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Effects of chelating agents on the Ca^{2+}-stimulated ATPase of rat liver plasma membranes

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Using strictly controlled ionic conditions we have demonstrated, in agreement with previous findings (Lotersztajn et al. (1981) J. Biol. Chem. 256, 11209–11215; Lotersztajn, S. and Pecker, F. (1982) J. Biol. Chem. 257, 6638–6641) a Ca^{2+}-stimulated ATPase in rat liver plasma membranes which is detectable at low free Mg^{2+} concentrations (normally fulfilled by endogenous levels) but not at free Mg^{2+} concentrations greater than about 10^{-5} M. The findings reported here also suggest that this (Ca^{2+} + Mg^{2+})-ATPase is activated by EGTA or one of its liganded species. Furthermore, this is probably an intrinsic property of the enzyme as it was found to be independent of the isolation technique. The stimulation by EGTA appears to be a function both of free Ca^{2+} concentration and of one or more liganded species of EGTA and it is also inhibited at high free Mg^{2+} concentrations (approx. 10^{-5} M). The specificity of the EGTA effect on ATPase activity is studied with respect to other, widely used, chelating agents namely HEEDTA, EDTA and CDTA. Of these, only CDTA shares the effect, although the concentration dependence of the activation is different from EGTA, suggesting that there is some degree of structural specificity involved rather than a generalised effect of complexed Ca^{2+}.

Introduction

Changes in free cytosolic Ca^{2+} have been invoked as the mediator of the action of certain hormones and other external stimuli on metabolic responses [1–5]. The cytoplasmic free calcium ion concentration is approximately 0.1 μM, which is 10^4-fold lower than that in the extracellular fluid [6,7].

The Ca^{2+} concentrations found in the cytosol are often mimicked in vitro by the use of EGTA as a specific calcium-chelating agent. Experimental findings are generally interpreted on the basis that EGTA or indeed the liganded form of calcium, Ca-EGTA, is biologically inert. However, for example, Sarkadi et al. [8] have described a 100-fold difference in calcium concentrations, half-maximally stimulating calcium uptake by inside-out red cell membrane vesicles, between experiments conducted in media containing unbuffered calcium and in media containing EGTA. Additional findings with red cell vesicles [9–12] and the calcium pump of isolated sarcoplasmic reticulum vesicles [13] show that in at least two well-characterised transport systems either EGTA or a liganded species of EGTA exerts an activating effect on calcium sensitive processes.

In the present study we report such an effect on the (Ca^{2+} + Mg^{2+})-ATPase of rat liver plasma

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HEEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid.

membranes. Furthermore, this is probably an intrinsic property of the enzyme as it was found to be independent of the isolation technique. The specificity of the effect of EGTA in its activation of ATPase activity is studied with respect to other, widely used, chelating agents. Of these, only CDTA shares this effect although the concentration dependence of the activation is different from EGTA. Our observations suggest that the stimulation by EGTA appears to be a function both of free Ca^{2+} concentration (as distinct from total Ca^{2+}) and one or more liganded species of EGTA.

When EGTA is used to buffer the ionic conditions, the free Ca^{2+} concentrations (also the free Mg^{2+} conditions) are directly related to changes in the total added Ca^{2+} (or Mg^{2+}). This simple relationship, however, is not maintained in experiments that involve chelating agents with high affinities for more than one reacting ionic species (notably Ca^{2+} and Mg^{2+} in this case). In such conditions an increase in total added Ca^{2+} (or Mg^{2+}) would release a proportion of the chelated Mg^{2+} (or Ca^{2+}) into the assay media, thereby elevating the concentration of the free species. For example, any stimulation of ATPase activity attributed to an increase in total added $CaCl_2$ may really be caused or enhanced by a concomitant increase in free Mg^{2+} concentration or vice-versa. This is important with $(Ca^{2+} + Mg^{2+})$-ATPases especially as Mg^{2+} appears to have a dual role related to its free concentration in the assay mixture. In the experiments described in this study we have controlled the concentrations of both free Ca^{2+} and Mg^{2+} to avoid this problem.

Several studies [14,15] have shown a low Mg^{2+} requirement of the liver $(Ca^{2+} + Mg^{2+})$-ATPase for Ca^{2+}-stimulation (normally fulfilled by traces of Mg^{2+} present endogenously in the membrane preparation and in nominally Mg^{2+} free solutions) which is contrasted with an inhibitory effect associated with high levels of Mg^{2+} [16]. Further studies on rat kidney basolateral membranes [17] have shown a similar marked decrease in Ca^{2+}-stimulated ATP hydrolysis at high (10^{-5}–10^{-4} M) free Mg^{2+} concentrations. In agreement, this report shows a similar dual effect of free Mg^{2+} and it also goes on to show that the activation of $(Ca^{2+} + Mg^{2+})$-ATPase activity by EGTA is also inhibited at high free Mg^{2+} concentrations.

