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CALCIUM AND MAGNESIUM ATPases OF THE SPECTRIN FRACTION OF HUMAN ERYTHROCYTES

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Using a rapid method of preparation, spectrin has been isolated from human erythrocytes and its ATPase activity investigated. The ATPase activity with calcium has two distinct components, one with optimal activity when calcium and ATP are of equal concentration (low-Ca-ATPase) and another which is activated above 1 mM $CaCl_2$ and is maximal at 100 mM $CaCl_2$. There is also a Mg-ATPase with maximal activity at 10 mM $MgCl_2$. The high-Ca-ATPase of spectrin, but not the low-Ca-ATPase, is inhibited by magnesium, while the Mg-ATPase is inhibited by Ca in excess of ATP. None of these activities exhibits the calcium-stimulated magnesium-dependent activity characteristic of the red cell calcium pump.

During the last decade several groups have studied the proteins extracted from erythrocyte membranes at low ionic strength. Marchesi et al. (1–3) showed that elution of these proteins, which they named spectrin, correlated with removal of a layer of fibrils from the cytoplasmic surface of the membrane and reported that the extracted protein could be repolymerized into actin-like fibrils (3). Nicolson et al. (4) used ferritin-labeled antispectrin antibodies to show that spectrin was derived from the extractable fibrillar material lining the inside of the membrane.

Mazia and Ruby (5) isolated a similar protein fraction from erythrocyte ghosts which they called tektin. Clarke (6) found that tektin had a molecular weight greater than myosin, was highly assymetric, and was probably present in solution as a dimer containing one of each of the two high-molecular weight bands found by SDS-gel electrophoresis.

Harris (7, 8) and Haggis (9) also isolated water-soluble protein from human ghosts. Harris studied the electron microscopic appearance of the water soluble protein in the absence of cations and found small ring-shaped structures which he called "torus protein." Haggis (9) observed both torus and fibrillar forms.

Rosenthal et al. (10) extracted water-soluble proteins with dilute EDTA and studied the ATPase activity of this fraction. They found a calcium-stimulated ATPase which was inhibited by magnesium and which appeared to be nearly maximal at 6 mM $CaCl_2$. Clarke and Griffith (11), Peter and Wolf (12), and Avissar et al. (13) have also reported ATPase activities in this fraction. We have shown elsewhere (14) that the above protein fractions from red cell ghosts are essentially equivalent in peptide composition.

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In prior publications from this laboratory (15–17) and elsewhere, it has been hypothesized that spectrin is involved in the regulation of red cell membrane deformability since formation of fibrillar aggregates of spectrin on addition of calcium could account for the observed effects (15–17) of calcium on red cell membranes. Involvement of an ATPase in this system could also account for reports of red cell "contraction" (18) and energized vesicle formation (endocytosis) (19, 20) and also explain reports of red cell "actomyosin" (21, 22). To put these speculations on a firmer foundation, we began our studies by reinvestigation of the results of Rosenthal et al. (10) on spectrin ATPase. We have found that the ATPase activity of spectrin is more complex than their observations indicate.

METHODS

Human blood was used within a week after collection in ACD. After removal of plasma, cells were washed three times in 10 vol of 0.16 M NaCl, with aspiration of buffy coat at each step. EDTA (1 mM, pH 7.2) was included in the first wash to remove extracellular calcium (23).

Red cell membranes (ghosts) were prepared by the procedure of Hoogeveen et al. (24), modified to use carbonate buffer as described by Bramley et al. (25). A fresh stock solution of $NaHCO_3$ (250 mM) was adjusted to pH 7.2 with HCl to make the carbonate buffer; packed cells were lysed in about 10 vol of 30 mM carbonate buffer containing 1 mM EDTA. The membranes were sedimented at 20,000 g for 20 min; washed again in the same buffer, and then washed twice in 18 mM carbonate buffer and twice with 12.5 mM carbonate buffer. Small tightly packed pellets (below the fluid ghost layer) were discarded at each stage to eliminate leukocytes and platelets (26). Omission of EDTA from the lysis steps, or increasing the carbonate concentrations to 18 mM for the final two steps, resulted in increased retention of hemoglobin but no alteration of spectrin ATPase activity.

