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A high-affinity plasma membrane Ca^{2+}-ATPase in *Dictyostelium discoideum*: its relation to cAMP-induced Ca^{2+} fluxes

Roswita Böhme, Johann Bumann, Susanne Aeckerle and Dieter Malchow

Fakultät für Biologie, Universität Konstanz, Konstanz (F.R.G.)

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Chemotactic stimulation of *Dictyostelium discoideum* induces an uptake of Ca^{2+} by the cells followed by a release of Ca^{2+}. In this study we investigated the mechanism of Ca^{2+} release and found that it was inhibited by La^{3+}, Cd^{2+} and azide. Ca^{2+} release occurred in the absence of external Na^+, indicating that an Na^+/Ca^{2+} exchange was not involved. Plasma membranes contained high- and low-affinity ATPase activities. Apparent $K_{0.5}$ values were 8 μM for the major Mg^{2+}-ATPase and 1.1 μM for the high-affinity Ca^{2+}-ATPase, respectively. The Mg^{2+}-ATPase activity was inhibited by elevated concentrations of Ca^{2+}, whereas both Ca^{2+}-ATPases were active in the absence of added Mg^{2+}. The activities of the Ca^{2+}-ATPases were not modified by calmodulin. The high-affinity Ca^{2+}-ATPase was competitively inhibited by La^{3+} and Cd^{2+}; we suggest that this high-affinity enzyme mediates the release of Ca^{2+} from *D. discoideum* cells.

Introduction

Cells of the cellular slime mold *Dictyostelium discoideum* are chemotactic to folic acid and cyclic AMP (cAMP). cAMP mediates the intercellular communication that leads to aggregation and fruiting body formation (for reviews see Ref. 1). cAMP binds to cell surface receptors (for reviews see Refs. 2–4) and induces a rapid increase in the intracellular cGMP concentration [5,6], a transient uptake and release of Ca^{2+} [7,8], and synthesis and relay of cAMP [2–4].

The release of Ca^{2+} following the cAMP-induced Ca^{2+} uptake could be mediated by Na^+/Ca^{2+} exchange [9] and/or by a plasma membrane-bound ATPase [10]. The findings reported here indicate that a Ca^{2+}-ATPase can account for the Ca^{2+} release.

Correspondence: D. Malchow, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, F.R.G.

Materials and Methods

Materials

cAMP, ATP, oligomycin, luciferin and luciferase were purchased from Boehringer (Mannheim, F.R.G.). Aprotinin, concanavalin A agarose and methyl α-mannoside were obtained from Sigma (München, F.R.G.), and 5 μm pore-size polycarbonate filters from Nuclepore (Pleasanton, CA, U.S.A.). Sodium azide and $CaCl_2 \cdot 2H_2O$, analytical grade, were purchased from Merck (Darmstadt, F.R.G.) and [^{3}H]cAMP (1.52 TBq/mmol) from Amersham Buchler (Braunschweig, F.R.G.).

Culture conditions

Dictyostelium discoideum, strain Ax-2, was cultivated in the presence of 1.8% maltose [11]. Growth-phase cells were harvested at $(3–8) \cdot 10^6$ cells per ml and washed twice in 17 mM Sørensen phosphate buffer (pH 6.0). To allow differentiation to a state of aggregation competence, $(1–2) \cdot$

10^7 cells per ml were shaken for 6–7 h at 150 rpm and 23° C in the same buffer.

Preparation of plasma membranes

150 ml of $5 \cdot 10^7$ cells per ml in 50 mM glycine (pH 8.5) containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 0.08 units/ml aprotinin were lysed by passage through Nuclepore filters [12]. ATP was added to 1 mM to remove myosin [13] and the lysate was centrifuged for 20 min at $5000 \times g$. The pellet, resuspended in 15 ml 50 mM Tris-HCl (pH 7.5) (buffer A), was stirred for 60 min at 4° C with 10 ml concanavalin A agarose beads, which had been washed four times in buffer A. Following aspiration of the supernatant, the beads were washed successively at 4° C with 20 ml buffer A, 15 ml 1 M NaCl in buffer A for 15 min, 20 ml buffer A, and were then eluted with 15 ml 154 mM methyl α-mannoside in buffer A at 23° C for 15 min. This eluate was combined with a further wash of 10 ml buffer A and was centrifuged for 20 min at $38000 \times g$ at 4° C. The pellet was resuspended to a density of 1–3 mg protein/ml. Plasma membrane-bound folate deaminase activity [14] ranged from 60 to 75 nmol per min per mg protein and succinate dehydrogenase activity [15] amounted to 23 ± 14 ($n = 5$) nmol/min per mg protein at 25° C. The membranes were thus enriched 30–40-fold based on the increase in the specific activity of membrane-bound folate deaminase [14]. The specific activity of succinate dehydrogenase activity decreased 6-fold. Membranes were also prepared according to Condeelis and Taylor [16].