Experimental

Materials

Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The chelating agents EGTA, CDTA and HEEDTA were obtained from Sigma while EDTA was from BDH, Poole, Dorset, U.K. The Nalgene tubes were supplied by Baird and Tatlock, London, U.K. ATP (disodium salt), including the special quality type (divalent metal free), came from BCL, Lewes, Sussex, U.K. All other reagents were analytical or equivalent grade.

Preparation of plasma membranes

The plasma membrane fraction was prepared from the livers of 350 g male albino Wistar rats. They were killed by decapitation before excision of the liver. Two different membrane preparations were used, as in Refs. 18 and 19. In the latter preparation a slight modification in the flotation step was the use of a SW25-2 swinging bucket rotor instead of the stipulated SW25-1 type. A modification in the Epping and Bygrave [18] preparation involved the use of 18-ml Nalgene tubes, rather than 15-ml Corex tubes to hold the discontinuous Percoll gradient. Both plasma membrane fractions were stored at 4°C and the protein concentration was determined by the method of Lowry et al. [20] using bovine serum albumin as a standard.

Standard ATPase assay

The ATPase activity of the plasma membrane fraction was determined by measurement of P_i release from ATP, at a temperature of 37°C. The incubation mixture contained, in a total volume of 1 ml: 100 mM KCl, 20 mM Hepes/KOH buffer (pH 7.0); various amounts of $CaCl_2$ and $MgCl_2$ and chelating agent (either CDTA, EGTA, EDTA, HEEDTA) to give the required free metal ion concentration and approximately 0.05 mg of protein from the plasma membrane fraction. Following preincubation at 37°C for two min, the reaction was started by the addition of 2.5 mM ATP. After 10 min or 30 min the assay was stopped by addition of 0.5 ml of 5% (w/v) trichloroacetic acid. Centrifugation for two min at $15000 \times g$ removed the precipitated protein and P_i was measured on a 1 ml sample of the supernatant by the

TABLE I

THE LOGARITHM OF THE ASSOCIATION CONSTANTS OF EGTA, CDTA, EDTA, HEEDTA AND ATP WITH H^+, Ca^{2+} AND Mg^{2+}

Ligand complex	Ligand				
	EGTA	EDTA	CDTA	HEEDTA	ATP
H-L	9.46	10.23	11.70	9.72	6.50
HH-L	18.31	16.39	17.82	14.97	10.55
HHH-L	20.99	19.06	21.34	17.01	14.59
HHHH-L	22.90	21.05	23.77	–	18.65
Ca-L	11.00	10.59	12.50	8.14	3.60
CaH-L	14.79	13.74	–	11.10	8.30
Mg-L	5.21	8.69	10.32	5.78	4.00
MgH-L	12.83	12.51	–	11.15	8.50

method of Fiske and SubbaRow [21]. Appropriate blanks were performed in all cases. The results obtained are expressed as micromoles of P_i liberated per mg of protein per assay time. Total Ca^{2+} and Mg^{2+} content of the assay mixtures and reagents was measured, after deproteinising with trichloroacetic acid, by atomic absorption spectrophotometry. Free Ca^{2+} and Mg^{2+} ion concentrations were computed at selected total metal cation concentrations by the method described by Dawson [22] based on the algorithm described by Storer and Cornish-Bowden [23].

Alternatively, the iterative algorithm COMICS (Concentration of Metal Ions and Complexed Species) was used as described by Epping and Bygrave [18] which is based on the method of Perrin and Sayce [24].

Both methods were found to give identical results. Table I shows the association constants of the chelating agents (including ATP) with H^+, Ca^{2+} and Mg^{2+} [25].

Results

Fig. 1 shows the results of an experiment that investigates the plasma membrane ATPase activity at constant free Ca^{2+} concentration as free Mg^{2+} varies. EGTA was used to maintain four different constant free Ca^{2+} levels over the Mg^{2+} range.

The data line at a free Ca^{2+} of 1.2 nM represents basal Mg-ATPase activity only, as at this concentration no significant Ca^{2+}-stimulation is