To prepare spectrin, 200 ml of packed ghosts were dialyzed overnight at 4°C against 6 liters of 0.1 mM EDTA (pH 7.2), and sodium azide (0.02 mM) was added to the dialysis solution to inhibit bacterial growth. EDTA could be omitted from the dialysis solution without effect on protein extraction, but traces of calcium and magnesium in the dialysis solution were then accumulated by the proteins whose divalent cation content rose from about 5 nmoles/mg protein (of each cation) to about 20 nmoles/mg. The dialyzed membranes were centrifuged 30 min at 30,000 g and the supernatant was recentrifuged at 100,000 g for 1 hr. The 100,000 × g supernatant, containing all the proteins solubilized by the dialysis procedure, was adjusted to pH 5.1 with HCl, and the material which flocculated was collected by centrifugation at 1,200 g for 10 min, at 4°C. (It is important to adjust the centrifugal force and time so that the flocculated material forms a loose pellet, since tightly packed pellets will not always go back into solution.) The pellet was resuspended in 5 mM HEPES (N-2-hydroxyethylpiperizine-N′-2-ethanesulfonic acid, pH 7.2) to a final protein concentration of 5–10 mg/ml, and 0.1 N NaOH was added dropwise to bring the pH to 7.2 while stirring at room temperature. The protein partially dissolved when HEPES was added and finished dissolving during pH adjustment.

Solubilization was judged to be complete when the material was optically clear and did not show trapped bubbles or other evidence of transparent aggregates; material judged soluble by these criteria also failed to sediment at 100,000 × g for 30 min.

ATPase activity was measured by a modification of the method of Berenblum and

Chain (27) with ATP-γ-^{32}P, essentially as described by Rosenthal et al. (10). Protein was incubated at 37°C with various concentrations of cations, 25 mM HEPES, 0.025 mM EDTA, and with tris-ATP (usually 1 mM or 0.25 mM) which contained ATP-γ-^{32}P, in a final volume of 0.2 ml. After incubation (usually one-half hour), the reaction was stopped with 2 vol of ice-cold 10% (weight/vol) trichloracetic acid. Precipitated protein was removed by centrifugation (2,000 g, 10 min, 4°C), 0.2 ml of molybdate-sulfuric acid (2.5 g ammonium molybdate-4H_2O in 100 ml 0.7 N H_2SO_4) and 0.8 ml isobutanol were added to 0.2 ml aliquots of the supernatant, and the tube was mixed vigorously with a Vortex mixer) for 30 sec. After standing 3 min, an 0.4 ml aliquot of the top (isobutanol) layer was mixed with 10 ml of scintillation fluid (1 liter toluene, 1 liter ethanol, 8 gm PPO, 100 mg POPOP) and counted. The extraction efficiency of this method was measured by substituting sodium phosphate-^{32}P for ATP-γ-^{32}P, and the value obtained (87% efficiency) was used to calculate maximum possible counts. It was verified with ^{14}C-ATP that ATP was not significantly extracted into isobutanol by this procedure. Protein was measured by the biuret method of Gornall et al. (28) as modified by Jacobs et al. (29). Optical density was read at 540 nm after 15 min incubation at room temperature and was calibrated with bovine serum albumin.

ATP-γ-^{32}P (Amersham-Searle, 1.2 Ci/nmole) was in 50% ethanol. The final concentration of ethanol in the reaction mixture was 0.05%; addition of more ethanol (to 0.1%) did not affect spectrin ATPase activity. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was done by the method of Fairbanks et al. (30). Concentrations of calcium and magnesium in ghosts and spectrin were determined by atomic absorption spectroscopy (31).

Electron microscopy was performed on a JEOL 100B electron microscope in the transmission mode at 80 kV. Drops of dilute protein were placed on carbon-stabilized formvar-coated grids (200 mesh). After 1 min the solution was drained well with filter paper, and a drop of 1% uranyl acetate was placed on the grid for 1 min and drained. Uranyl acetate solutions were centrifuged at 2,000 X g for 10 min immediately before use to remove any crystals or aggregates.

Dilute protein samples were prepared by adding 5 mM HEPES, pH 7.2, to concentrated fresh spectrin to obtain final protein concentrations of 0.05–0.5 mg/ml. The diluted spectrin was then filtered through a 0.22 micron Millipore filter in a syringe adaptor to eliminate any aggregates formed by surface denaturations of spectrin during preparation. Appropriate amounts of $CaCl_2$ stock solutions were added to aliquots of the filtered spectrin, and incubated 15–30 min at room temperature before placement of samples on the grid.

