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MODULATION OF THE CALCIUM-TRANSPORT ATPase IN HUMAN ERYTHROCYTES BY ANIONS

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Certain polyanionic agents, such as poly(L-aspartic acid) (M_r = 20000) and poly(L-glutamic acid) (M_r = 20000 and 26000) were shown to increase the Ca^{2+} sensitivity of (Ca^{2+} + Mg^{2+})-ATPase activity in erythrocytes by converting the biphasic (high and low affinity) Ca^{2+} activation observed in Tris-maleate buffer to a predominantly hyperbolic Ca^{2+} activation of high affinity. Aspartic acid and glutamic acid, however, were ineffective. The enzyme also exhibited a single high Ca^{2+} affinity state in the presence of the alicyclic sulfonic acids, sodium Hepes (55 mM) or sodium Mes (55 mM), but not with the aliphatic sulfonic acid, sodium Tes (55 mM). This effect of both the poly(L-carboxylic acid)s and the sulfonic acid buffers did not require the presence of an intact membrane, as it was also observed in a Triton X-100 solubilized (Ca^{2+} + Mg^{2+})-ATPase preparation. In contrast to the polyanions and the sulfonic acids, the anionic Ca^{2+} binding protein, calmodulin, increased the maximum velocity of the enzyme as well as its Ca^{2+} sensitivity. Certain aromatic carboxylic acids (benzoic acid and salicyclic acid) also increased the Ca^{2+}-sensitivity of the (Ca^{2+} + Mg^{2+})-ATPase, whereas a variety of inorganic anions tested were ineffective at 66 mM concentrations. Trifluoperazine (30 μM) antagonized activation of the enzyme by calmodulin and poly(L-asparatic acid), but not by sodium Hepes or sodium Mes. The activation by poly(L-carboxylic acid)s or sodium Hepes was not additive to a similar activation produced by trypsin. These results suggest that the modulation of the Ca^{2+} sensitivity of (Ca^{2+} + Mg^{2+})-ATPase by calmodulin is associated with its anionic properties, and that this property can be mimicked by other anions, probably by interaction at an anion-regulatory site on the enzyme.

Introduction

The ATP-driven calcium pump in plasma membranes has been implicated in maintaining low intracellular calcium concentrations in many excitable and non-excitable cells [1]. The Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase ((Ca^{2+} + Mg^{2+})-ATPase) is the biochemical expression of the calcium pump, which uses energy from ATP hydrolysis for active calcium transport [2]. Both (Ca^{2+} + Mg^{2+})-ATPase activity and calcium transport are stimulated by the anionic protein, calmodulin, found in the cytosol of the red blood cell [3]. Much attention has been recently directed to the calcium sensitivity of the calcium pump ATPase and its modulation by calmodulin [4]. Thiry et al. [5] and Gagnon et al. [6] reported that

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-*N*-(morpholino)ethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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amidation or methylation of carboxyl groups on calmodulin reduce the activation properties of calmodulin on phosphodiesterase, suggesting that carboxyl groups of calmodulin have an important functional role in the control of phosphodiesterase activity. Recently, we found that carboxymethylated calmodulin failed to activate the $(Ca^{2+}+Mg^{2+})$-ATPase of human erythrocyte membranes (Al-Jobore, A., Roufogalis, B.D. and Gagnon, C., unpublished observation). These findings prompted us to investigate further the role of free carboxyl groups and other anions in the modulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity. Our results indicate that certain anionic and polyanionic compounds modulate the $(Ca^{2+}+Mg^{2+})$-ATPase activity, primarily by affecting the sensitivity of the $(Ca^{2+}+Mg^{2+})$-ATPase to calcium.