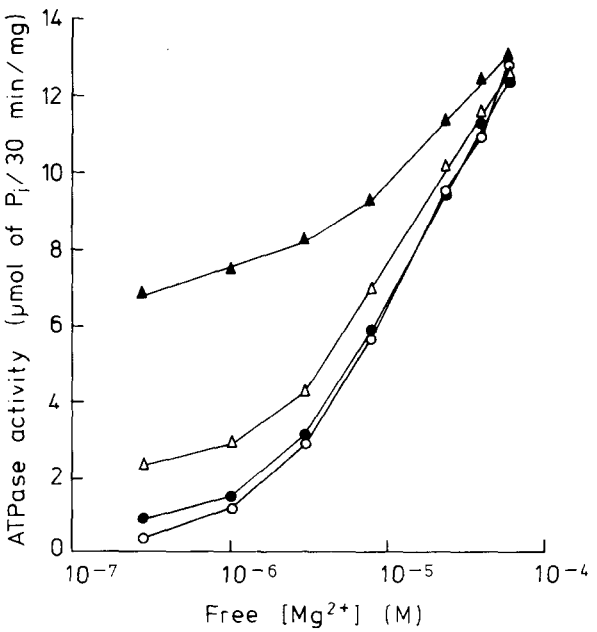

Fig. 1. Effect of Mg^{2+} on plasma membrane ATPase activity using constant free Ca^{2+} concentrations buffered by EGTA. ATPase activities were assayed, as described in the Experimental section, in the presence of four constant free Ca^{2+} concentrations namely, (○) 1.2 nM (corresponding to endogenous, 10 μM, total Ca), (●) 40 nM (0.55 mM total Ca), (△) 200 nM (1.7 mM total Ca) and (▲) 800 nM (2.75 mM total Ca). The free Mg^{2+} range (0.28 μM to 60 μM) corresponded to total Mg from endogenous, 6 μM, to 0.85 mM. The EGTA concentration was 3.5 mM. The protein concentration was 0.04 mg/ml and activity is expressed as μmol of P_i liberated per mg of protein per 30 min.

detected (results not shown). It is clear from the data shown in Fig. 1 that at low (approx. 10^{-7} M) Mg^{2+} concentrations there is stimulation by Ca^{2+}. Increasing Mg^{2+} concentrations increase total ATPase activity, but there is also a progressive decrease in Ca^{2+} stimulation, indicated by the merging of the four lines of data, representing the different constant Ca^{2+} conditions, at a free Mg^{2+} of approx. 60 μM. At this point the ATPase activity of the data line at a free Ca^{2+} concentration of 1.2 nM is similar to that at the largest free Ca^{2+} concentration (800 nM). This implies that at a high free Mg^{2+} concentration (60 μM) the ATPase activity can largely be attributed to the basal Mg^{2+}-ATPase activity as any contribution by Ca^{2+}-stimulated ATPase activity appears to be very small.

To establish whether or not the Ca^{2+}-stimula-

tion was Mg^{2+}-dependent it was necessary to buffer free Mg^{2+} at a lower concentration than that obtained using EGTA and ATP. EDTA with its high affinities for both Ca^{2+} and Mg^{2+} ions was chosen for this purpose. However, the inclusion of EDTA, as compared to EGTA, in the assay mixture brought with it an inherent problem of keeping the free Ca^{2+} constant while increasing the Mg^{2+} concentration. This was overcome by systematically reducing the total added Ca^{2+} in a manner that allowed a constant free Ca^{2+} concentration to be maintained while free Mg^{2+} was increased.

Fig. 2 shows that at a free Mg^{2+} concentration of approx. 60 nM, and a Ca^{2+} concentration of 1.2

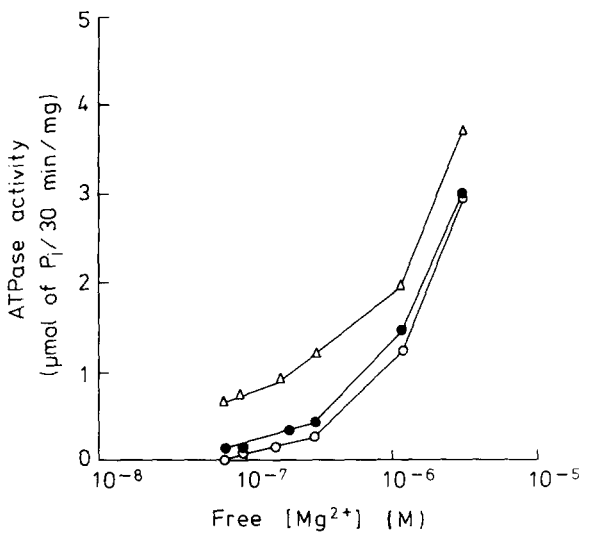

Fig. 2. Effect of Mg^{2+} on plasma membrane ATPase activity using constant free Ca^{2+} concentrations buffered by EDTA. ATPase activities were assayed, as described in the Experimental section, in the presence of three constant free Ca^{2+} concentrations over the given range of free Mg^{2+}. Experimental condititions: (○), free Ca^{2+} 1.2 nM, EDTA 0.82 mM; (●), free Ca^{2+} 40 nM, EDTA 1.4 mM; (△), free Ca^{2+} 200 nM, EDTA 2.55 mM. Constant free Ca^{2+} was maintained by systematically decreasing the total Ca as the total and free Mg^{2+} was increased. (○), free Ca^{2+} constant at 1.2 nM (endogenous, 10 μM, total Ca) as total Mg was increased from 6 μM (endogenous) to 0.4 mM; (●), free Ca^{2+} maintained at 40 nM by systematically decreasing total Ca from 0.6 mM to 0.42 mM while total Mg increased from 16 μM to 0.47 mM; (△), free Ca^{2+} maintained at 200 nM by decreasing total Ca from 2 mM to 1.75 mM while total Mg increased from 11 μM to 0.38 mM. Total protein concentration was 0.048 mg/ml. Activity is expressed as μmol of P_i liberated per mg of protein per 30 min.

nM ATPase activity is eliminated. Furthermore, below this Mg^{2+} concentration, no decrease in ATPase activity is observed at either 40 nM or 200 nM free Ca^{2+} (results not shown). It was not possible, using EDTA to buffer the ionic conditions, to obtain the highest free Ca^{2+} concentration (800 nM) over the stipulated Mg^{2+} range.