RESULTS

Spectrin purified by the method described consists almost entirely of a doublet of high molecular weight peptides which have an apparent molecular weight slightly greater than that of the heavy chain of myosin (Fig. 1). The most prominent bands in the water-soluble extracts, (Fig. 1, gel 3) besides the high molecular weight doublet, are the peptide of 40,000 daltons [band 5 (30)] and hemoglobin. Band 5 is left in the supernatant after pH precipitation (Fig. 1, gel 4), but hemoglobin precipitates with spectrin.

As can be seen in Fig. 1, gels 5–11, traces of other bands are usually present, which may represent proteolytic breakdown products of the high-molecular weight peptides. The prominence of these nonspectrin bands varies among preparations without any correlation with specific activity. Ammonium sulfate fractionation by the method of

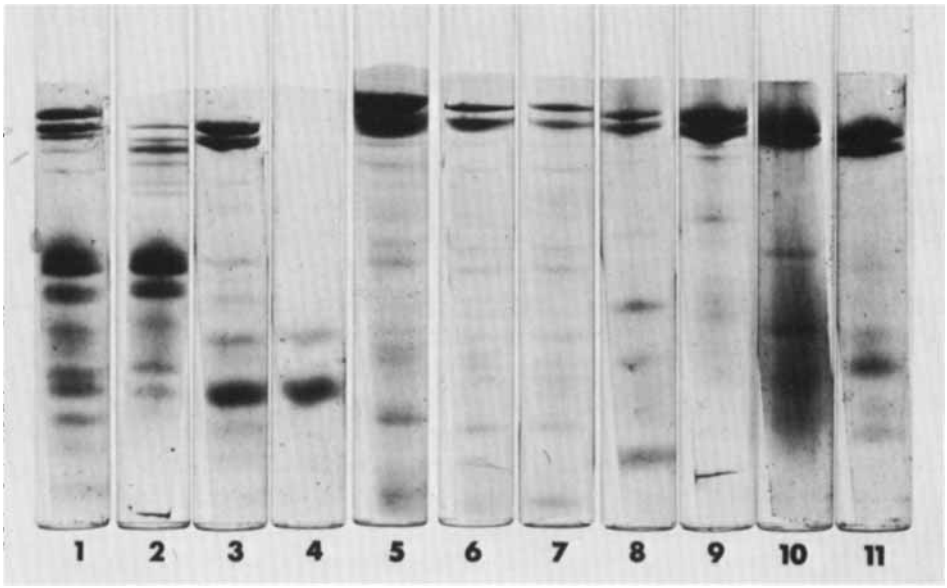

Fig. 1. Analysis of peptide composition by SDS-polyacrylamide gel electrophoresis. 1. Ghosts (40 μg). 2. "Residue," pellet of 30,000 × g centrifugation, washed once in 5 mM HEPES, pH 7.2 (40 μg). 3. Supernatant of 100,000 × g centrifugation ("Water-soluble proteins"), ca 20 μg. 4. Supernatant of pH precipitation (same volume as 3). 5–11. Seven different preparations of spectrin (15–40 μg).

Marchesi et al. (3) does not remove the presumed breakdown products once formed. Proteolysis may be due to proteases present in the red cell membrane fraction, as recently illustrated by Hulla (32).

The formation of fibrils by purified spectrin was studied by negative staining electron microscopy. Under various conditions, all the forms of spectrin described in the literature can be observed, except the actin-like form reported by Steers and Marchesi (3) after trypsin digestion. In the absence of divalent cations, spectrin appears as dispersed or partially aggregated particles, some of which resemble the torus structures described by Harris (7, 8), with no evidence of formation of fibrils (Fig. 2A, B). Addition of divalent cations causes spectrin to polymerize into fibrils, as described by others (2, 3, 6, 9, 10–12). Concentrations of calcium of 1–3 mM appear to produce maximal amounts of fibrils, and low concentrations of protein (0.05–0.2 mg/ml) produce fibrils that are separated, as shown in Fig. 2C, D. Higher concentrations of protein tend to produce large aggregates with tight side-by-side binding of fibrils (Fig. 2E, F), as reported by Clarke and Griffith (11). The ends of large bundles of fibers (Fig. 2E) give patterns comparable to Fig. 2C, D.