Materials and Methods

Human blood, not more than 4 days old, was obtained from the Canadian Red Cross blood bank. Erythrocytes, collected by centrifugation at $2500 \times g$ were washed three times with isotonic sodium phosphate buffer (pH 7.4) and then lysed and washed by the procedure of Dodge et al. [7]. The ghosts were stored at −20°C and used within 4 days. $(Ca^{2+}+Mg^{2+})$-ATPase was solubilized from calmodulin-deficient ghosts by Triton X-100 (1 mg/mg protein), as described by Carafoli et al. [8]. $(Ca^{2+}+Mg^{2+})$-ATPase activity was assayed in ghosts and solubilized preparations by following the rate of $^{32}P_i$ release from [γ-^{32}P]ATP, as described previously [9,10]. The assay medium contained (unless indicated otherwise) 66 mM NaCl, 6.5 mM $MgCl_2$, 0.1 mM ouabain, 0.1 mM EGTA, various concentrations of free calcium and 55 mM of either Tris, Hepes, Mes or Tes adjusted to the required pH (pH 6.9 or 7.2) in the assay medium at 37°C by addition of maleate (Tris-maleate), HCl (Tris-HCl) or sodium hydroxide (sodium-Hepes, sodium-Mes, sodium-Tes and sodium maleate). The effects of poly(L-carboxylic acid)s (sodium as counterion) were studied in Tris-maleate buffer and in the presence of NaCl (66 mM). Free Ca^{2+} concentrations were calculated by solving a series of quadratic equations, as described previously [11], and checked directly by a calcium selective electrode at the appropriate pH (6.9 or 7.2, see legends), as previously described [12]. Dodge 'ghosts' (0.18 mg protein) or solubilized $(Ca^{2+}+Mg^{2+})$-ATPase (0.02 mg protein) were assayed with 2 mM ATP (disodium salt) for 60 min ('ghosts') or with 0.5 mM ATP (Tris salt) for 10 min (solubilized ATPase) at 37°C. Purified human erythrocyte calmodulin (0.6 μg/0.6 ml) (kindly supplied by Dr. Fred Larsen) or other anions and poly(L-carboxylic acid)s were added to the enzyme preparation 10 min prior to starting the reaction with ATP. The results shown are typical of at least three similar experiments.

All chemicals were of the highest purity available. Anions (sodium salts) and poly(L-carboxylic acid)s were purchased from Sigma. The poly(L-carboxylic acid)s were dialyzed against 5 mM EDTA, 10 mM Tris-maleate (pH 7.5 at room temperature) and then in EDTA-free medium, before assay. The calcium contamination of anions and polyanions was tested by atomic absorption spectrophotometry. In all compounds tested the total calcium in stock solutions was less than 4 μM (representing 10^{-8} M or less free Ca^{2+} in the assay medium); this level of Ca^{2+} did not increase the basal Mg^{2+}-ATPase activity.

Results and Discussion

Calcium activation of $(Ca^{2+}+Mg^{2+})$-ATPase in erythrocyte ghosts was biphasic, occurring over three logarithmic cycles, when the enzyme activity was assayed in 55 mM Tris-maleate, pH 7.2 (Fig. 1). The kinetics of calcium activation are described by assuming a mixed population of high and low calcium affinity states of the enzyme [12]. The $(Ca^{2+}+Mg^{2+})$-ATPase was activated at lower calcium concentrations and shifted to a form showing hyperbolic Ca^{2+} activation of high affinity in the presence of poly(L-aspartic acid) (M_r = 20000) and poly(L-glutamic acid)s (M_r = 20000 and M_r = 26000) (Fig. 1). In an attempt to delineate the mechanism and kinetics of the activation by these polyanions, we studied their effects on Triton X-100 solubilized $(Ca^{2+}+Mg^{2+})$-ATPase. It was shown that poly(L-aspartic acid) (M_r = 20000) and poly(L-glutamic acid)s (M_r = 20000 and 26000) increased the detergent solubilized $(Ca^{2+}+Mg^{2+})$-ATPase activities by affecting the sensitivity of the pump for Ca^{2+} (Fig. 2).

