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CALMODULIN AND THE TARGET SIZE OF THE (Ca^{2+} + Mg^{2+})-ATPase OF HUMAN RED-CELL GHOSTS

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An average target size of 251 kDa has been obtained for the (Ca^{2+} + Mg^{2+})-ATPase of calmodulin-depleted erythrocyte ghosts by radiation inactivation with 16 MeV electrons. This is close to twice the size of the purified calcium-pump polypeptide. When calmodulin was included during the ATPase assay, a component of about 1 MDa appeared in addition to the activated dimer.

In the presence of Ca^{2+}, calmodulin stimulates the (Ca^{2+} + Mg^{2+})-ATPase activity of human red-cell membranes, as well as active Ca^{2+} transport by membrane vesicles [1–3]. It would appear that one calmodulin binds per ATPase polypeptide [4]. The stimulation seems to result from an increase in both the Ca^{2+} affinity and the maximal turnover rate of the enzyme [5,6]. Furthermore, the dependence of the activity on the ATP concentration has been found to change, and it has been suggested that the disclosure of low-affinity ATP sites accompanies calmodulin activation [7].

The purpose of the present study has been to determine the molecular size of the red-cell (Ca^{2+} + Mg^{2+})-ATPase in the intact red-cell membrane and to find out whether the effects of calmodulin are associated with different states of aggregation of the enzyme. Radiation inactivation by 16 MeV electrons has been used in order to assess the target size of the particle. Freeze-dried samples of calmodulin-depleted ghosts have been irradiated under vacuum. After reconstitution, each sample has been assayed for its (Ca^{2+} + Mg^{2+})-ATPase activity, both with and without added calmodulin. Target sizes have been calculated with the empirical formula of Kepner and Macey [8] from the dose necessary to reduce the enzymic activity to $1/e$ of the control ($dose_{37}$).

The result of one experiment with radiation doses up to 6 Mrad is shown in Fig. 1A. The upper curve shows that the disappearance of the basal (Ca^{2+} + Mg^{2+})-ATPase activity can be adequately described as a single exponential. From the fitted line, a $dose_{37}$ of 2.37 Mrad and a target size of 270 kDa can be calculated. Since the enzyme purified from red cells seems to consist of a single polypeptide of 135–150 kDa [10,11] the present target size suggests that a homodimer would be the species catalyzing hydrolysis in the absence of calmodulin. The result of the assay in the presence of calmodulin, however, shows that more than 60% of the control activity has been lost under 1 Mrad and that only from that point is there a decay that could be described by a single exponential. The target size calculated for this second component is 273 kDa, i.e., similar to that for the calmodulin-depleted membranes.

An experiment to explore the initial part of the

Abbreviations: EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

inactivation curves is shown in Fig. 1B. The data for the depleted membranes have been fitted as a single exponential which gave a target size of 236 kDa. Considering the trend in the experiment of Fig. 1A, the calmodulin-stimulated activities were treated as if they followed a multiple exponential decay. This decay was resolved into two components by drawing a straight line tangentially to the