Very high levels of calcium (10–100 mM) do not further increase the aggregation of spectrin into fibrils. If protein concentration is greater than 1 mg/ml, calcium causes visible flocculation of protein. These aggregates are too electron-dense to be easily interpretable, but large fiber bundles or dispersed fibrils are rare, and these aggregates are predominantly amorphous. Occasional isolated clumps of precipitate at high calcium resemble Fig. 2B rather than 2C or 2F.

The ATPase activity of spectrin at 1 mM ATP is shown in Fig. 3. The specific activities shown in this paper for spectrin are only slightly higher (about 10%) than the specific activities obtained with unfractionated water-soluble proteins, and the pattern of

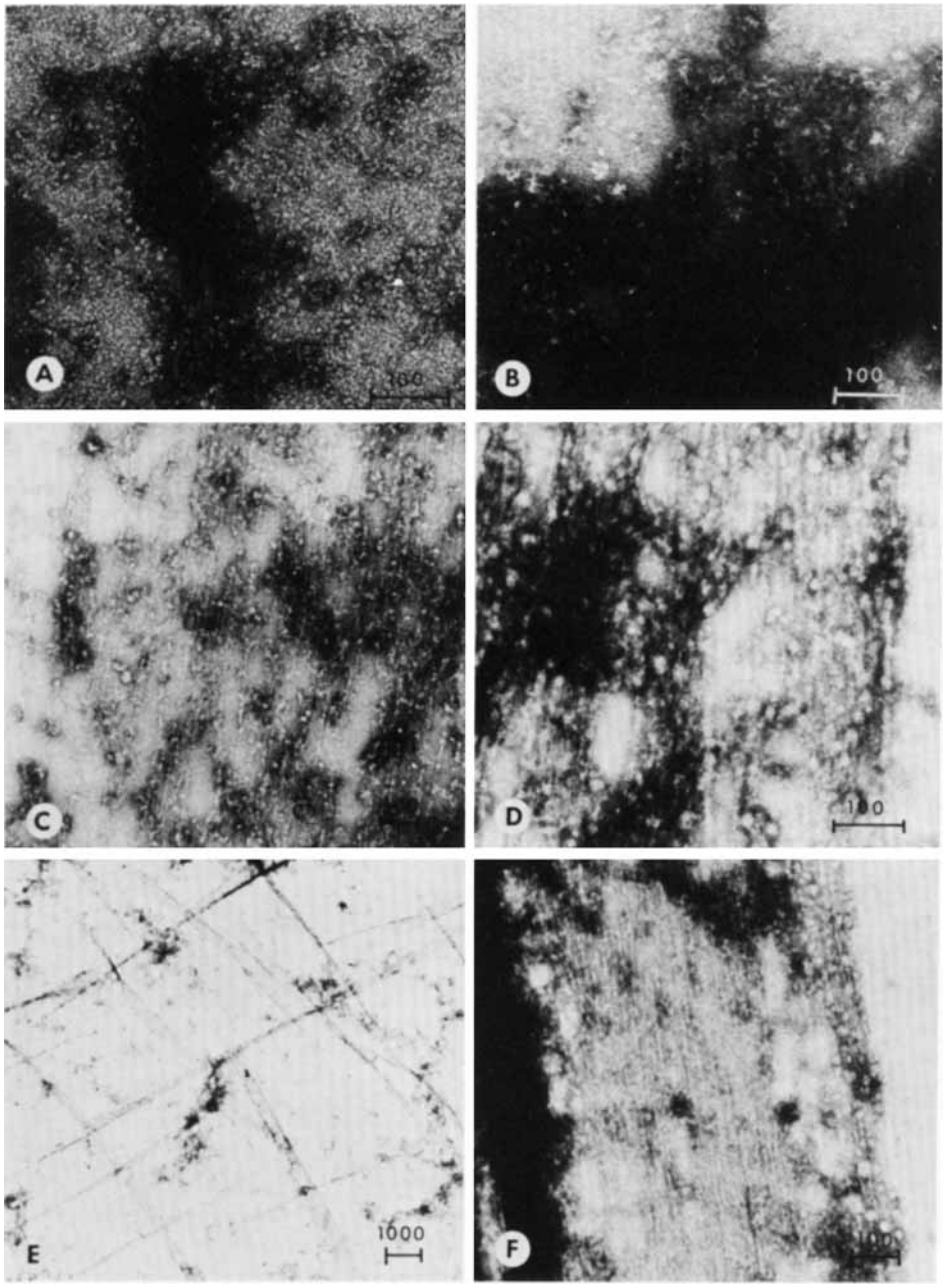

Fig. 2. Electron micrographs of negatively stained spectrin. Droplets of dilute spectrin were placed on carbon-stabilized formvar-coated grids for 1 min and drained, and the grids were stained for 1 min with 1% uranyl acetate. A. Spectrin, 0.1 mg/ml, in absence of divalent cations (120,000 ×); B. Spectrin, 0.2 mg/ml, no calcium (105,000 ×); C. Spectrin, 0.05 mg/ml, 3 mM $CaCl_2$ (56,000 ×); D. Spectrin, 0.10 mg/ml, 1 mM $CaCl_2$ (105,000 ×); E. Spectrin, 0.5 mg/ml, 1 mM $CaCl_2$ (5,000 ×); F. Edge of a thin bundle in E (70,000 ×). Bar: 100 nm, except E which is 1,000 nm.

cation stimulation observed is identical. This indicates that removal of band 5 does not affect spectrin ATPase activity.