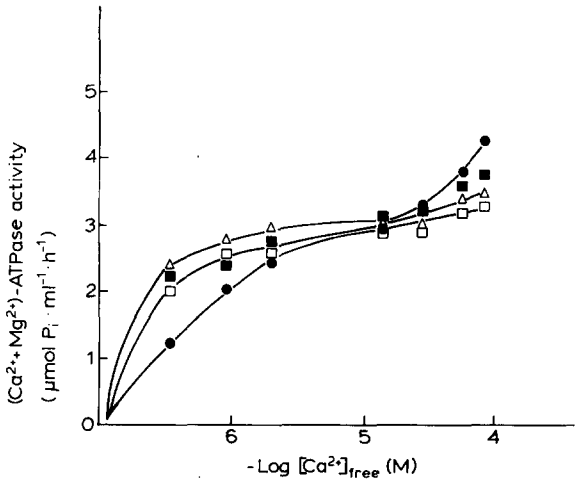

Fig. 1. Effect of poly(L-carboxylic acid)s on Ca^{2+} activation of human erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPase activity. The enzyme activity was measured in a medium containing 55 mM Tris-maleate, pH 7.2, 0.1 mM EGTA, 0.1 mM ouabain, 6.5 mM $MgCl_2$, 66 mM NaCl, 2 mM ATP and various concentrations of free calcium as indicated. Enzyme activity was measured in the absence (●) and in the presence of 81 μM poly(L-aspartic acid), $M_r = 20000$ (△), 57 μM poly(L-glutamic acid), $M_r = 20000$ (□), and 44 μM poly(L-glutamic acid), $M_r = 26000$ (■).

An increase in the apparent calcium sensitivity of ($Ca^{2+} + Mg^{2+}$)-ATPase was also observed in the presence of certain sulfonic acids, including sodium-Hepes (55 mM) or sodium-Mes (55 mM)

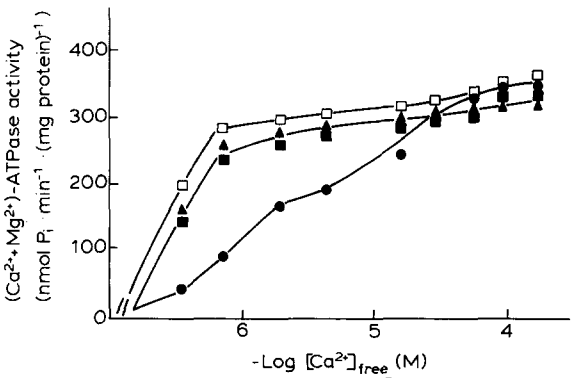

Fig. 2. Effect of poly(L-carboxylic acid)s on Ca^{2+} activation of Triton X-100 solubilized ($Ca^{2+} + Mg^{2+}$)-ATPase from human erythrocyte membranes. The enzyme activity was measured in the absence (●) and in the presence of 81 μM poly(L-aspartic acid), $M_r = 20000$ (▲), 57 μM poly(L-glytamic acid), $M_r = 20000$ (□), and 44 μM poly(L-glutamic acid), $M_r = 26000$ (■). The assay medium was identical to that in Fig. 1 except that the concentration of ATP was 0.5 mM and the assay time 10 min.

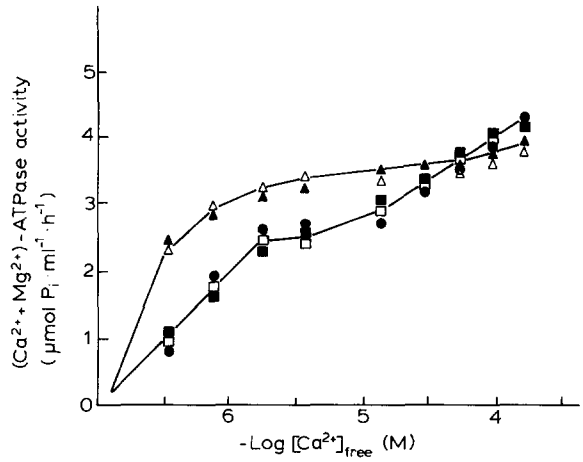

Fig. 3. Effect of sulfonic acid anions on the Ca^{2+} activation of ($Ca^{2+} + Mg^{2+}$)-ATPase in human erythrocyte membranes. Ghosts were assayed in 55 mM Tris-maleate buffer (●), 55 mM Tris-HCl (□), 55 mM sodium maleate (■), 55 mM sodium Hepes (△) and 55 mM sodium Mes (▲). The pH of the assay mixtures was 6.9 at 37°C. The assay mixture contained the buffer (as indicated), 0.1 mM EGTA, 0.1 mM Ouabain, 66 mM NaCl, 6.5 mM $MgCl_2$, and various concentrations of free calcium.