Inspection of Figs. 1 and 2 shows that for both EGTA and EDTA the data is qualitatively similar over the same Mg^{2+} range. However, there is a marked difference in the absolute ATPase activity between the two sets of data. It can be seen that under the same free Ca^{2+} and Mg^{2+} conditions, (Mg-ATP values being the same irrespective of the chelator used), greater overall specific activity of the ATPase is found in the presence of EGTA.

These and subsequent experiments were performed on the plasma membrane fraction from the method according to Epping and Bygrave [18] unless otherwise stated in the text.

This apparent enhancement of ATPase activity in the presence of EGTA, compared to EDTA, was investigated further at two different, constant, free Mg^{2+} concentrations (namely, 0.27 μM and 8.0 μM) over a range of free Ca^{2+} (Fig. 3). Ca^{2+}-stimulation is observed at both Mg^{2+} concentrations but particularly at the lower constant free Mg^{2+} concentration when EGTA is used to buffer the Ca^{2+} concentration. However, when EDTA is used (at a similar chelator concentration as EGTA), Ca^{2+}-stimulation is observed at the lower, but not the higher free Mg^{2+} concentration. Even at the lower Mg^{2+} concentration, Ca^{2+} stimulation is decreased compared with that seen in the presence of EGTA.

It is not possible, on the basis of the above data, to distinguish between an inhibitory effect of EDTA and an activating effect of EGTA. This problem was resolved by an experiment designed to give the same free Mg^{2+} and Ca^{2+} conditions in the presence and absence of EDTA. Where EDTA was absent in the assay mixture, ATP was the species that buffered the cations (total ATP was the same whether EDTA was present or absent). The results from this experiment (Table II) show that the ATPase activities obtained in the presence of EDTA are very similar to those obtained in its absence, suggesting the EDTA is not inhibitory, and hence that EGTA activated the enzyme.

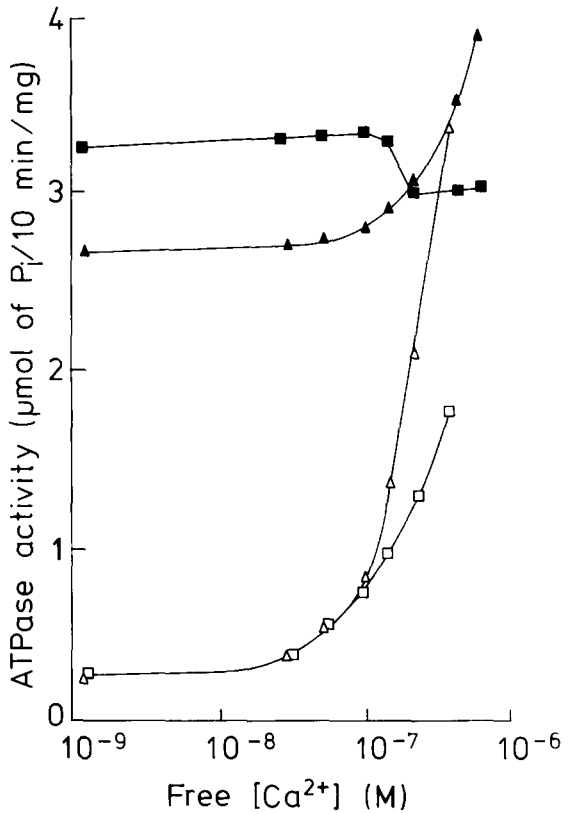

Fig. 3. A comparison of plasma membrane ATPase activity in the presence of EGTA and EDTA. ATPase activities were assayed as described in the Experimental section at two different, constant, free Mg^{2+} concentrations over the given range of free Ca^{2+} using either EDTA or EGTA to buffer free Ca^{2+} and Mg^{2+}. Experimental conditions: (□), free Mg^{2+} 0.27 μM, EDTA 3.5 mM; (■), free Mg^{2+} 8.0 μM, EDTA 2.5 mM; (△), free Mg^{2+} 0.27 μM, EGTA 3.5 mM; (▲), free Mg^{2+} 8.0 μM, EGTA 3.5 mM. For EDTA: (□), free Mg^{2+} maintained at 0.27 μM by decreasing total Mg from 0.23 mM to 0.03 mM as total Ca increased from 0.07 mM to 3 mM; (■), free Mg^{2+} maintained at 8.0 μM by decreasing total Mg from 1.83 mM to 0.49 mM as total Ca increased from 10 μM to 2.0 mM. For EGTA: (△), free Mg^{2+} constant at 0.27 μM (6 μM total) as total Ca was increased from 10 μM to 2.2 mM; (▲), free Mg^{2+} constant at 8.0 μM (0.15 mM total) as total Ca was increased from 10 μM to 2.55 mM. Protein concentration was 0.044 mg/ml for the low Mg^{2+} (□, △) and 0.073 mg/ml for the high Mg^{2+} (■, ▲) condition. ATPase activities are expressed as μmol of P_i liberated per mg of protein per 10 min.