In order to find the level of calcium causing maximum activity, the calcium concentration was increased above the level used by Rosenthal et al. (10), and an additional activity optimum at very high levels of calcium was found. The optimum value of $CaCl_2$, 100 mM, is extremely high and unphysiological and correlates with the concentrations at which calcium exhibits "chaotropic" effects similar to those caused by thiocyanate or guanidine (33). This high-calcium-ATPase is not an artifact of the assay procedure, since incubation of ATP with 100 mM $CaCl_2$ does not produce additional hydrolysis compared to incubation in water. It is also not due to a nonspecific phosphatase, since p-nitrophenyl phosphate is not hydrolyzed by spectrin. Moreover, the high-calcium-ATPase activity has a linear Lineweaver-Burk plot (Fig. 4) with a "Km" for calcium of about 20 mM. This probably corresponds to the lowest affinity class of calcium-binding sites of spectrin described earlier (34).

Addition of magnesium in the absence of calcium shows that there is also a Mg-ATPase activity in this fraction, with an optimal magnesium concentration of about 3–10 mM. The Mg-ATPase is inhibited by $CaCl_2$, and the high-Ca-ATPase is inhibited by $MgCl_2$, as shown in Table I. This agrees with the results of others (10, 11). The activity with other divalent cations is shown in Fig. 5. The order of activity is dependent on concentration. At 60 mM the order is Ca > Sr > Mn, Mg, Ba, while at 1 mM the order is Mn > Mg > Ca, Sr, Ba. These results may indicate that two distinct ATPases are present (Mg-ATPase, Ca-ATPase), but the present data do not definitively prove this.