in erythrocyte ghosts (Fig. 3). In the absence of sulfonic acids non-hyperbolic Ca^{2+} activation of the enzyme was observed in 55 mM Tris-maleate, 55 mM Tris-HCl or 55 mM sodium maleate, pH 7.2 (Fig. 3). The sulfonic acids also increased the Ca^{2+} sensitivity of the Triton X-100 solubilized enzyme (Fig. 4). In Tris-maleate buffer the Ca^{2+} activation was best fitted to the equation for biphasic Ca^{2+} activation described previously [12] and in Table I, with a K_d of 2.2 ± 0.7 μM for the high Ca^{2+} affinity component and a K_d of 40 μM or more for the low Ca^{2+} affinity component. In sodium-Hepes or sodium-Mes (55 mM in anion) the Ca^{2+} activation was best fitted by a single hyperbolic high Ca^{2+} affinity component ($K_d = 0.35 \pm 0.08$ μM). Fig. 4B shows the fit of the data by a computer-generated nonlinear curve fitting program, in the form of Eadie-Hofstee plots. As in ghosts, the maximum velocity obtained was not significantly different in Tris or sulfonic acid buffers. The addition of calmodulin (0.6 μg/0.6 ml assay medium) in the presence of sodium-Hepes buffer increased the maximum velocity of the ($Ca^{2+} + Mg^{2+}$)-ATPase without further affecting its apparent Ca^{2+} affinity ($K_d = 0.3 \pm 0.04$ μM)

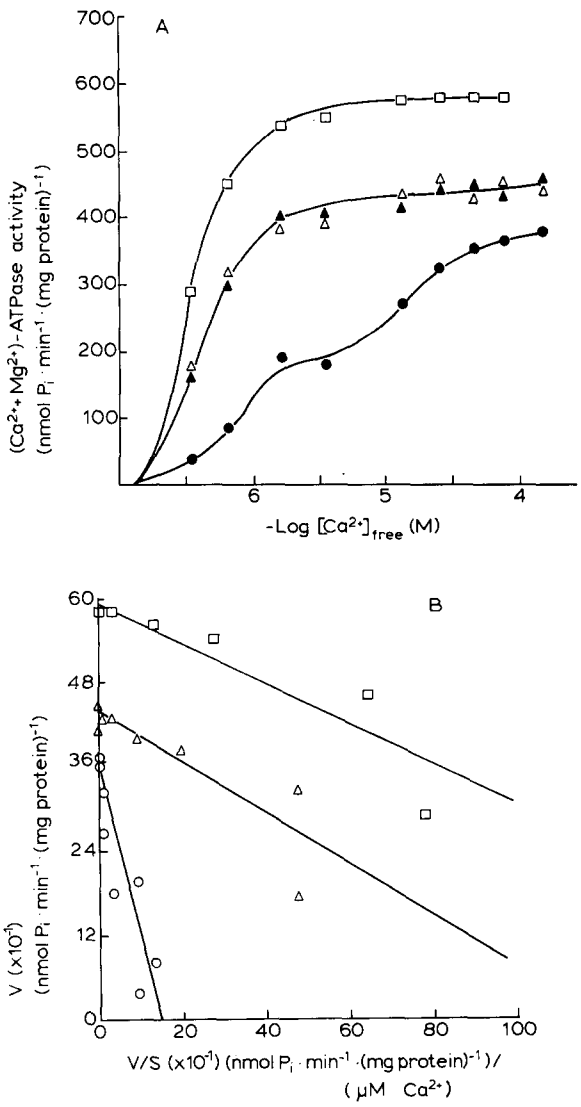

Fig. 4. Effect of sulfonic acid anions in the presence and absence of calmodulin on Ca^{2+} activation of $(Ca^{2+}+Mg^{2+})$-ATPase in Triton X-100 solubilized $(Ca^{2+}+Mg^{2+})$-ATPase from human erythrocyte membranes. (A) The solubilized enzyme was assayed in 55 mM Tris-maleate buffer (●,○), 55 mM sodium-Hepes (△), 55 mM sodium-Mes (▲) and 55 mM sodium-Hepes plus 0.6 μg calmodulin/0.6 ml (□). The pH of the assay mixture was 7.2 at 37°C. The other components of the assay mixture were identical to those shown in Fig. 3. In (B), the curves were fitted by a nonlinear curve-fitting computer program (see text and Table I).

(Figs. 4A and 4B). This is in contrast to the effect of calmodulin in Tris-maleate buffer, which increases both the apparent Ca^{2+} affinity and the maximum velocity of $(Ca^{2+}+Mg^{2+})$-ATPase, both in ghosts and solubilized $(Ca^{2+}+Mg^{2+})$-ATPase preparations [12].