ATPase determination

50–200 μg freshly prepared protein were incubated in buffer A with 2.75 μg/ml oligomycin, 1.6 mM ATP, salts and drugs as specified for 10–60 min in a total volume of 300 μl at 25° C. The reaction was started by the addition of ATP. At intervals, 85 μl of the mixture were inactivated with 50 μl of 5% perchloric acid. 100 μl of the cleared supernatant were used to determine the concentration of P_i by colorimetry [17]. Duplicate assays were performed for two different incubation times. The reaction was essentially linear under these conditions. Oligomycin at the concentration used inhibited mitochondrial ATPase activity (not shown). For calculation of Mg^{2+}- and Ca^{2+}-ATPase activities the values obtained in the presence of 1 mM EDTA or 1 mM EGTA, respectively, were subtracted. Free Ca^{2+} and free Mg^{2+} concentrations were calculated as described by Bulos and Sacktor [18] with the association constants given in Refs. 18, 19.

Alternatively, ADP was determined in the neutralized perchloric acid supernatants [20]. In three independent experiments Mg^{2+}-ATPase activity amounted to 68.8 ± 11.4 and 67.4 ± 9.4 ($\pm$S.D.) nmol per min per mg protein for ADP and P_i generation at 1 mM $MgCl_2$, respectively, and Ca^{2+}-ATPase activity to 14.9 ± 3.0 and 15.5 ± 3.5 ($\pm$S.D.) nmol per min per mg protein at 1 mM $CaCl_2$.

Other measurements

Calmodulin was prepared from vegetative cells of *D. discoideum* [21]. Extracellular Ca^{2+} measurements were performed with a Ca^{2+}-sensitive electrode as described [8,22]. The ATP concentration was determined before and after addition of sodium azide to a cell suspension of $2 \cdot 10^7$ cells per ml. 20 μl were withdrawn and were inactivated with 20 μl of 2 M $HClO_4$. After addition of 5 μl of 100 mM EDTA, the sample was neutralized with 8.5 μl 3 M potassium carbonate and centrifuged. Supernatants were analyzed for ATP by the luciferin/luciferase method [23]. cAMP-binding to $5 \cdot 10^7$ cells per ml was measured by incubation for 15 s at room temperature in 5 mM Tricine buffer (pH 7.0) containing 5 mM KCl, 10 nM [^{3}H]cAMP, 10 μM 5′-AMP and 10 mM dithiothreitol. Non-specific binding was determined in the presence of 100 μM cAMP and was less than 0.1%. The cells were separated and counted as described [24].

Results

In aggregation competent cells cAMP induces an uptake of Ca^{2+} followed by a release of Ca^{2+} [7,8]. Both uptake and release are inhibited by 0.3 mM azide (Fig. 1). Ca^{2+} uptake recovered upon continued incubation; Ca^{2+} release also recovered, but much more slowly and to a lesser extent. In separate experiments we found that the reduction in ATP concentration caused by sodium azide was small and fell to $92 \pm 9\%$ and $74 \pm 18\%$ ($n = 4$) of

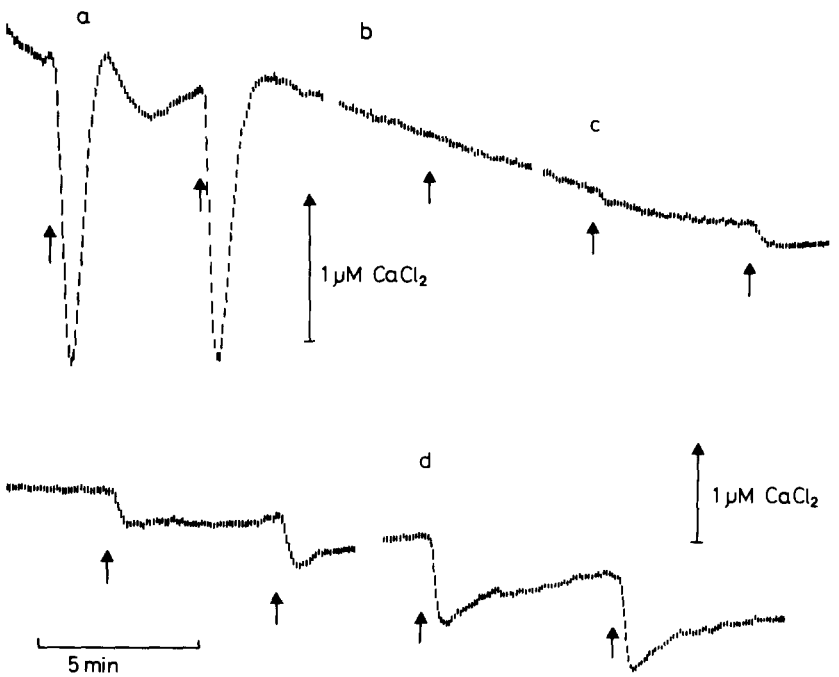

Fig. 1. Azide inhibition of the cAMP-induced uptake and release of Ca^{2+}. The extracellular Ca^{2+} concentration in a suspension containing $1 \cdot 10^8$ cells per ml was recorded with a Ca^{2+}-sensitive electrode [8,22] before (a) and 20 min (b), 30 min (c) or 80 min (d) after addition of 0.3 mM sodium azide. Stimulation of the cell suspension with 0.1 μM cAMP is indicated by the small arrows. Large arrows represent calibration pulses of 1 μM calcium chloride. Note that Ca^{2+} release was absent during the first three pulses of cAMP in (c). The experiment was performed in 5 mM Tricine buffer (pH 7.0)/5 mM KCl with cells starved for 6 h in 17 mM Sørensen phosphate buffer (pH 6.0).