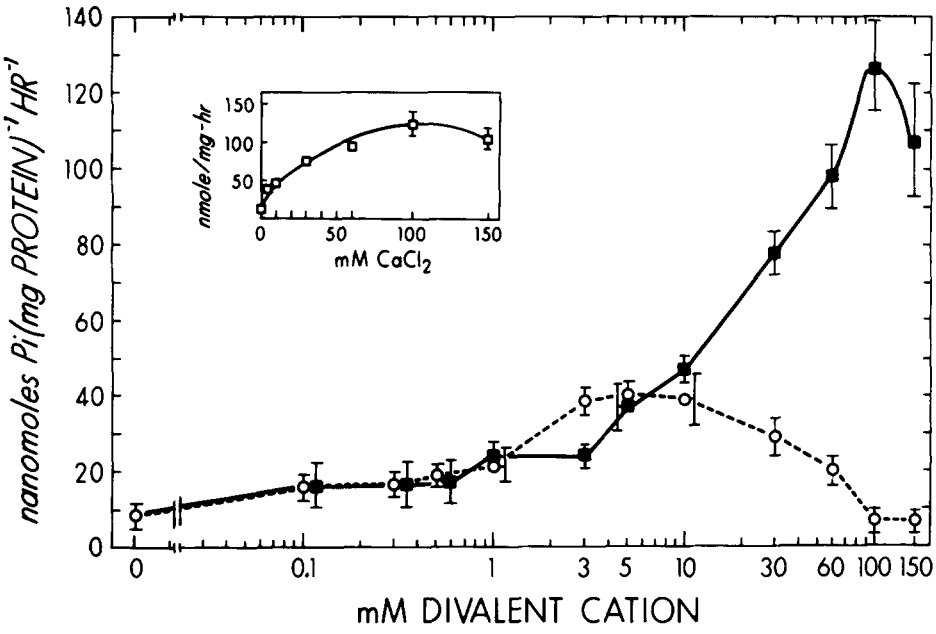

Fig. 3. ATPase activity of spectrin in presence of calcium and magnesium at 1 mM ATP. ATPase activity was measured as described under Methods at 1 mM ATP and various amounts of divalent cations. Incubations were 0.5 hr. Error bars represent standard deviation of means of 10–12 experiments. Inset: a linear plot of the calcium-ATPase data shown in the main figure. ■ $CaCl_2$; ○ $MgCl_2$; □ $CaCl_2$ (inset).

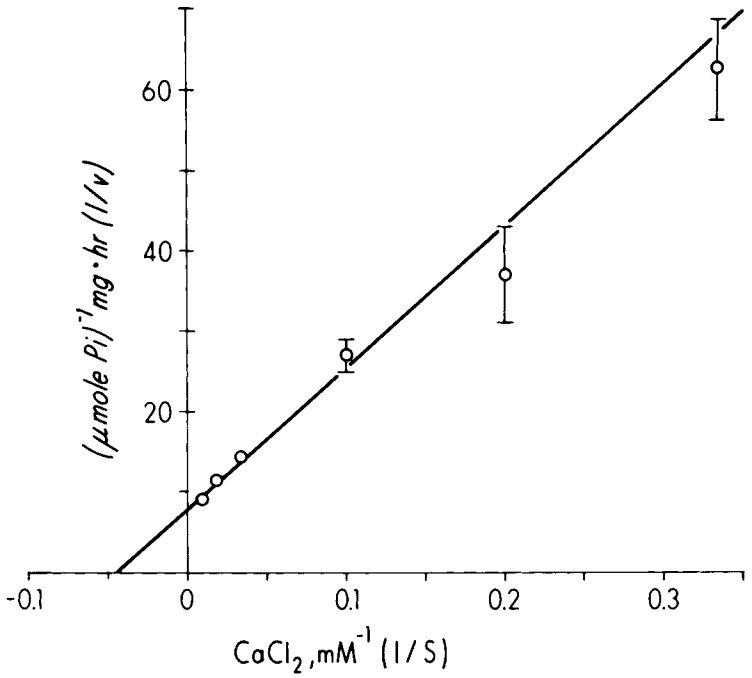

Fig. 4. Lineweaver-Burk plot of high-calcium ATPase. The reciprocal of specific activity is plotted vs reciprocal of calcium concentration between 3–100 mM $CaCl_2$. The intercept with the horizontal axis is about 20 mM^{-1}.

The Lineweaver-Burk plot of the high-Ca-ATPase is not linear below 5 mM Ca, and a plateau can be seen in the Ca-ATPase between 1–5 mM Ca (Fig. 3). If the ATP concentration is reduced, the plateau can be resolved into two peaks of activity (Fig. 6). At low ATP concentration, Ca-ATPase has a maximum in activity when the concentration of Ca and ATP are approximately equal, and as the calcium concentration is increased above this level, the activity declines. At about 2 mM, activity rises again due to activation of the high-Ca-ATPase.

If Mg-ATP is added instead of ATP, the activity is increased by addition of calcium to the same extent as in the absence of magnesium – that is, if the activity caused by the addition of Mg is subtracted, then the stimulation by calcium is identical in the presence and absence of magnesium. There was sufficient "background" ATPase in the spectrin preparations that in some preparations the rise in activity of low-Ca-ATPase above background was less than the experimental error of measurement of the activity (e.g., control,

TABLE I. Simultaneous Effects of Calcium and Magnesium on Spectrin ATPase Activity*

$[Ca^{2+}]$ mM		0	1	10	30	60	100
$[Mg^{2+}]$, mM:	0	23	24	56	65	87	130
	1	71	76	28	23	40	56
	10	81	62	37	19	22	35
	30	49	38	16	19	15	20
	60	31	21	29	29	19	14

*Conditions as in Fig. 3. Average of two experiments, expressed in nmoles Pi/mg/hr.