The shift in Ca^{2+} affinity of the enzyme in the presence of sodium-Hepes or sodium-Mes cannot be attributed to a change in the concentration of sodium (the counter ion in the sulfonic acid buffers), because no activation was obtained in sodium maleate buffer at a similar sodium concentration (see Fig. 3). In addition, deletion of NaCl from the assay medium in the presence of Tris-maleate buffer did not alter the biphasic Ca^{2+} kinetic pattern observed in 'ghosts' or solubilized preparations (results not shown). It is also unlikely that the low Ca^{2+} affinity component resulted from the presence of Tris, as it was also seen in sodium maleate buffer. The fact that these anions shifted the Ca^{2+} affinity of solubilized enzyme preparations suggests that they act directly on the Ca^{2+}-transport ATPase complex, rather than by an indirect effect on membrane structure. Furthermore, to test the possibility that polyanions shifted the affinity of the $(Ca^{2+}+Mg^{2+})$-ATPase indirectly by mobilizing tightly bound calmodulin (i.e. calmodulin not removed by hypotonic lysis in the absence of Ca^{2+}), membranes were preincubated with polyanions and washed twice with 20 mM

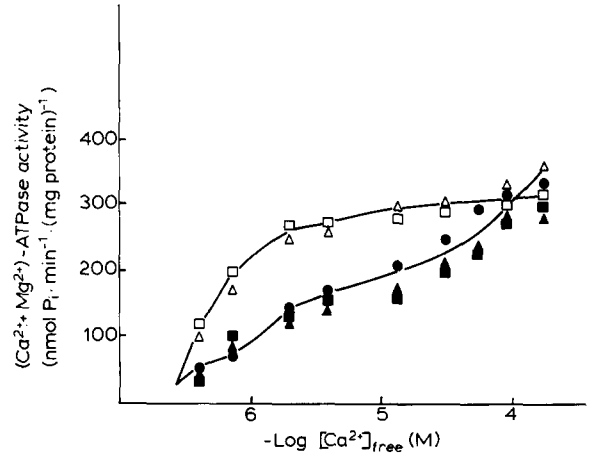

Fig. 5. Effect of inorganic anions and aromatic carboxylic acids on Ca^{2+} activation of Triton X-100 solubilized $(Ca^{2+}+Mg^{2+})$-ATPase from human erythrocytes. $(Ca^{2+}+Mg^{2+})$-ATPase activity was assayed in a medium containing 55 mM Tris-maleate, pH 7.2, 0.1 mM EGTA, 0.1 mM ouabain, 6.5 mM $MgCl_2$, various concentrations of free calcium and 66 mM NaCl (●), 66 mM sodium bicarbonate (■), 66 mM sodium carbonate (▲), 66 mM sodium benzoate (△) or 66 mM sodium salicylate (□).

sodium phosphate, pH 7.4. The washed membranes still retained their sensitivity to polyanion stimulation of $(Ca^{2+}+Mg^{2+})$-ATPase, similar to the untreated membranes (results not shown). It was determined that neither polyanions nor sulfonic acids altered the calcium sensitivity by altering the sensitivity of the enzyme to $MgCl_2$ or ATP (results not shown).

To study the nature of anion affects on the modulation of the Ca^{2+} sensitivity of the calcium transport ATPase, two classes of anions (water structure 'makers' and 'breakers') were used (Table I). Of the range of inorganic and organic anions tested, only the aromatic carboxylic acids, benzoic acid and salicylic acid, increased sensitivity and shifted the biphasic kinetics to a predominantly single high Ca^{2+} affinity state (Table I, Fig. 5). Aliphatic carboxylic and sulfonic acids (sodium gluconate, hippurate, aspartate, glutamate and Tes) were ineffective at the same concentrations (Table I). Similarly, none of the inorganic anions tested increased the Ca^{2+} sensitivity of the enzyme at 66 mM, while F^- and to a lesser extent NO_3^- slightly inhibited the enzyme activity (Table I).