Having established the likelihood of EGTA activation of ATPase activity, the effect of Ca^{2+} and Mg^{2+} were studied at different concentrations of EGTA. Fig. 4 shows the variation in ATPase

TABLE II

EFFECT OF Ca^{2+} ON PLASMA MEMBRANE ATPase ACTIVITY USING CONSTANT FREE Mg^{2+} CONCENTRATIONS BUFFERED IN THE PRESENCE AND ABSENCE OF EDTA

ATPase activities were assayed, as described in the Experimental section, at three constant free Mg^{2+} concentrations namely, 0.024 μM, 1.0 μM and 8.2 μM over a limited free Ca^{2+} range.

Free ionic conditions (concn., μM)		Specific activity (μmol P_i per mg of protein per 10 min)	
Mg^{2+}	Ca^{2+}	− EDTA	+ EDTA
0.024	0.14	–	0.45
0.024	0.16	0.30	–
0.024	0.31	–	0.68
0.024	0.38	0.53	–
1.00	0.15	–	1.40
1.00	0.17	1.52	–
1.00	0.4	1.68	1.64
8.2	0.2	3.47	3.32
8.2	0.41	3.51	3.31
8.2	0.58	3.60	3.28

activity at two constant Ca^{2+} concentrations in the presence of high (3.5 mM) and low (10 μM) total EGTA over a range of free Mg^{2+}. At a free Ca^{2+} concentration of 0.05 μM, Mg^{2+} stimulation of ATPase activity is very similar at both EGTA concentrations. Increasing the Ca^{2+} concentration to 0.5 μM has only a small effect on ATPase activity at 10 μM EGTA, but at 3.5 mM EGTA there is, in agreement with Fig. 1, considerable stimulation of ATPase activity at the lower, but not the higher, Mg^{2+} concentrations. In addition it is seen that at high free Mg^{2+}, regardless of the total added EGTA or Ca^{2+} concentrations in the assay mixture, a similar ATPase activity is obtained. This and subsequent experiments used the special quality type ATP which, being free of divalent metal cations, contributed particularly to lower endogenous levels of Ca^{2+} and Mg^{2+}. It allowed greater control of total ionic conditions in the presence of a low buffering capacity such as 10 μM total chelator concentration.

Fig. 5 shows how the ATPase activity varies with change in free Ca^{2+} under conditions of two constant free Mg^{2+} concentrations at high (3.5 mM) and low (10 μM) total added EGTA. In agreement with the data shown in Fig. 4, there is a

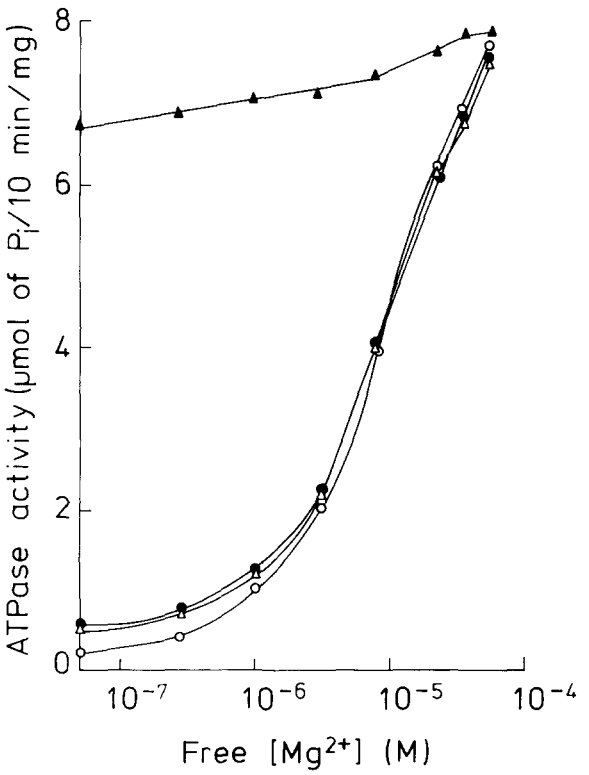

Fig. 4. The effect of Mg^{2+} on plasma membrane ATPase activity at different concentrations of EGTA. ATPase activities were assayed as described in the Experimental section at two different, constant, free Ca^{2+} concentrations over the given range of free Mg^{2+} using either 10 μM EGTA (○, ●) or 3.5 mM EGTA (△, ▲) to buffer free Ca^{2+} and Mg^{2+}. Experimental conditions: (○), free Ca 0.05 μM, EGTA 10 μM; (●), free Ca^{2+} 0.5 μM, EGTA 10 μM; (△), free Ca^{2+} 0.05 μM, EGTA 3.5 mM; (▲), free Ca^{2+} 0.5 μM, EGTA, 3.5 mM. For 10 μM EGTA: (○), free Ca^{2+} constant at 0.05 μM (endogenous, 2.5 μM, total Ca) while total Mg increased from 1 μM (endogenous) to 0.85 mM; (●), free Ca^{2+} constant at 0.5 μM (12 μM total Ca) while total Mg increased from 1 μM to 0.85 mM. For 3.5 mM EGTA: (△), free Ca^{2+} constant at 0.05 μM (0.7 mM total Ca) while total Mg increased from 1 μM to 0.85 mM; (▲), free Ca^{2+} constant at 0.5 μM (2.45 mM total Ca) while total Mg increased over the same range. Due to the weak binding of Mg^{2+} to EGTA, the main Mg^{2+} buffering species under these conditions is ATP. Protein concentration was 0.053 mg/ml and ATPase activities are expressed as μmol of P_i liberated per mg of protein per 10 min.