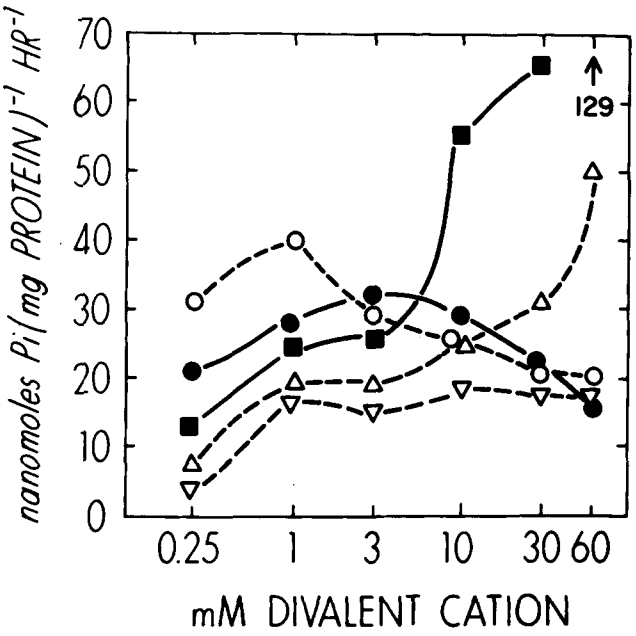

Fig. 5. Spectrin ATPase activity in presence of various divalent cations. ATP concentration is 1 mM, except that at 0.25 mM cation, ATP = 0.25 mM. Points are means of two experiments. ○ $MnCl_2$; ●$MgCl_2$; ■ $CaCl_2$; △ $SrCl_2$; ▼ $BaCl_2$.

5.8, 6.3; experimental, 6.0, 6.4). The background ATPase could not be suppressed even with 10 mm EDTA or EGTA. Hence the probability that the ATPase was actually stimulated by Ca^{2+} was analyzed statistically by the Wilcoxon rank order test (35) and found to be significantly different from zero ($p < 0.01$).Above the optimal Ca:Mg:ATP ratio, calcium inhibited both the low-Ca-ATPase and the Mg-ATPase activities, and the presence of Mg prevented the rise of the high-Ca-ATPase.

Experiments on the effects of monovalent cations have been done, and show that isotonic Li, Na, K, and tris-chloride do not stimulate ATPase activity in the absence of divalent cations, or in the presence of $MgCl_2$, or at any concentration of $CaCl_2$ up to 10 mM. However, all of the above cations except Li stimulate the high-Ca-ATPase (at 60–100 mM Ca) by about 70%, in agreement with the results of Avissar et al. (13).

DISCUSSION

The high-Ca-ATPase of spectrin, as observed in these studies, corresponds in level of activity to the activity observed by Rosenthal et al. (10) and Clarke and Griffith (11), and similarly this activity is inhibited by Mg^{2+}. Both groups found that calcium stimulation of ATPase activity leveled off above 2 mM Ca^{2+}. Our curve is also flat in the vicinity of 1–5 mM $CaCl_2$, due to superposition, at 1 mM ATP, of the decline of the low-Ca-ATPase and the rise of the high-Ca-ATPase. Since none of the calcium levels observed to stimulate spectrin Ca-ATPase activity are physiologically reasonable, it is premature to assign a functional role to these activities of spectrin.

Because the low-Ca-ATPase is not inhibited by Mg^{2+}, and the Mg^{2+}-ATPase is not inhibited by low levels of Ca^{2+}, these two activities could be interpreted as the actions

