical pyrex tubes. A range of free Ca^{++} concentrations was established in this medium by means of an EGTA-CaEGTA or HEDTA-CaHEDTA buffer system as described by Porzig [25]. For each particular pH we calculated the concentrations of ionized Ca ($[Ca^{++}]$) from the pK′ values (negative ^{10}log of the apparent dissociation constants) and the given Ca/EGTA or Ca/HEGTA ratios according to equations given by Portzehl, Caldwell and Rüegg [23]. The pH values 8.5, 8.0, 6.8 and 6.0 which were used in the binding assay corresponded to the pK′ values 9.49, 8.60, 6.26, and 4.66 for Ca EGTA and 6.89, 6.41, 5.21, and 4.35 for Ca HEDTA. The calculation was based on the "true" pK values of 8.14 for Ca HEDTA [35] and 10.97 for Ca EGTA [36]. However, the choice which had to be made for the "true" pK values from the range of values offered in the literature introduces some uncertainty into the calculated values of $[Ca^{++}]$ and, consequently, into the experimentally determined Ca-membrane dissociation constants. Since the present work attempts to compare Ca binding data to functional effects of Ca, we preferred to use the same dissociation constants chosen in the study on $[Ca^{++}]$-dependent permeability changes in red cells [25]. Moreover, in control experiments we found good agreement between the Ca-membrane binding constants determined at a given pH either in Ca HEDTA or in Ca EGTA buffer systems if the above pK values were used. These pK values have been determined at an ionic strength of 0.1 M [35, 36]. The ionic strength in all our experiments was 0.16 M. This difference, which may have caused an increase in K' under our conditions by not more than a factor of 1.5 (*see* [6]), was disregarded in view of more important possible errors involved in the selection of true K values.

The total Ca concentration in the system was usually kept at 0.05 or 0.1 mM. 2 μCi of ^{45}Ca in a volume of 20 μl were added to each tube. Finally, 1 ml of the membrane stock suspension was added to make up a total volume of 6.020 ml. The specific activity of the experimental suspension varied between 6,000 and 12,000 cpm/nmole Ca. In experiments designed to test the effect of ATP on Ca binding, enough ATP and Mg was added to make up final concentrations of 1.7 and 2.5 mM, respectively. After thorough mixing the suspension was kept at 22 °C for 60 min. Subsequently, the samples were centrifuged under refrigeration at 14,000 × *g* for 5 min. A 0.5-ml sample of the supernatant was transferred to counting vials. The rest of the supernatant was removed as completely as possible by careful suction. The tubes containing the pellets were weighed immediately so that the wet weight of the membrane sediment could be calculated. In a first approximation, the wet weight of the sediment was assumed to equal the weight of the supernatant that could not be removed. Therefore, the term "sediment" as it is used here includes all of the incubation medium trapped within the membrane pellet.

A major advantage of this procedure is that it avoids washing the membranes in non-radioactive solution prior to the determination of bound Ca. Hence, the binding equilibrium

established during the incubation period remains undisturbed, and the amount of Ca found to be associated with the membrane fraction corresponds exactly to the value at equilibrium.

The membrane pellet was dissolved in 1 ml of a 1:20 dilution of Triton X-100 and was quantitatively transferred into counting vials. A total of 3 ml bidistilled H_2O was used to rinse the assay tubes and was mixed together with the Triton-dissolved membranes into 10 ml scintillation fluid. This fluid contained a 1:1 mixture (v/v) of Triton X-100 and xylol as well as 4 g/l Omnifluor (NEN premixed scintillator composed of 98% PPO and 2% bis-MSB[2]). The counting efficiencies for membrane sediment and supernatant were made identical by adding 3 ml of bidistilled water and 1 ml of the 1:20 Triton dilution together with 10 ml of the scintillation fluid to each 0.5 ml aliquot of the supernatant.

Calculation of the Results

The amount of Ca bound per ml of membrane sediment was calculated from the specific activity and the difference in counts between 1 ml supernatant and 1 ml sediment as defined above. Under conditions where no Ca was bound ($[Ca^{++}] < 10^{-8}$ M) this difference was a negative number, i.e., the sediment contained less Ca per unit of volume than the supernatant. With a stepwise increase in $[Ca^{++}]$ to concentrations where binding did occur, the negative difference diminished gradually and turned into positive values. This finding suggested that part of the sediment space was not accessible for Ca. The size of this space, as calculated from the difference in ^{45}Ca counts between sediment and supernatant, depended on the protein concentration and varied from 0.04 to 0.1 ml/ml membrane sediment. Its apparent volume was not changed significantly in the presence of the detergent saponine and therefore corresponds most likely to the membrane volume, rather than to a population of sealed vesicles within the membrane sediment.

The sensitivity of the method rests essentially on the combination of high specific activity with high membrane protein concentrations in the assay medium. However, it was a major drawback of this procedure that the amount of Ca bound had to be calculated as the difference of two large numbers. Under conditions where the total amount of Ca bound to high affinity sites was low (pH < 7), the large scatter of the experimental data did not allow us to identify saturable high affinity Ca binding sites in about one third of the experiments.

The protein concentration in the membrane stock suspension was measured with the biuret method [11] after solubilization of the membrane proteins with a small amount of Triton X-100. The same amount of Triton was added to the standards. Labtrol was used as a protein standard.

Determination of ATPase Activities

In some of the membrane preparations the activity of Ca-Mg-ATPase was measured under the same conditions used for Ca binding assays in the presence of ATP. Inorganic phosphate was determined after an incubation period of 30 or 60 min at 22 or 37 °C, either according to Schwartz *et al.* [33] or to the simpler method of Eibl and Lands [7]. Both methods yielded identical results. The Na-K-ATPase activity was almost completely inhibited in the standard K-containing binding medium (*soln. IV* or *V*), and no significant ouabain-inhibitable activity could be detected.

All chemicals used in the experiments were of the highest purity commercially available. EGTA and HEDTA were obtained from Merck, Darmstadt, Germany. Triton X-100 is a trade mark of Röhm AG, Darmstadt, Germany, and was purchased from Siegfried, Zofingen,

2 PPO = 2,5-diphenyloxazole; bis-MSB = *p*-bis-(*o*-methylstyryl)benzene.

Table 1. Composition of solutions (mM)

Solution No.	NaCl	KCl	Tris	HEPES	PIPES	EGTA	pH	Use
I	10	–	10	–	–	1	7.6	Hypotonic washing
II	–	150	10	–	–	1	7.6	Isotonic washing
III	–	150	10	–	–	–	7.6	Isotonic washing
IV	–	150	–	10	–	–	6.8–8.5	Incubation medium
V	–	150	–	–	10	–	6.0	Incubation medium
VI	–	–	–	10	–	1	7.6	Hemolyzing medium

Switzerland. Labtrol is a trade mark of American Hospital Supply Corp., Miami, Fla. Na_2ATP was purchased from Boehringer Mannheim, Mannheim, Germany. ^{45}Ca was obtained from the Eidgenössisches Institut für Reaktorforschung, Würenlingen. Propranolol was a gift of ICI, Macclesfield, UK. Tetracaine was the Pharmacopoea Helvetica preparation. All solutions were prepared with deionized, doubly quartz-distilled water and are generally identified by their respective number in Table 1. The Ca stock solution was prepared from $CaCO_3$. The EGTA and HEDTA stock solutions were prepared from the acids and titrated with Tris to pH 7. The Ca contamination in these stock solutions was about 75 μmoles/mole as determined by atomic absorption spectrophotometry. Shortly before use all glass and plastic labware was thoroughly rinsed with diluted HCl, followed by multiple washings in deionized water.