In an attempt to investigate the site and mechanism of action of these anions and polyanions in relation to calmodulin, two types of experiments were performed. Firstly, the enzyme was treated with trypsin. Trypsin treatment of ghosts increased both the maximum velocity and the calcium sensitivity of the $(Ca^{2+}+Mg^{2+})$-ATPase (Fig. 6),

TABLE I

EFFECT OF ANIONS ON THE Ca^{2+} SENSITIVITY OF TRITON X-100 SOLUBILIZED $(Ca^{2+}+Mg^{2+})$-ATPASE

The assay medium contained 55 mM Tris-maleate (pH 7.2 at 37°C), 0.1 mM EGTA, 6.5 mM $MgCl_2$, 0.1 mM ouabain, various concentrations of free calcium and 66 mM in anion, tested as the sodium salts. The kinetics of Ca^{2+} activation were examined over a range of Ca^{2+} concentrations in experiments similar to those shown in Fig. 5. The Ca^{2+} activation was fitted to the equation for biphasic Ca^{2+} activation [12]: $v=((V_1[Ca^{2+}])/(K_1+[Ca^{2+}]))+K_2'[Ca^{2+}]$ where V_1 and K_1 are the maximum velocity and Ca^{2+} dissociation constant of the high Ca^{2+} affinity component and $K_2'=V_2/K_2$ (the corresponding maximum velocity and Ca^{2+} dissociation constant of the low Ca^{2+} affinity component). The criterion used for deciding whether the kinetics fitted the biphasic activation or a hyperbolic function (left hand term of equation only) was the correlation coefficient of the fit (r) and the magnitude of the standard deviation of the estimates of the constants. V_1 is in μmol P_i/h per mg protein and K_1 is in μM. K_2' is the ratio of V_2 and K_2.

Anion	Kinetics of Ca^{2+} activation			
	Ca^{2+} activation	Kinetic constants		
		V_1	K_1	K_2'
Cl^-	Biphasic	322±27	2.2 ±0.7	0.3 ±0.02
Br^-	Biphasic	315±25	2.1 ±0.6	0.32±0.02
I^-	Biphasic	320±26	2.1 ±0.65	0.3 ±0.02
F^-	Biphasic	308±18	2.5 ±0.72	0.27±0.02
SO_4^{2-}	Biphasic	315±26	2.3 ±0.66	0.29±0.02
CO_3^{2-}	Biphasic	318±29	2.1 ±0.65	0.29±0.02
CH_3COO^-	Biphasic	320±26	2.3 ±0.67	0.30±0.03
NO_3^-	Biphasic	305±20	2.6 ±0.78	0.26±0.02
Gluconate	Biphasic	325±26	2.3 ±0.69	0.29±0.03
Hippurate	Biphasic	318±18	2.2 ±0.71	0.31±0.03
Salicylate	Hyperbolic	340±32	0.6 ±0.08	–
Benzoate	Hyperbolic	330±29	0.62±0.08	–
Aspartate	Biphasic	315±23	2.2 ±0.08	0.29±0.02
Glutamate	Biphasic	320±26	2.0 ±0.85	0.28±0.02
Taurine [a]	Biphasic	315±25	2.2 ±0.68	0.30±0.02
Tes [a]	Biphasic	309±18	2.1 ±0.71	0.32±0.02

[a] Taurine and Tes were used at a concentration of 55 mM.

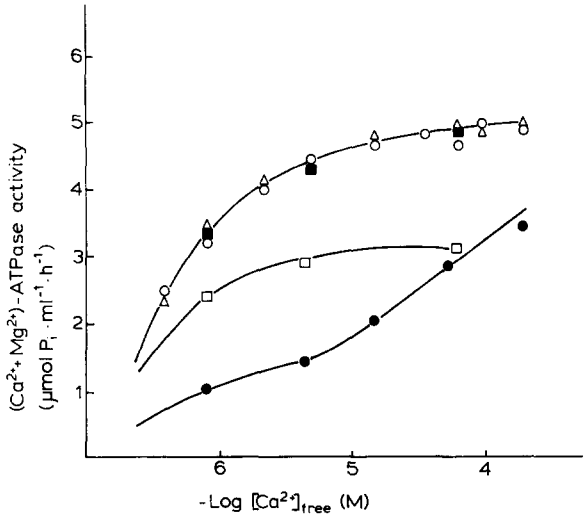

Fig. 6. Effect of poly(L-carboxylic acid)s and sulfonic acids on trypsin treated membranes. $(Ca^{2+} + Mg^{2+})$-ATPase activity of non-trypsin treated (control) membranes was assayed in Tris-maleate buffer (55 mM) (●). Trypsin-treated membranes were assayed in Tris-maleate (55 mM) (○) or sodium-Hepes (55 mM) (△). In the presence of 56 μM poly(L-aspartic acid) ($M_r = 20000$) $(Ca^{2+} + Mg^{2+})$-ATPase activity was assayed before (□) and after trypsin treatment (■) in Tris-maleate (55 mM).