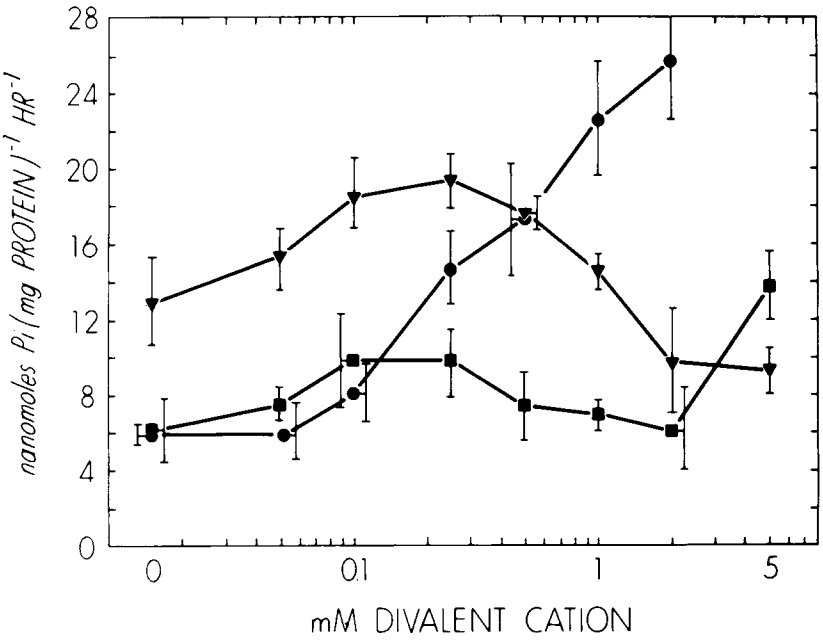

Fig. 6. Spectrin ATPase activity with calcium, magnesium, and calcium plus magnesium, at 0.25 mM ATP. Conditions are as in Fig. 3, except ATP concentration is 0.25 mM. Lesser ATP concentrations show essentially this pattern, while ATP concentrations greater than 0.25 mM show increasing overlap of the stimulation of ATPase by calcium, seen here at 0.25 mM and at 5 mM. Standard deviation of means of 6–8 experiments. ■ $CaCl_2$; ▼ $CaCl_2$ in presence of 0.25 mM $MgCl_2$; ● $MgCl_2$.

of different ions on the same site. However, there have been several recent reports (36–38) that each of the two bands of spectrin (on SDS gels) has several N-terminal amino acids and are immunochemically (39) and isoelectrically (40) heterogeneous. It is possible that spectrin could contain several kinds of peptides, each having a different ATPase activity. This hypothesis is supported by our recent observation that the low-Ca-ATPase is absent in hereditary spherocytosis, while the Mg-ATPase and high-Ca-ATPase are normal (41). In addition, preliminary experiments indicate that it is possible to separate the Ca-ATPase and Mg-ATPase activities of spectrin (Woods and Kirkpatrick, unpublished observations).

Smaller peptides seem normally to be associated with enzymatically active spectrin fractions, as shown by Clarke (6) and Avissar et al. (13), as well as by our results. The major identifiable peptides, band 5 and hemoglobin, can be removed without any effect on spectrin ATPase activity. However, the other peptides cannot be separated from the high-molecular weight bands under nondenaturing conditions. We have attempted separation using isoelectric precipitation, ammonium sulfate fraction according to Marchesi et al. (3), and gel permeation chromatography on Sephadex G-200 (3), Biogel P-300 (6), and Glycophase G-250. It is our opinion that these peptides are primarily the result of the action of proteases, extracted from the membrane fraction (32), on isolated spectrin. This hypothesis would explain the observed variability, in size and amount, of these peptides. The same hypothesis would also account for the large number of N-terminal amino acids and immunochemical and isoelectric components seen in these fractions by other workers

(36–40). The large number of components observed seems to make the "subunit" hypothesis (38) unlikely. However, the limited number of peptides seen by Clarke (6) and the results of Avissar et al. (13) may indicate that some minor peptides may play a role in regulation of spectrin ATPase activity. Since we have not been able to separate the ATPase activity from the high-molecular weight peptides, we attribute the ATPase activity to these peptides; however, attempts to fractionate spectrin are continuing. It should be noted that spectrin ATPase never exhibits calcium stimulation of a magnesium-dependent activity. Since such stimulation is a good diagnostic test for the "calcium pump" ATPase of red cells, we agree with Schatzmann's recent assessment (42) that spectrin ATPase is not related to the calcium pump enzyme.

It is possible that spectrin ATPase activity reflects turnover of phosphoprotein. Recent work has shown (43, 44) that a protein kinase activity is associated with the water-soluble proteins of erythrocyte membranes, and this activity has cation requirements similar to the low-Ca-ATPase described here. The two systems may be identical, with the liberated phosphate representing turnover of phosphoprotein, but further work is needed to test this hypothesis.

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