Results

Previous studies suggested that at least two membrane sites sensitive to low concentrations of Ca are involved in the control of the cation permeability of human red cell ghosts (*see* [25]). The apparent Ca dissociation constants of these functionally defined sites were shown to range between 3×10^{-7} and 2×10^{-6} M at pH 7.2. In the present investigation we have tested whether individual Ca binding sites can be identified in fragmented red cell membranes whose properties (in terms of affinities, pH dependency, and interaction with local anesthetics) are comparable to those of the functional sites.

In the first set of experiments, we studied Ca binding to membrane fragments which were prepared by a freeze-thawing procedure under strictly isotonic conditions (*see* Methods). In the second series of experiments, we prepared white membranes by combining hypotonic hemolysis and repeated freeze-thawing cycles under isotonic conditions. This preparation was used to study effects of pH and local anesthetics on Ca binding and, in the last set of experiments, to test the effects of Ca-ATPase activity on high affinity Ca membrane binding.

Ca Binding to Membranes Prepared at Normal Ionic Strength ($I=0.16$)

In Fig. 1*A* the amount of Ca bound to membranes which were prepared under isotonic conditions is plotted semilogarithmically against the free Ca concentration in the medium. The membranes were suspended in a KCl medium (*soln. IV*) which contained 6.7×10^{-8} to 8.4×10^{-5} M [Ca^{++}]. This range of concentrations covered only the lower end of the complete S-shaped binding isotherm. The low affinity binding sites were still far from being saturated. However, since we were mainly interested in Ca sites with high affinities, we made no attempt to complete this curve. A measurable amount of Ca binding was consistently observed with [Ca^{++}] at or above 6×10^{-7} M. In Fig. 1*B* the data of Fig. 1*A* were plotted according to Scatchard [29]. It is obvious from this graph that saturable high affinity Ca binding sites did, in fact, contribute to the total Ca binding in this preparation.

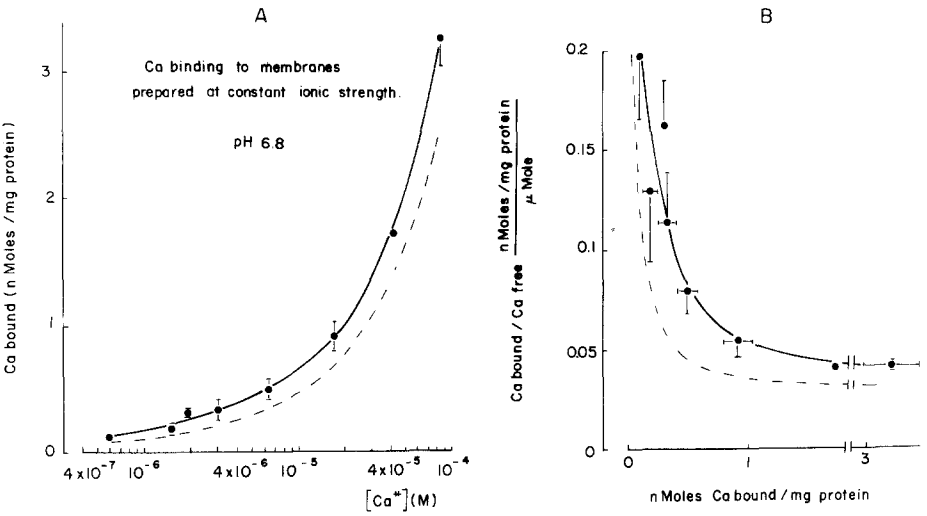


Fig. 1. (*A*): Ca binding to red cell membranes prepared by multiple freeze-thawing cycles at constant ionic strength ($I=0.16$ moles/liter). The binding was measured in soln. IV containing EGTA-buffered Ca ion concentrations ranging from 6×10^{-7} to 6.3×10^{-6} M. No buffering system was used for Ca concentration above 1.5×10^{-5} M. Mean values of 4–5 measurements in 5 experiments (except for a single value at 4.2×10^{-5} M). (*B*): Scatchard plot of the same data. The smooth curves fitting the experimental points in the two graphs were calculated from the following constants: high affinity site: $K'=1.5\times10^{-6}$ M, $n=0.34$ nmoles/mg protein; low affinity site: $K'=10^{-3}$ M, $n=37$ nmoles/mg protein. The broken lines were taken from Fig. 3*D*. They represent binding curves obtained under comparable conditions with another membrane preparation (*see* legend to Fig. 2 and text). The bars give ±SEM

The binding of Ca to membrane components with m classes of independent binding sites can be described by the general equation [16]

$$r=\sum^{m} \frac{n_i[Ca^{++}]}{K_i'+[Ca^{++}]} \tag{1}$$

which is derived from the law of mass action. r represents moles of Ca bound per mg protein, n_i the number of binding sites in an individual class of sites (=capacity). In the form

$$r/[Ca^{++}]=\sum^{m} \frac{n_i}{K_i'+[Ca^{++}]} \tag{2}$$

this equation can be used to fit data which were plotted according to Scatchard as it has been done in Fig. 1*B*. A first estimate of the numerical values of the binding parameters n and K' was obtained from a graphical evaluation of the Scatchard plot. A binding curve was produced with these estimated values and compared with the experimental points. Systematic corrections were then applied such as to yield by trial and error a "best fit" to the experimental points. A more detailed consideration of the principles and the reliability of our fitting procedure follows in the discussion. A critical survey of this and other procedures to determine binding parameters from Scatchard plots has been given by Weder *et al.* [41].

The whole of the low affinity sites was treated as a single site with variable n and a fixed K' value of 10^{-3} M. The smooth curve which fits the experimental points in Fig. 1*B* represents the equation

$$r/[Ca^{++}]=\frac{3.4\times10^{-10}}{1.5\times10^{-6}+[Ca^{++}]}+\frac{3.7\times10^{-8}}{10^{-3}+[Ca^{++}]}. \tag{3}$$

The same numerical values were then used to construct the smooth curve fitting the points in Fig. 1*A*. Hence, it seemed that the binding of Ca to this membrane preparation could be described adequately by assuming only two binding sites, one with high affinity and low capacity and one with low affinity and high capacity. The quality of the fit could not be improved when three, rather than two, classes of independent binding sites were assumed.

Ca Binding to Membranes Prepared by Hypotonic Hemolysis

In functional studies on the effects of Ca on the cation permeability of human red cell membranes resealed red cell ghosts were prepared by reversal of hypotonic hemolysis. Some evidence suggested that the exposure to media of low ionic strength modified the accessability for Ca of the Ca-sensitive membrane sites (*see* [25]). Therefore, we extended our binding assay to a preparation of membrane fragments that were obtained by combining hypotonic hemolysis and freeze-thawing cycles under isotonic conditions (*see Materials and Methods*). In a first set of pilot experiments we compared the Ca binding to membranes that were prepared either in the absence or in the presence of a Ca complexing agent. Reports in the literature had suggested a decrease of the Ca affinity of the Ca-ATPase from red cells if the enzyme was prepared at very low Ca ion concentrations ($[Ca^{++}] < 10^{-8}$ M) [30, 43]. However, the Ca-binding parameters of the present membrane preparation were not altered significantly if EGTA was present during the initial steps of the preparation procedure.