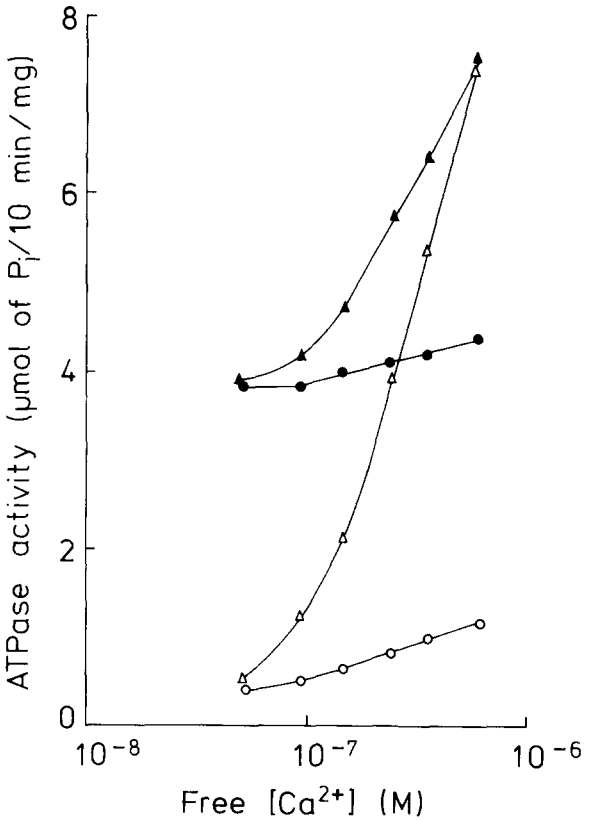

Fig. 5. The effect of Ca^{2+} on plasma membrane ATPase activity at different concentrations of EGTA. ATPase activities were assayed as described in the Experimental section at two different, constant, free Mg^{2+} concentrations over the given range of free Ca^{2+} using either 10 μM EGTA (○, ●) or 3.5 mM EGTA (△, ▲) to buffer free Ca^{2+} and Mg^{2+}. Experimental conditions: (○), free Mg^{2+} 0.27 μM, EGTA 10 μM; (●), free Mg^{2+} 8.0 μM, EGTA 10 μM; (△), free Mg^{2+} 0.27 μM, EGTA 3.5 mM; (▲), free Mg^{2+} 8.0 μM, EGTA 3.5 mM. For 10 μM EGTA: (○), free Mg^{2+} constant at 0.27 μM (5.5 μM total Mg) while total Ca increased from 2.5 μM to 13 μM; (●), free Mg^{2+} constant at 8.0 μM (150 μM total Mg) while total Ca increased from 2.5 μM to 13 μM. For 3.5 mM EGTA: (△), free Mg^{2+} constant at 0.27 μM (5.5 μM total Mg) while total Ca increased from 0.7 mM to 2.6 mM; (▲), free Mg^{2+} constant at 8.0 μM (150 μM total Mg) while total Ca increased from 0.7 mM to 2.6 mM. Due to the weak binding of Mg^{2+} to EGTA, the main Mg^{2+} buffering species under these conditions is ATP. Protein concentration was 0.053 mg/ml and ATPase activities are expressed as μmol of P_i liberated per mg of protein per 10 min.

greater response to increasing Ca^{2+} in the presence of 3.5 mM compared to 10 μM EGTA. A similar shift in Ca^{2+} sensitivity was observed by Al-Jobore and Roufogalis [10] in their work on the (Ca^{2+} + Mg^{2+})-ATPase in erythrocyte membranes, on increasing the total EGTA concentration from 0.1 mM to 3 mM. Furthermore, as in Fig. 3, a greater response to increase in free Ca^{2+} is observed at the lower constant free Mg^{2+}.

The results shown in Figs. 4 and 5 reflect the ATPase activity found in the plasma membrane fraction from the method according to Emmelot et al. [19]. Repeats of those experiments in Figs. 4 and 5 performed on the Epping and Bygrave [18] plasma membrane fraction gave the same information (results not shown). The only difference between the results from the two membrane fractions was a greater overall ATPase activity exhibited by the Emmelot et al. fraction which can be attributed to a greater degree of organelle contamination (evidence from marker enzyme analysis and polyacrylamide gel electrophoresis, results not shown).

The concentration dependence of the activation by EGTA is shown in Fig. 6 as well as a comparison with EDTA over the same concentration range.