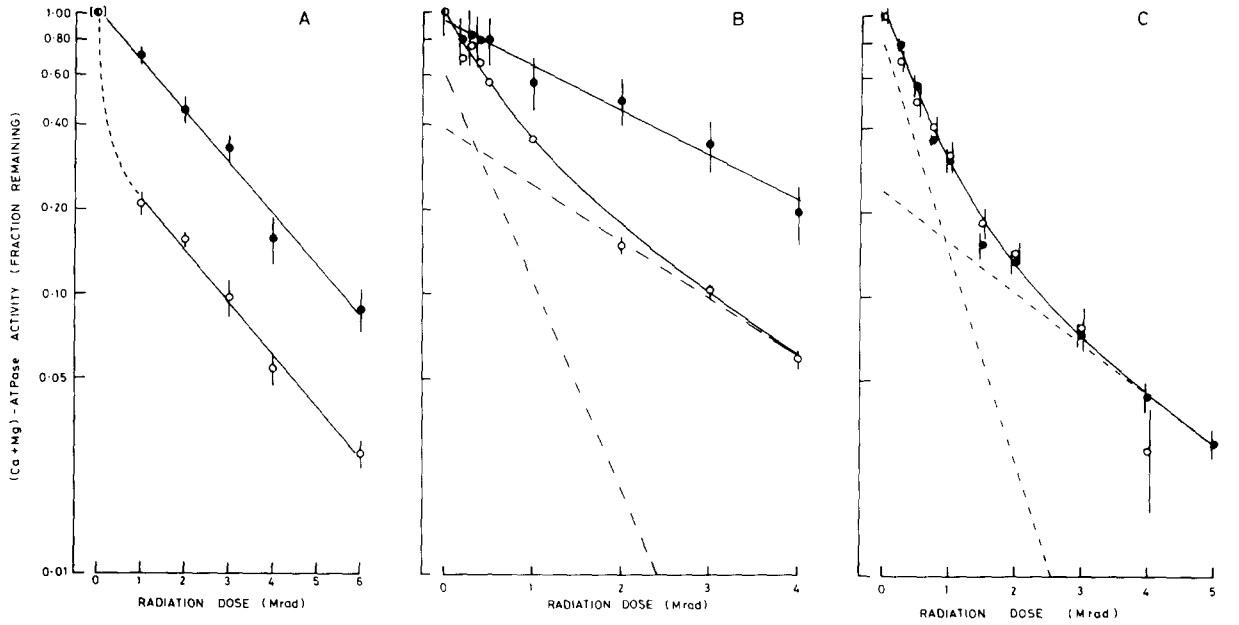

Fig. 1. Inactivation of the red-cell ($Ca^{2+} + Mg^{2+}$)-ATPase by irradiation with 16 MeV electrons at the doses shown (semi-logarithmic plots). Red-cell ghosts were prepared and depleted of endogenous calmodulin according to Table I of Muallem and Karlish [6], except that EGTA was used at 10 mM and that Tris-Hepes (pH 7.4) was at 5 mM together with 0.1 mM $MgCl_2$ ($MgCl_2$/Hepes). The membranes were stored at −20°C. When samples were freeze-dried and reconstituted with cold distilled water, the recovery was 101% for the basal ($Ca^{2+} + Mg^{2+}$)-ATPase activity and 92% for the calmodulin-stimulated activity. Freeze-dried aliquots of ghosts were irradiated with a linear electron accelerator (Addenbrooke's Hospital, Cambridge) under vacuum, at a rate of 2 Mrad/min and at 20–30°C. These samples plus the non-irradiated controls were reconstituted with cold distilled water and diluted conveniently with cold $MgCl_2$/Hepes containing ouabain. Two equal aliquots of each membrane suspension (at a haematocrit of 75% on the original cell volume) were taken. One of them received a calmodulin solution, while the control was made up with a similar volume of distilled water. In addition, they both received enough concentrated $CaCl_2$ to obtain 50 μM Ca^{2+}. The calmodulin, calcium and ouabain concentrations at this stage were 2.5-times higher than those during the ATPase assay. All suspensions were incubated for 5 min at 37°C to accelerate the binding of calmodulin to the membranes. After cooling on ice, the membranes were prepared for the ATPase assay at 2 mM ATP (containing [γ-^{32}P]ATP), 20 μM $CaCl_2$, 1.5 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl, 5 mM Hepes (pH 7.4), 0.1 mM ouabain, with or without calmodulin (7 μg/ml), and with or without 100 μM EGTA, in triplicate initial and final tubes. The total volume was 50 μl and the membranes represented a final haematocrit of 12% on the original cell volume. The finals were incubated at 37°C for periods between 15 min and 4 h; linear time-courses were obtained by keeping the total ATP hydrolysis under 20%. After stopping the reaction by cooling on the ice-bath, the released $^{32}P_i$-label was extracted as the phosphomolybdate complex as described by Brown [9], using 2 mM P_i as carrier. The initial tubes were extracted at the time when the respective finals started their incubation. All activities are expressed as a fraction of that of the controls. All vertical bars indicate ±1 S.E. of triplicate determinations.

(A) ●, depleted membranes; ○, membranes incubated with calmodulin during the ATPase assay. Control ($Ca^{2+} + Mg^{2+}$)-ATPase values (mmol/l original cells per h): depleted membranes 1.01 ± 0.06 (mean ± S.E.); calmodulin-stimulated membranes, 3.58 ± 0.10. Straight lines fitted by linear regression. (B) An experiment to expand the initial part of the curves. Symbols as in (A). The magnitude of the errors on the first four calmodulin-stimulated activities is comprised within the size of the symbols. Control values (mmol/l original cells per h): depleted membranes, 0.77 ± 0.14; calmodulin-stimulated membranes, 7.51 ± 0.11. The uppermost line has been fitted by linear regression, and the curved continuous line represents the sum of the two exponentials shown as discontinuous lines. (C) Test of the effect of free-radical scavengers. Membrane samples were irradiated as described in Fig. 1A in the presence (●) or in the absence (○) of 100 mM sucrose and 10 mM dithiothreitol. The irradiated samples were assayed only in the presence of calmodulin. Control values (mmol/l original cells per h): with additions, 5.69 ± 0.23, no additions, 4.62 ± 0.29. The continuous line represents the sum of the two exponentials drawn as discontinuous lines.

lowest points of the semi-logarithmic plot and fitting the differences with the actual data to a second straigth line. The two exponentials (discontinuous lines) gave target sizes of 297 kDa and 1.06 MDa. Their sum is shown as the continuous lower line. It should be noted that at 3 Mrad, the contribution of the large size component should be not more than 0.42% of the total, and yet the enzyme activities are 0.766 and 0.262 mmol/l cells per h, with and without calmodulin, respectively.