Effect of pH on high affinity binding. In a preceding study, the two functionally defined high affinity Ca binding sites in red cell ghosts were affected differently by a change in pH. The Ca affinity of the site mediating an increase in the K permeability of the membrane decreased as the pH was shifted from 8.5 to 6. A similar pH shift did not change the affinity of the site promoting the resealing of ghosts after osmotic hemolysis [25]. Therefore, we first studied the pH dependence of Ca binding to the membrane preparation.

In Fig. 2 the Ca binding at pH 8.5 and 6.8 is plotted against the free Ca concentration in the medium. The experimental points represent mean values of 9 experiments. The total amount of Ca bound at each free Ca concentration was reduced by more than 40% as the pH was lowered from 8.5 to 6.8. A further decrease in pH to 6.0 caused only a small additional reduction of total Ca binding (not shown in Fig. 2). The overall Ca binding capacity at pH 6.8 was about 20% smaller than the corresponding value in the membrane preparation obtained at constant ionic strength. The decrease in binding capacity which is reflected in a small parallel shift of the binding curve (*see* broken line in Fig. 1*A*) may be related to a loss of membrane associated proteins. Hypotonic washing is likely to elute part of the spectrin polypeptides (*see* [38]). In Fig. 3*A–D* Scatchard plots were constructed from the mean values of Ca binding at

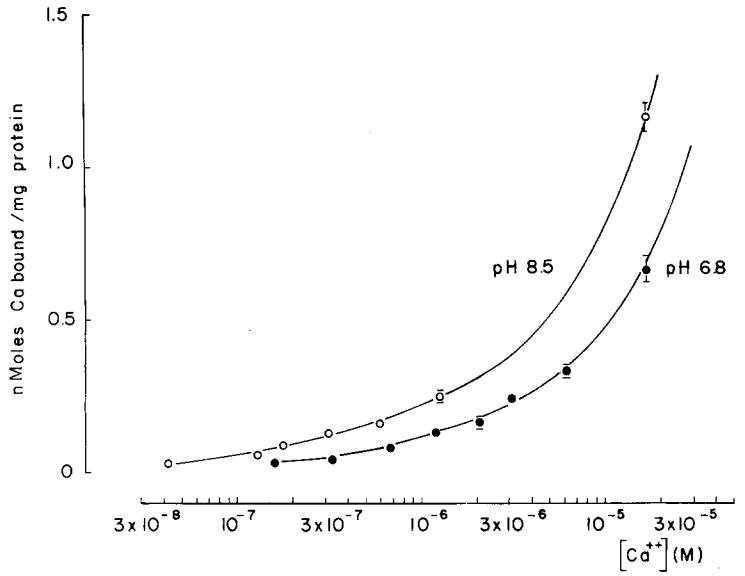


Fig. 2. Effect of pH on total Ca binding to red cell membranes. The membranes were prepared by a method combining hypotonic hemolysis and freeze-thawing cycles (*see Materials and Methods*). Incubation as in Fig. 1, except that a Ca-HEDTA buffering system was used. Mean values of 4–9 measurements from 9 experiments. Vertical bars give ±SEM. No bar indicates a variation smaller than the symbol. The smooth curves were calculated from the constants given in Table 2 for the respective pH values

four different pH values. The graphs were evaluated exactly as described for Fig. 1*B*. The K' values as well as the capacities of the Ca binding sites are listed in Table 2 and were used to calculate the smooth curves fitting the experimental points. The same parameters were also determined separately from Scatchard plots of each individual experiment in order to get an idea of their statistical variation. The resulting mean values (and standard errors) are also summarized in Table 2. They agree reasonably with the numerical values of the binding constants obtained from the standard evaluation procedure described above.

Several important observations could be made in these experiments. (i) At all pH values tested, an optimal fit of the experimental points required the assumption of three independent binding sites: two high affinity, low capacity sites and one low affinity, high capacity site. (ii) The pH-dependent decrease in total Ca binding capacity could be attributed mainly to a reduction of low affinity Ca binding. (iii) The site with the highest affinity for Ca ("site I") had a K' value close to 2×10^{-7} M in the pH range between 8.5 and 6.8. As the pH was lowered further to 6.0, K'

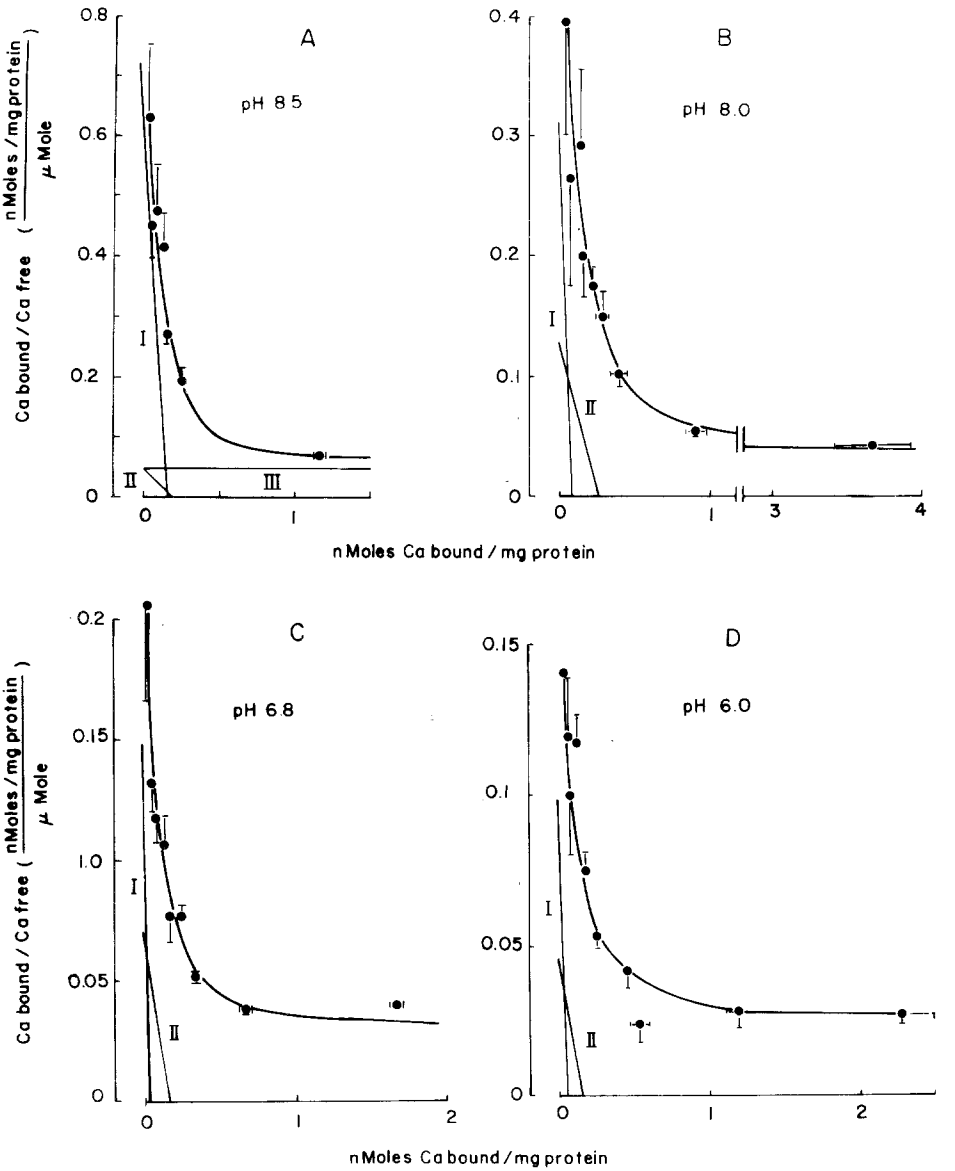


Fig. 3. Scatchard plots of membrane Ca binding at different pH values. (*A*): 8.5; (*B*): 8.0; (*C*): 6.8; (*D*): 6.0. Membrane preparation as in Fig. 2. The binding was measured in soln. IV and V containing HEDTA-buffered Ca ion concentrations between 10^{-8} and 10^{-5} M. Mean values from 6–9 measurements in 9 experiments (*A*), 5–6 measurements in 6 experiments (*B*), 4–9 measurements in 9 experiments (*C*), and 3–4 measurements in 4 experiments (*D*). The bars indicate ±SEM. The smooth curves were calculated from the binding constants summarized in Table 2. Note that the scale expansion of the ordinate increases successively from *A* to *D*. The straight lines represent individual Scatchard plots for the two high affinity sites (*I* and *II*). With the high scale expansion used here, the respective individual plot for the low affinity site (*III*) results almost in a parallel to the abscissa and is only shown in *A*