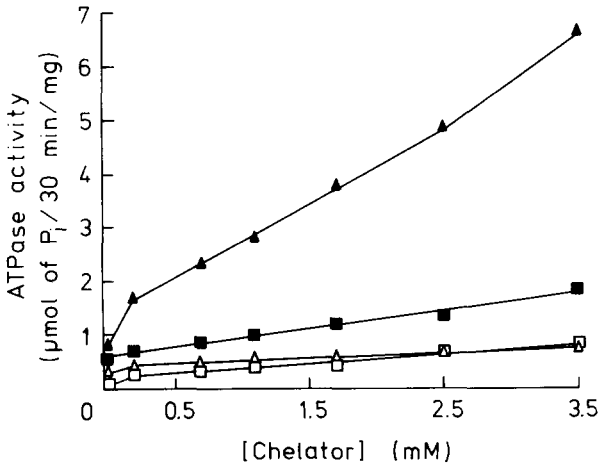

Fig. 6. The concentration dependence of the activation effect shown by EGTA on plasma membrane Ca^{2+}-ATPase activity and its comparison with EDTA. ATPase activities were assayed as described in the Experimental section, at a constant free Mg^{2+} concentration of 0.27 μM. Free Ca^{2+} concentration was: (□, △), 0.05 μM; (■, ▲), 0.5 μM. Chelator was: (□, ■), EDTA; (△, ▲), EGTA. Experimental conditions: (□), free Ca^{2+} 0.05 μM, total Ca varying from 5.5 μM to 1.75 mM with increasing EDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.8 μM to 117 μM. (■), free Ca^{2+} 0.5 μM, total Ca varying from 14 μM to 3.15 mM with increasing EDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.5 μM to 25 μM. (△), free Ca^{2+} 0.05 μM, total Ca varying from 2.5 μM to 0.72 mM with increasing EGTA; free Mg^{2+} 0.27 μM, total Mg 5.5 μM. (▲), free Ca^{2+} 0.5 μM, total Ca varying from 12 μM to 2.5 mM with increasing EGTA; free Mg^{2+} 0.27 μM, total Mg 5.5 μM. Protein concentration was 0.026 mg/ml and ATPase activities are expressed as μmol of P_i liberated per mg of protein per 30 min.

In order to study only the effect of variation in chelator concentration on ATPase activity, a limited set of conditions was kept throughout. This used a constant free Mg^{2+} concentration of 0.27 μM (cf. Fig. 5) while Ca^{2+}-stimulation at a determined chelator concentration was detected by comparison of ATPase activity between 0.05 μM and 0.5 μM free Ca^{2+}. Fig. 6 shows that at the lower free Ca^{2+} neither EDTA nor EGTA have much effect on ATPase activity. However, at the higher Ca^{2+} concentration (0.5 μM) there is a greater degree of stimulation produced in the pres-

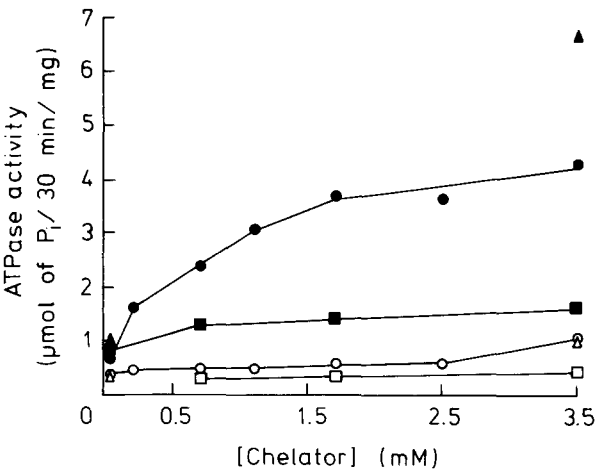

Fig. 7. Effects of CDTA and HEEDTA on plasma membrane Ca^{2+}-ATPase activity. ATPase activites were assayed as described in the Experimental section at a constant free Mg^{2+} concentration of 0.27 μM. Free Ca^{2+} concentration was: (□, ○, △), 0.05 μM; (■, ●, ▲), 0.5 μM. Chelator was: (□, ■), HEEDTA; (○, ●), CDTA; (△, ▲), EGTA. EGTA was included to allow comparison with data in Fig. 6. Experimental conditions: (□), free Ca^{2+} 0.05 μM, total Ca varying from 10 μM to 49 μM with increasing HEEDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.7 μM to 6.6 μM. (■), free Ca^{2+} 0.5 μM, total Ca varying from 7.0 μM to 0.42 mM with increasing HEEDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.5 μM to 6.5 μM. (○), free Ca^{2+} 0.05 μM, total Ca varying from 8.0 μM to 2.55 mM with increasing CDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.7 μM to 92 μM. (●), free Ca^{2+} 0.5 μM, total Ca varying from 15 μM to 3.2 mM with increasing CDTA; free Mg^{2+} 0.27 μM, total Mg varying from 5.5 μM to 160 μM. (△), free Ca^{2+} 0.05 μM, total Ca 2.5 μM and 0.72 mM at 10 μM and 3.5 mM EGTA, respectively; free Mg^{2+} 0.27 μM, total Mg 5.5 μM. (▲), free Ca^{2+} 0.5 μM, total Ca 12 μM and 2.5 mM and 10 μM and 3.5 mM EGTA, respectively; free Mg^{2+} 0.27 μM, total Mg 5.5 μM. Protein concentration was 0.026 mg/ml and ATPase activities are expressed as μmol of P_i liberated per mg of protein per 30 min.