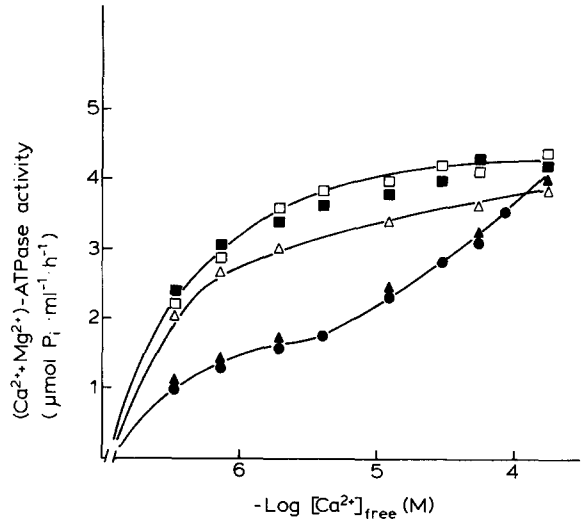

Fig. 7. Effect of trifluoperazine on human erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence of poly(L-carboxylic acid)s or sodium-Hepes buffer. The enzyme activity was measured in sodium-Hepes buffer either in the absence (□) or in the presence of 30 μM trifluoperazine (■). The enzyme was assayed in 55 mM Tris-maleate buffer (●) and in the presence of 56 μM poly(L-aspartic acid) ($M_r = 20000$) without trifluoperazine (△) or with 30 μM trifluoperazine (▲).

probably by cleaving the calmodulin binding site on the $(Ca^{2+} + Mg^{2+})$-ATPase [13–15]. Addition of either poly(L-aspartic acid) or sodium-Hepes to the trypsin-treated membranes failed to further affect the Ca^{2+} sensitivity or activity of the enzyme compared to that in Tris-maleate buffer alone. These results suggest that pretreatment of the membrane with trypsin leads to a change in the enzyme similar to that produced by binding of the anions. However, unlike anions, but similarly to calmodulin, trypsin treatment has the further effect of increasing the maximum velocity obtained. While these results show that the anions tested have similar effects to calmodulin and trypsin treatment, we cannot conclude from the kinetic analysis that the various anions and calmodulin necessarily affect the same site.

Secondly, the effect of trifluoperazine on the activation by anions was examined, since it is known to block the calmodulin activation of $(Ca^{2+} + Mg^{2+})$-ATPase. Whereas 30 μM trifluoperazine blocked $(Ca^{2+} + Mg^{2+})$-ATPase activation by poly(L-aspartic acid), it did not antagonize the effect of sodium-Hepes on the enzyme activity (Fig. 7). It is not known at present if trifluoperazine inhibits the polyanion activation of $(Ca^{2+} + Mg^{2+})$-ATPase by binding to the polyanion through ionic interaction in the absence or presence of calcium or to the enzyme. Until the mechanism of the calmodulin blockade by trifluoperazine is established, it is not possible to conclude whether the increased Ca^{2+} sensitivity of the $(Ca^{2+} + Mg^{2+})$-ATPase by poly(L-carboxylic acid)s and sulfonic acids occurs by the same or a different mechanism.

These studies suggest that distinct structural features of calmodulin may contribute to the increase in the Ca^{2+} sensitivity and maximum velocity of $(Ca^{2+} + Mg^{2+})$-ATPase. The results obtained with polyanions and some of the other anions examined suggest that the acidic nature of calmodulin may be involved in altering the Ca^{2+} sensitivity (K activation). The mechanism of K activation by the anions may be through allosteric modulation of the enzyme conformation to a high Ca^{2+} affinity state, by affecting a regulatory site on the enzyme. It was proposed that calmodulin and phosphatidylserine bind to overlapping but

distinct regions on the regulatory site, thereby altering its conformation, and removing the constraint on Ca^{2+} activation of the $(Ca^{2+} + Mg^{2+})$-ATPase [16]. The ability of calmodulin to also increase the maximum velocity (V activation) may be due to its hydrophobic nature, as demonstrated by similar (K + V) activation by certain acidic phospholipids [15,16] which are both negatively charged and hydrophobic. It remains to be determined if endogenous anions in the red cell affect the state of the calcium pump in vivo.

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