Table 2. Effect of pH on the parameters of Ca binding to fragmented red cell membranes

pH of incubation medium		8.5	8.0	6.8	6.0
Apparent dissociation constants (M)	K'_1	2.5×10^{-7} ($2.5 \pm 1.3 \times 10^{-7}$)	2.4×10^{-7} ($2.3 \pm 0.6 \times 10^{-7}$)	2×10^{-7} ($4.2 \pm 0.7 \times 10^{-7}$)	6×10^{-7} ($7.2 \pm 0.9 \times 10^{-7}$)
	K'_2	4×10^{-6} ($3 \pm 0.45 \times 10^{-6}$)	2×10^{-6} ($2.8 \pm 0.6 \times 10^{-6}$)	2.5×10^{-6} ($2.3 \pm 0.4 \times 10^{-6}$)	3.2×10^{-6} ($3 \pm 1 \times 10^{-6}$)
	K'_3	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Binding capacities (nmoles/mg protein)	n_1	0.16 (0.12 ± 0.02)	0.08 (0.082 ± 0.014)	0.04 (0.064 ± 0.007)	0.055 (0.086 ± 0.029)
	n_2	0.2 (0.11 ± 0.01)	0.26 (0.29 ± 0.13)	0.17 (0.13 ± 0.2)	0.15 (0.15 ± 0.01)
	n_3	50	40	30	26
Number of experiments		9	6	9	4

Values of K' and n without a SE represent parameters obtained by fitting plots of the mean ratios Ca bound/Ca free *vs.* the mean amount of Ca bound at each particular $[Ca^{++}]$ from all experiments. These constants were used to calculate the smooth curves in Fig. 3*A–D*. The numbers in brackets represent the mean values ±SEM of individual estimates of K' and n values in each particular experiment.

increased to 6×10^{-7} M. The Ca binding capacity of the same site decreased between pH 8.5 and 6.8 from 0.16 to 0.04 nmoles/mg protein but remained almost constant between pH 6.8 and 6.0. (iv) The second high affinity site ("site II") had a K' value around 3×10^{-6} M and a capacity close to 0.2 nmoles/mg protein. Both parameters did not show any systematic pH dependence within the tested range.

It must be concluded from these results that the two high affinity Ca binding sites have the same affinities as predicted for those sites which mediate the effects of Ca on cation permeability. Moreover, the Ca binding parameters of site I vary with the pH in a way that is compatible with the observed pH sensitivity of the site which mediates the Ca-dependent increase in K permeability.

The effect of local anesthetics on high affinity Ca binding. Another independent means to test the presumed relationship between Ca binding and function consisted in studying the effects of certain local anesthetics. It had been shown in an earlier investigation [24] that propranolol, in contrast to tetracaine, was capable of inhibiting the Ca-activated K flux.

Moreover, some evidence suggested that the inhibitory compound was the propranolol base, rather than the ionized drug [24]. If one of the high affinity Ca binding sites corresponded to the site in resealed ghosts which mediates the increase in K permeability, propranolol, but not tetracaine, should be able to alter the Ca binding constants of that site. Figs. 4 and 5 are based on experiments which were designed to test this point. We assessed Ca binding always at pH 8.5 for two reasons: (i) The fraction of site I Ca binding was highest at that pH value. (ii) More than 10% of the total concentration of propranolol (pK = 9.45) were present in the free base form.

Figure 4 presents Scatchard plots from a single experiment. The effects of propranolol on Ca binding were first compared to the effects of a detergent, saponin. This was an essential control because most local anesthetics, including the two compounds used here, show some surface activity. Therefore, the question to answer was whether this property would influence the Ca-binding characteristics of the membrane. Saponin is known to open permanent holes in the red cell membrane [34]. If membrane vesicles would have been formed in our preparation, excluding Ca from part of its binding sites, one might have predicted an increase in Ca binding in the presence of saponin. In this experiment, the rather high concentration of 67 μg/ml saponin caused, indeed, an increase in site II Ca binding capacity from 0.12 to 0.3 nmoles/mg protein. However, the binding capacities of the two other sites remained unchanged. Saponin did not affect the K' values of any of the Ca binding sites.

By contrast, propranolol *reduced* the binding capacity of site II to 0.08 nmoles/mg protein and increased K' of site I by a factor of more than 5 from 7×10^{-8} to 4×10^{-7} M. (The alternative assumption that propranolol reduced or eliminated Ca binding to site I did not yield an equally good fit of the theoretical curve to the experimental points.) The major fraction of the propranolol-induced decrease in the total binding capacity was apparently due to an inhibition of low affinity binding. These results cannot be explained by a detergent effect, but must reflect some more specific interaction of propranolol with high affinity Ca binding.

Figure 5*A* is a Scatchard plot that summarizes the results with propranolol. The points represent mean values of the ratio Ca bound/Ca free which were obtained in 3 independent experiments. In a similar plot, Fig. 5*B* shows mean values of the results of 2 experiments with tetracaine. The concentration of the free base form of the two compounds