ence of EGTA compared to the condition where EDTA is used. The large stimulation seen in the presence of EGTA increases even though the free Ca^{2+} and Mg^{2+} levels are kept constant throughout. Within the limits of this experiment it was found that this effect shown by increasing EGTA was not saturated at or below a concentration of 3.5 mM.

Fig. 7 investigates two more chelators, namely HEEDTA and CDTA, under the same experimental conditions. For comparison with data in Fig. 6 two concentrations of EGTA were also included (these are shown as isolated triangles in Fig. 7). The results show that at low Ca^{2+} there is little significant difference in ATPase activity in the presence of CDTA and HEEDTA compared with EGTA and EDTA in Fig. 6, all four chelators showing a common profile. However, at the higher level of free Ca^{2+} (0.5 μM) this common profile is no longer seen. HEEDTA and EDTA are both rather ineffective at activating the ATPase activity while CDTA behaves in a rather similar fashion to EGTA, although the activation by CDTA appears to be saturated at total chelator concentrations of greater than 1.7 mM, unlike the situation for EGTA (see above).

Discussion

Rat liver plasma membranes contain a (Ca^{2+} + Mg^{2+})-stimulated ATPase and also a Mg^{2+}-ATPase which appears to be Ca^{2+} independent [14,25]. When studying effects of changing free Ca^{2+} concentration, it is therefore important to ensure that free Mg^{2+} is held constant over the working Ca^{2+} range, and vice-versa if the effect of Mg^{2+} is to be studied. Using these strictly controlled conditions, we have demonstrated, in agreement with previous findings, that rat liver plasma membrane Ca^{2+}-stimulated ATPase is detectable at low [14,15] but not high [16] free Mg^{2+} concentrations. The findings reported here also suggest that the (Ca^{2+} + Mg^{2+})-ATPase of rat liver plasma membranes is activated by EGTA or one of its liganded species. Similar observations have been made by Al-Jobore and Roufogalis [10] on the (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes. Stimulation by EGTA was found to be very similar in plasma membranes prepared both by the method of Epping and Bygrave [18] and by that of Emmelot et al. [19], suggesting that it is an intrinsic property of the enzyme rather than an artefact introduced by a particular isolation technique.

Several reports have suggested that Ca^{2+}/EGTA buffers modify the behaviour of Ca^{2+}-dependent systems by increasing the apparent affinity of Ca^{2+}-binding sites [8,10–13,27] and in some cases without increasing the maximal rate [8,10,12,13]. Because of the great sensitivity of the rat liver plasma membrane Ca^{2+}-ATPase to Mg^{2+} concentrations and the consequent need to control the concentrations of both ions, we were restricted to working over a free Ca^{2+} range where this could be achieved. For this reason we cannot say whether in this case K_m or V_{max} is affected, although it would appear (Figs. 3 and 5) that Ca^{2+} activation occurs over a similar Ca^{2+} concentration range in the presence of either EDTA or EGTA, suggesting primarily an effect in V_{max}.

Stimulation by EGTA appears to be a function both of free Ca^{2+} concentration and of one or more liganded species of EGTA [11,13] rather than total Ca^{2+} concentration [8]. This may be seen in Fig. 5 where at 50 nM Ca^{2+} (free) the activity is the same in the presence of 10 μM EGTA (2.5 μM Ca total) and 3.5 mM EGTA (0.7 mM Ca total). It should be noted that as free Ca^{2+} concentration increases, the concentrations of free Mg^{2+}, free ATP, Mg-ATP and Ca-ATP remain constant. A similar situation is seen in Fig. 3, where a difference between EGTA and EDTA (at the lower free Ca^{2+} concentration) is only observed at free Ca^{2+} concentrations greater than 0.1 μM. In this case total Ca is two to four times higher in the presence of EDTA compared with EGTA, suggesting that total Ca, as distinct from Ca-EGTA, has no influence by itself on the system.

The activating effect of EGTA is also observed with CDTA. The nature of the activation is, however, not clear. There seem to be two main possibilities; firstly, that EGTA (or Ca-EGTA) interacts with a Ca^{2+}-binding site on the enzyme to cause activation [10]; secondly that activation results from the non-specific interaction between EGTA (or one of its liganded states) and the enzyme, or the membrane [12].

Whatever the mechanism involved, the finding that EGTA and CDTA (or their complexes) are activators, while EDTA and HEEDTA are not, suggests that there is some degree of structural specificity involved, rather than a generalised effect of complexed Ca^{2+}. This raises the possibility that EGTA and CDTA may mimic a naturally occurring cytosolic Ca^{2+}-ligand.

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