This analysis implies the presence of independent targets: the dimers, the activity of which could be stimulated 3-fold by calmodulin, and one or more components of larger size, which should be present at the moment of irradiation but should become active only in the presence of calmodulin (and which account for most of the stimulated activity in these experiments). Two exponentials seem sufficient to describe the data in this case, but it is possible that a more complex picture could arise from more detailed experiments. An alternative interpretation is that the calmodulin-stimulated activity was the sum of a calmodulin-independent ATPase, represented by the top curve, and another ATPase that operated only in the presence of calmodulin. From a plot based on the difference data, which should represent the assumed calmodulin-dependent $(Ca^{2+} + Mg^{2+})$-ATPase, a target size of 511 kDa could be approximated. Although the possibility of a tetrameric configuration seems appealing, such a plot (not shown) was still bent upwards.

It is possible that the observed curvature was the result of artefacts. Multiple exponentials can be rendered single after including free-radical scavengers in the samples to be irradiated [12]. This possibility was tested by including 100 mM sucrose and 10 mM dithiothreitol before freeze-drying and irradiating. The result is presented in Fig. 1C and shows that this set of samples cannot be distinguished from that without additions, although there is a 25% increase in the activity throughout. It is then unlikely that secondary inactivation of the enzyme can explain the curvature. A double-exponential fitting of the combined data gave target sizes of 266 kDa and 1.09 MDa.

Other possible explanations for the curvature are that the irradiation was greatly reducing the enzyme's affinity for calmodulin or releasing from the samples a material which avidly bound calmodulin, thus competing with $(Ca^{2+} + Mg^{2+})$-ATPase for the activator. To test these alternatives, the effect of a 10-fold increase in calmodulin concentration was investigated. Table I shows the result of this experiment, done at a single dose of 2 Mrad. It is apparent that the higher calmodulin concentration was effective in further increasing the ATPase activity in both control and irradiated samples. It failed, however, to increase the irradiated/control ratio from 0.16 to about 0.5, the characteristic value for the lower slope and the target size of the basal activity. Therefore, an explanation along these lines is also unlikely.

Target sizes obtained in four experiments (including those in Fig. 1) average to 251 ± 10 kDa (mean ± S.E., $n = 3$) for the basal activity, 270 ± 11

TABLE I

INACTIVATION OF RED CELL $(Ca^{2+} + Mg^{2+})$-ATPase BY 2 Mrad IRRADIATION

Lack of effect of a 10-fold increase in the calmodulin concentration on the fractional inactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity by a dose of 2 Mrad. Erythrocyte ghosts were irradiated as described in Fig. 1. Aliquots of the reconstituted membranes were preincubated without calmodulin, or with 7 or 70 μg calmodulin/ml (final concentration in the ATPase assay) and assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity. The figures show the mean ± S.E. of triplicate determinations.

Calmodulin concentration (μg/ml)	$(Ca^{2+} + Mg^{2+})$-ATPase activity (mmol/l original cells per h)		Fraction remaining (irradiated/control)
	Control	Irradiated	
0	0.39 ± 0.05	0.20 ± 0.04	0.51 ± 0.12
7	4.57 ± 0.21	0.71 ± 0.07	0.16 ± 0.02
70	6.52 ± 0.22	1.15 ± 0.09	0.18 ± 0.02

kDa ($n = 4$) for the second component of the calmodulin-stimulated activity and 1.1 MDa for the first component. The slightly higher mean for the second component of the calmodulin-stimulated activity with respect to the basal activity is probably due to chance, but it is in the right direction considering that at least one calmodulin (M_r 16 500) must bind per dimer.

The data strongly suggest that the dimer is the enzymatically active form of $(Ca^{2+} + Mg^{2+})$-ATPase in the red-cell membrane and that this form can be stimulated by calmodulin. There is also a case for an association of $(Ca^{2+} + Mg^{2+})$-ATPase units to other particles or for them forming clusters in the membrane, although the possibility of unforeseen artefacts cannot be ruled out. Besides the active monomer, larger forms of the purified Ca^{2+}-pump polypeptide have been observed in chromatographic runs in the presence of detergents [10]. It is possible that the monomer itself can be active in those conditions, but it may also be that dimerization occurs during the reconstitution of the enzyme before the enzymatic and transport assays.

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