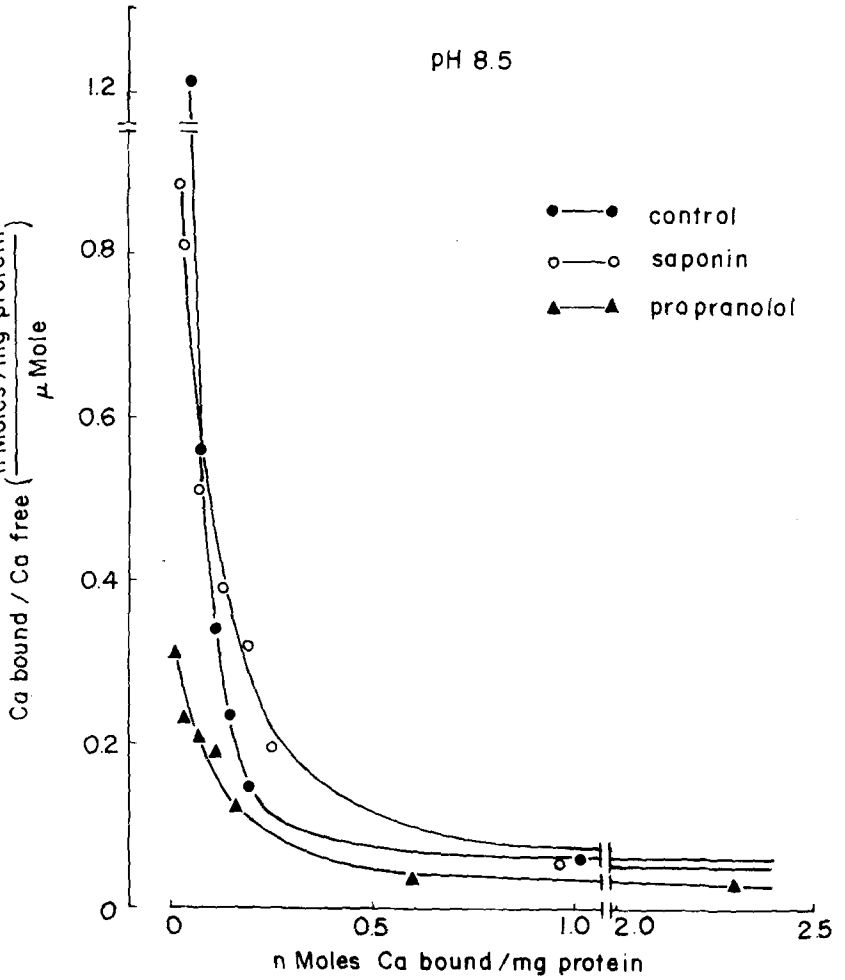


Fig. 4. The effect of saponin and of propranolol on Ca binding to red cell membranes. Scatchard plot of data from a single experiment. Same membrane preparation as in Fig. 2. Same binding medium as in Fig. 3*A*. The smooth curves were calculated assuming 3 independent binding sites. ●—●: control (K' values of sites *I–III*: 7×10^{-8}, 4×10^{-6}, 10^{-3} M; n values of sites *I–III*: 0.1, 0.12, 50 nmoles/mg protein). ○—○: saponin (67 μg/ml) (K' values: 10^{-7}, 2×10^{-6}, 10^{-3} M; n values: 0.09, 0.3, 50 nmoles/mg protein). ▲—▲: propranolol (2 mM) (K' values: 4×10^{-7}, 4×10^{-6}, 10^{-3} M; n values: 0.1, 0.2, 25 nmoles/mg protein). Similar results were obtained in 4 other experiments with propranolol and 2 other experiments with saponin

at pH 8.5 was 0.2 mM for propranolol and 0.5 mM for tetracaine. It should be noted that there is a considerable difference in the scale of the two ordinates. The binding parameters used to construct the smooth curves in Fig. 5*A* and *B* are listed in Table 3. The data of Fig. 5*A* confirmed the effects of propranolol which were already described for the single experi-

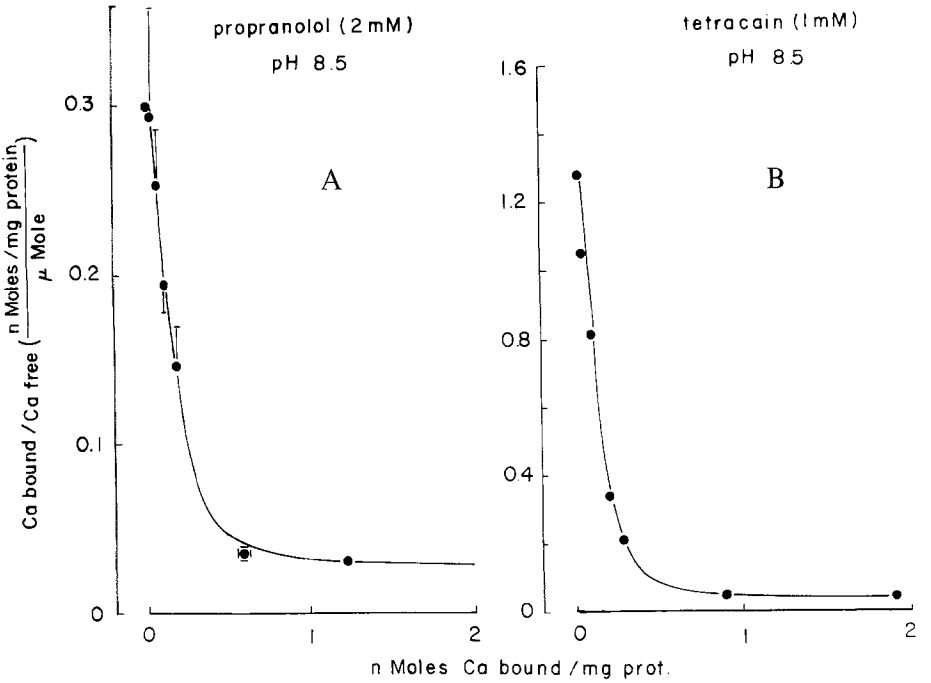


Fig. 5. Scatchard plots of Ca membrane binding in the presence of propranolol (*A*) and tetracaine (*B*). Same conditions as in Fig. 4. Mean values of 3 experiments (*A*) and 2 experiments (*B*). The binding constants used to construct the smooth curves are listed in Table 3. Note that the scale of the ordinates differs by a factor of 5

Table 3. Effect of propranolol and tetracaine on Ca binding to fragmented red cell membranes

		Ca binding in the presence of 2 mM propranolol	Ca binding in the presence of 1 mM tetracaine
Apparent dissociation constants (M)	K'_1	6.5×10^{-7}	1.5×10^{-7}
	K'_2	4×10^{-6}	4×10^{-6}
	K'_3	10^{-3}	10^{-3}
Binding capacities (nmoles/mg protein)	n_1	0.19	0.21
	n_2	0.08	0.15
	n_3	25	35
pH of incubation		8.5	8.5
Number of experiments		3	2

The K' and n values were estimated by fitting Scatchard plots of the mean ratios Ca bound/Ca free *vs.* the mean values of Ca bound at each particular $[Ca^{++}]$.

ment of Fig. 4. The increase in the K' value of site I without a change in the binding capacity suggested a competitive antagonism between propranolol and Ca at this particular site. Equation (2) on p. 123 was derived for a Scatchard plot of Ca binding to m classes of binding sites. For the binding to a single class of sites it can be rearranged to the form

$$r/[Ca^{++}] = \frac{1}{K'}(n-r) \tag{4}$$

and in the presence of a competitive inhibitor

$$r/[Ca^{++}] = \frac{1}{K'}\frac{K''}{K''+X}(n-r). \tag{5}$$

K'' is the apparent dissociation constant of the inhibitor and X is the free concentration of the inhibitor.

By contrast, tetracaine had barely any influence on the Ca binding parameters of the high affinity sites (compare the values in Table 3 with those in the first column of Table 2). However, like propranolol, it reduced low affinity Ca binding significantly. We have also tested the effect of the local anesthetic lidocaine (2 mM) on high affinity Ca binding. At pH 8.5 this drug is better soluble than tetracaine. Nevertheless, lidocaine behaved exactly like tetracaine and left the Ca binding parameters of the high affinity sites unchanged.

At pH 6.8 where tetracaine and propranolol both exist predominantly in the ionized form, the two drugs (2 mM) had no significant effect on the high affinity sites but seemed to inhibit Ca binding to the low affinity site. However, the small contribution of site I Ca binding to the overall Ca binding at pH 6.8 made a reliable estimate of changes in its binding constants rather difficult. Not enough experiments have been done to allow a definite conclusion.

Ca Binding in the Presence of Mg and ATP

Human red cell membranes possess a Ca-Mg-activated ATPase which is involved in the outwardly directed ATP-dependent Ca transport [32]. The apparent Ca dissociation constant for this enzyme at pH values close to 7 has been determined from direct measurements [42] as well as from transport studies [31] to be close to 10^{-6} M. The similarity

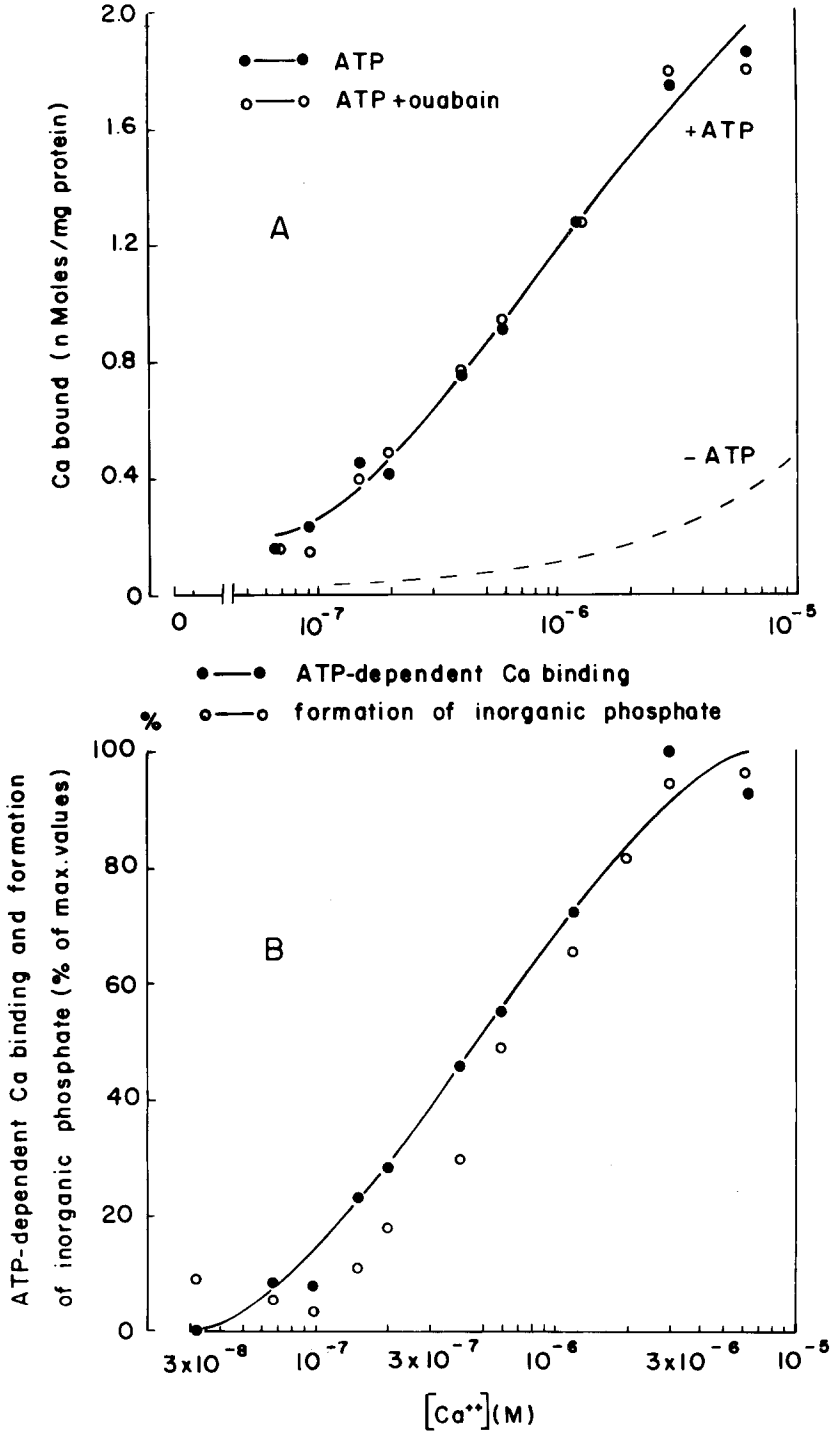
of the K' values made it impossible to evaluate the fraction of high affinity Ca binding to site II which might be due to a binding of Ca to this enzyme in the absence of ATP. Therefore, we studied Ca binding in the presence of ATP hoping that we would be able to observe at least ATP-dependent changes in the Ca binding constants of the enzyme. First, control experiments were conducted to assess the effect of 1 mM Mg^{++} on the Ca binding constants in the absence of ATP (*see* broken curve in Fig. 6*A*). We calculated that the addition of 1 mM Mg^{++} to the Ca-EGTA buffer media increased the free $[Ca^{++}]$ by less than 5%. This effect was small enough to be neglected. In a Scatchard plot (not documented by a figure) the mean values of 3 experiments at pH 6.8 could be fitted by the equation

$$r/[Ca^{++}] = \frac{1.7 \times 10^{-10}}{6.5 \times 10^{-7} + [Ca^{++}]} + \frac{2.5 \times 10^{-8}}{10^{-3} + [Ca^{++}]}. \quad (6)$$

Hence, only one high affinity Ca binding site was detectable under these conditions. The K' value of this site for Ca (6.5×10^{-7} M) was higher than the respective value of site I and lower than the value of site II under control conditions. Moreover, the Ca binding capacity of this single high affinity site was lower than the sum of the capacities of sites I and II in the controls. These findings suggested a mixed competitive-noncompetitive interaction between Ca and Mg at the membrane Ca binding sites.

In the presence of Mg (2.5 mM) and ATP (1.7 mM) we observed a considerable increase in the total amount of Ca bound to the membrane fragments (Fig. 6*A*). The ATP-dependent Ca binding was measured in the presence of 150 mM KCl, i.e., in the same medium which was used for

Fig. 6. (*A*): Ca membrane binding in the presence of Mg and ATP. Membrane preparation as in Fig. 2. Binding was measured after 60 min in soln. IV containing EGTA-buffered Ca ion concentrations ranging from 3×10^{-8} to 6×10^{-6} M, 1.7 mM Na_2 ATP, 2.5 mM $MgCl_2$ and either none (closed circles) or 10^{-4} g/ml ouabain (open circles). One of 7 experiments. The smooth curve was calculated assuming one high affinity site ($K' = 6.2 \times 10^{-7}$ M, $n = 1.8$ nmoles/mg protein) and one low affinity site ($K' = 10^{-3}$ M, $n = 50$ nmoles/mg protein). The broken line is a calculated fit for the mean values (3 experiments) of Ca bound in the absence of ATP, but in the presence of 1 mM Mg. The respective binding constants are given in the text. (*B*): Comparison between the ATP-dependent fraction of Ca binding (closed circles), data from *A*, and Ca-stimulated formation of inorganic phosphate (open circles). Incubation period, 60 min. Membrane preparation as in *A*. The two parameters were both normalized with respect to their maximal values at $[Ca^{++}] = 6.2 \times 10^{-6}$. Binding curve drawn by eye


ATP
ATP+ouabain
+ATP
A
- ATP
Ca bound (n Moles/mg protein)
2.0
1.6
1.2
0.8
0.4
0
10^{-7}
10^{-6}
10^{-5}
ATP-dependent Ca binding
formation of inorganic phosphate
%
B
ATP-dependent Ca binding and formation of inorganic phosphate (% of max. values)
100
80
60
40
20
$3x10^{-8}$
$3x10^{-7}$
$3x10^{-6}$
$[Ca^{++}](M)$

the standard binding assay. Under these conditions the Na-K-ATPase was almost completely inhibited. Hence, the ATP-stimulated Ca binding did not depend on the activity of the Na-K-ATPase. Moreover, the binding was not inhibited by ouabain (10^{-4} g/ml). ATP-stimulated Ca binding and the formation of inorganic phosphate occured in exactly the same range of buffered free Ca concentrations (Fig. 6*B*). Half maximal values were obtained with 5×10^{-7} M [Ca^{++}]. These findings may suggest that it is the occupancy of the Ca-Mg-ATPase by ATP which stimulated Ca membrane binding. However, the Ca binding capacity of the enzyme is probably much too small to account for a major fraction of the total amount of ATP-stimulated Ca binding (*see Discussion*, p. 139). In control experiments the addition of inorganic phosphate in the absence of ATP had no measurable effects on the Ca binding parameters. With [Ca^{++}] above 10^{-5} M, the ATP-stimulated component of Ca binding (the difference between the total amounts of Ca bound in the presence and in the absence of ATP) decreased in absolute as well as in relative terms. At 8.4×10^{-5} M [Ca^{++}], ATP-stimulated binding accounted for only 15–30% of the total Ca binding. In Fig. 7 the data from the

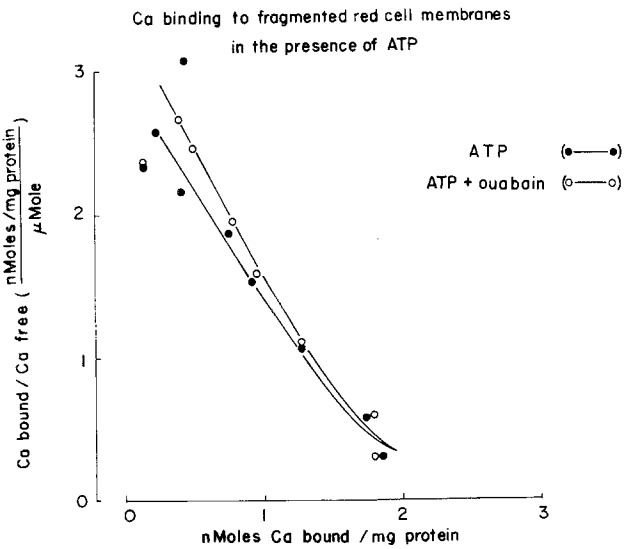


Fig. 7. Scatchard plot of ATP-stimulated Ca binding in the presence (○—○) or absence (●—●) of ouabain (10^{-4} g/ml). Same experiment as in Fig. 6*A*. The smooth curve fitting the ouabain points was calculated using the following constants: High affinity site: $K' = 5.4 \times 10^{-7}$ M, $n = 1.8$ nmoles/mg protein. Low affinity site: $K' = 10^{-3}$ M, $n = 50$ nmoles/mg protein. The curve across the filled circles was drawn according to the constants given in Fig. 6*A*

experiment of Fig. 6 were plotted according to Scatchard. The best fit of the experimental points required the assumption of a single high affinity Ca binding site with a K' close to 6×10^{-7} M and a capacity of 1.8 nmoles/mg protein. Thus, in the presence of ATP the capacity of high affinity Ca binding increased almost by a factor of 10. The increase in the Ca binding capacity was large enough to obscure any high affinity binding to additional sites with small capacities (sites I and II). Moreover, the amount of ATP-stimulated Ca binding was rather variable and scattered much more than the values for ATP-independent binding. Therefore, the Scatchard plots of only a few experiments could be evaluated individually. It was impossible to obtain a reliable fit of their mean values. Nevertheless, the experiments provided evidence for the presence of a saturable ATP-dependent component of high affinity Ca binding to fragmented membranes.

Discussion

Our results show that at least two independent high affinity Ca binding sites exist in fragmented red cell membranes exposed to a medium of physiological ionic strength. Several properties of these sites suggest that they might be equivalent to two Ca-sensitive sites involved in the regulation of membrane cation permeability. (i) In membranes subject to hypotonic hemolysis the K' values of the Ca binding sites I and II at pH 6.8 (2×10^{-7} and 2.5×10^{-6} M, respectively) agree well with those of the two functionally defined sites: The membrane site which mediates the Ca-dependent increase in K permeability ("K-site") has its K' value close to 3×10^{-7} M Ca. Another site which seems to control the resealing of ghosts after hypotonic hemolysis ("resealing site") had a K' value close to 2×10^{-6} M Ca [25]. (ii) A shift in pH from 8.5 to 6 affected the binding constants for Ca at site I, but not at site II. Correspondingly, the same decrease in pH caused the apparent dissociation constant of the K-site to increase but did not alter the respective value of the resealing site. (iii) Propranolol, which had been shown to inhibit the Ca-mediated K efflux [24], competed with Ca at binding site I. Tetracaine, which was not inhibitory at the K-site [24], also did not interfere with Ca binding to site I. (iv) Mg ions which were known to compete with Ca for the K-site and for the resealing site [25] caused a mixed competitive-noncompetitive inhibition of high affinity Ca binding. (v) The two high affinity Ca binding sites could not be observed simultaneously unless the

membranes were exposed temporarily to a medium of low ionic strength. In red cell ghosts it was the resealing site which became available only after hypotonic hemolysis. The K site seemed to be located close to the inner surface of the membrane and seemed readily accessible [25].

Nevertheless, closer inspection of the data reveals some inconsistencies. As noted earlier, competitive interaction of Ca with some other substance for a common binding site should result in an increase of K' for Ca at that site (*see* Eq. (5), p. 131). By contrast, noncompetitive interaction at a site should result in a decrease of the apparent Ca binding capacity. In functional studies, a competitive interaction between Ca and protons at the K-site was observed over the entire pH range from 8.5 to 6 [25]. Yet, the binding studies suggest a noncompetitive interaction at pH values above 6.8 (decrease in capacity of site I) and a competitive interaction in the acidic range (increase in K' of site I). In functional studies the competitive interaction between protons and Ca was rather weak in the pH range from 6.8 to 8.5. Probably this effect is too small to be detected in binding studies. Nevertheless, the decrease in Ca binding capacity would not have been predicted from functional studies. It is possible that the "site I" sites which are detectable at high pH values constitute an inhomogeneous population of which only a part contributes to the Ca-induced increase in K permeability. A similar difficulty arises with the effect of propranolol. The functional studies [24] pointed to a noncompetitive inhibitory action of propranolol at the K site. The binding assay suggests a competitive interaction of the drug with Ca at site I. However, in this case the pH value of the medium used for the binding studies (8.5) differed from the one used to demonstrate the inhibitory effect of propranolol on Ca-induced K permeability (7.2). Consequently the concentration ratio of the free base form to the ionized form of the drug differed by more than a factor of 10 under the two conditions. Possibly, the type of interaction between propranolol and the Ca binding sites is determined to some extent by this ratio. Unfortunately, the low Ca binding capacity of site I at pH 7.2 prevents a reliable test of this hypothesis with the method used in our experiments.

Another problem is the obvious difference in the Ca binding characteristics of the two different membrane preparations which were used in the present study (*see* Figs. 1*B* and 3*C*). The results of functional studies [25] led us to predict that the K site, but not the resealing site, would be accessible in membranes prepared at constant ionic strength, whereas both sites might be detectable in membranes exposed to hypotonic hemolysis. In fact, two high affinity sites were observed in the latter and

only a single site in the former preparation. However, contrary to our prediction, the Ca binding constants of this single high affinity site at pH 6.8 ($K' \sim 1.5 \times 10^{-6}$ M, $n \sim 0.34$ nmoles/mg protein) differed considerably from those of site I ($K' \sim 2.5 \times 10^{-7}$ M, $n \sim 0.04$ nmoles/mg protein) which we think corresponds to the K site in the second membrane preparation. More experiments are needed to characterize high affinity Ca binding in isotonically prepared membranes in terms of pH dependency and sensitivity to local anesthetics until the reason for this discrepancy may become clear.

It is an inherent weakness in the interpretation of the present results that we have to rely on the evaluation of binding constants from nonlinear Scatchard plots (*see* discussion in [41]). The resolution of this method is rather limited. If more than two high affinity Ca binding sites are assumed to exist in the membrane, or if the K' values of the two sites differ by less than a factor of 3, the influence of the variation of an individual variable on the goodness of the curve fit becomes too small to be readily recognized. Hence, we have tried to keep the number of variables to a minimum. The existence of two high affinity Ca binding sites was assumed only after the data could not be fitted satisfactorily by assuming a single high affinity site. The K' value of low affinity Ca binding was assumed to be constant at 10^{-3} M because the experimental design did not allow an exact estimate of Ca binding constants of individual low affinity sites. Therefore, any inhibition of low affinity Ca binding, irrespective of whether it was due to competitive or noncompetitive interactions, was always reflected in an apparent decrease of the binding capacity.

The "goodness of fit" was judged by eye. This is possible only if the scatter of the data is low; hence, the best fit can be identified as a curve which intersects most of the experimental points. Therefore, we usually preferred to fit the binding curves to the mean values for Ca binding of several identical experiments rather than to fit the data from individual experiments. Under our conditions a twofold change in any one of the binding constants listed in the tables and legends to the figures, would have caused a recognizable deviation from the "best fit". Several other statistical procedures have been applied to fit nonlinear Scatchard plots. However, their general superiority over a "fit by eye" has not been demonstrated (*see* [41]).

Even though our results suggested an identity of binding site I with the K-site, an alternative target for high affinity Ca binding could not be ruled out. It is possible that the Ca-Mg-ATPase of the red cell

membrane provides a set of high affinity Ca binding sites even in the absence of ATP. Pertinent observations were made on the sarcoplasmic reticulum Ca-ATPase. This enzyme, which shares a number of important properties with the red cell Ca-Mg-ATPase, was shown to bind Ca with high affinity ($K' \leqq 10^{-6}$ M) in the absence of ATP [4, 8, 13, 14, 22]. The pH dependence of ATP-independent Ca binding to the pump protein was similar to the relation observed in our system: The binding affinity decreased when the pH was lowered from 8 to 6 [22]. Moreover, propranolol, an antagonist of Ca binding to site I, is known to inhibit the Ca transport ATPase in red cell membranes [24] as well as in the sarcoplasmic reticulum [39]. It is intriguing that the characteristics of high affinity Ca binding to site I are consistent with predictions made for the K-site as well as with those made for Ca-pump sites. It is possible, but not at all proven, that the two biological receptors for Ca on the inside of the red cell membrane have a closely related structure.

The identification of binding site II with the resealing site has no likely alternative. However, the measured Ca binding capacity of this site may be an underestimate since we have no possibility of testing whether all of these functional sites are accessible for Ca under our experimental conditions. Hypotonic hemolysis at 22 °C in the presence of EGTA together with two hypotonic washes may prevent part, but not all, of the membranes from reconstituting into the state they maintain in resealed ghosts (where the resealing site is not readily accessible).

Except for a recent report by Romero [26], Ca binding to red cell membranes has not been determined previously in the presence of buffered low concentrations of ionized Ca^{++}. Nevertheless, several studies using different methods agreed in describing a high affinity Ca binding site with a K' close to 4×10^{-6} M [5, 26, 40]. This value corresponds well to the affinity of our binding site II, which was described in the present paper. The reported binding capacities vary between 250 nmoles/mg protein [40] and 0.2 nmole/mg protein (this paper). However, different assay conditions (nonequilibrium binding) as well as differences in the evaluation of the binding curves make a quantitative comparison of the data impossible. Cohen and Solomon [5] noted that the high affinity site was detectable in the presence of 0.1 M KCl, but not if an ionic strength of 0.01 was employed. The KCl-medium reduced considerably the large unspecific Ca binding which otherwise tended to obscure any high affinity binding (*see* [20]). The authors reported a binding capacity of ~ 0.9 nmole/mg protein for this site. This value is still somewhat higher than ours, but the difference in experimental

techniques may well explain the small discrepancy. In addition, these authors provided convincing evidence that this particular site was accessible only from the inside of the red cell membrane.

The high affinity binding site I, which we think is a correlate of the K-site in resealed ghosts, was also subject of the study by Romero [26]. He reported a K' value of 3×10^{-8} M and a binding capacity of 1.2 nmole Ca/μg lipid P (corresponding to about 32 nmoles Ca/mg membrane protein). These values deviate considerably from those found in the present study ($2-6 \times 10^{-7}$ M and 0.04–0.15 nmoles/mg protein, respectively). A number of factors may have contributed to this discrepancy: (i) In Romero's work, Ca binding was not measured under equilibrium conditions at constant ionic strength. (ii) The sequestration of some Ca EGTA into membrane vesicles was not completely excluded by the method used. (iii) The simple graphical evaluation of nonlinear Scatchard plots, which was applied, usually tends to overestimate considerably the binding capacities of high affinity sites (*see* Fig. 3). (iv) The protein/phospholipid ratio in the membrane preparation of Romero [26] may have been higher than in our case.

We have used our value for the Ca binding capacity of site I to estimate roughly the number of these Ca receptors per cell. If one ghost cell is assumed to contain 6.6×10^{-13} g protein [15], the amount of Ca which can be bound to site I at pH 6.8 is 3×10^{-20} moles. Under the assumption that two Ca ions are bound per site (*see* [25]) this value corresponds to ~8,000 sites per cell or 60 sites per μm^2. This figure sets only an upper limit to the number of functional sites at this particular pH because the contribution of unspecific binding is unknown.

ATP-Dependent Ca Binding

Our experimental results provided evidence for a saturable ATP-dependent component of high affinity Ca binding to red cell membranes. The capacity of the ATP-induced Ca binding site was smaller than 1.8 nmoles/mg protein.

What is the functional significance of ATP-stimulated Ca binding? The present experiments cannot answer this question conclusively. The stimulation of Ca binding by ATP and the activation of the membrane Ca-ATPase were observed within the same range of free Ca concentrations. However, this finding does not necessarily imply an ATP-induced increase in the Ca binding capacity of the Ca transport ATPase. ATP-dependent Ca binding, 1.8 nmoles/mg protein, corresponds to

~340,000 sites per cell, if the same assumptions are made as were used to estimate the number of site I receptors. Even though the number of Ca-pump sites in the human red cell membrane is unknown, it seems unlikely that it exceeds the number of Na-K-pump sites by three orders of magnitude. This conclusion is supported by the finding that the amount of Ca-phosphoprotein is not higher than the sum of the Mg- and Na-phosphoproteins [17]. It is conceivable that membrane proteins not related to the Ca pump are phosphorylated in the presence of ATP and thus provide additional high affinity Ca binding sites. On the other hand, Ca binding to the Ca-pump protein of the sarcoplasmic reticulum is barely affected by ATP [22].

This paper is dedicated to Prof. W. Wilbrandt on the occasion of his 70th birthday.
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