the control level at 0.3 and 0.6 mM azide concentration, respectively. Ca^{2+} release was also blocked by La^{3+} at concentrations where Ca^{2+} uptake was only partially reduced (Fig. 2). Similarly, Cd^{2+} completely inhibited Ca^{2+} release before uptake of Ca^{2+} was blocked (not shown). Under conditions where Ca^{2+} release is absent (Figs. 1 and 2) the time for Ca^{2+} uptake can be determined to last about 30 s.

Neither azide not La^{3+} inhibited cAMP-binding to cell surface receptors. Instead, an increase in binding was observed: 1 mM NaN_3 increased binding by $35 \pm 2\%$ ($n = 3$) and 1 mM La^{3+} by $315 \pm 12\%$ ($n = 2$). A similar enhancement of binding has been published for Ca^{2+} [25,26]. Taken together, these results indicate that the mechanisms of uptake and release of Ca^{2+} after chemotactic stimulation are different.

Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of plasma membranes increased about 30-fold in response to rising Ca^{2+} concentrations (Fig. 3). Two plateaux were reached, at about 0.01 and 2 mM Ca^{2+}. An Eadie-Hofstee plot yielded an apparent $K_{0.5}$ value

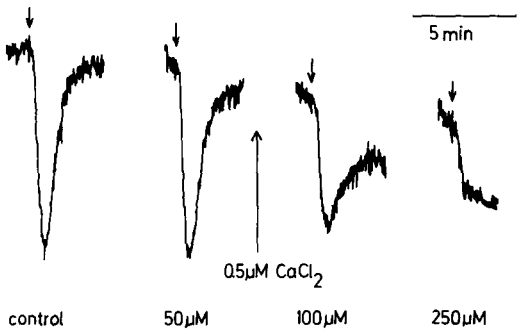

Fig. 2. Preferential inhibition of calcium release by La^{3+}. The extracellular calcium ion concentration was recorded in a suspension of $2 \cdot 10^8$ cells per ml before and after addition of $LaCl_3$. La^{3+} was added to the concentration indicated 2 min before a pulse of 0.1 μM cAMP (small arrow) was applied. The large arrow represents a calibration pulse with 0.5 μM $CaCl_2$. The experiment was performed in 15 mM Tricine buffer (pH 7.5) with cells starved for 9 h.

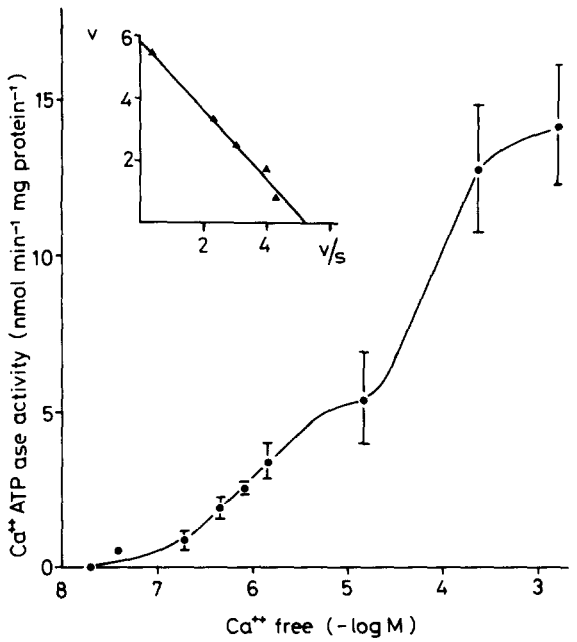

Fig. 3. Dependence of plasma-membrane Ca^{2+}-ATPase activity on Ca^{2+} concentration. Ca^{2+}-EGTA buffers containing up to 1 μM free Ca^{2+} were prepared in 50 mM Tris-HCl (pH 7.2) according to Ref. 18. In the Eadie-Hofstee plot, V is expressed in nmol P_i per min per mg protein; S, the concentration of Ca^{2+}, is expressed in μM (S = 0.02 to 15 μM). The mean ± S.D. of three independent experiments with plasma membranes prepared from vegetative cells is shown.

of 1.1 μM for the high-affinity component. The $K_{0.5}$ value of the low-affinity component was estimated to be about 80 μM. Addition of *D. discoideum* calmodulin (0.2–7.5 μg/ml) did not significantly alter enzyme activity when tested over the range 0.1–100 μM Ca^{2+}.

Plasma membranes from aggregation competent cells exhibited about the same Ca^{2+}-ATPase activity as those from vegetative cells. In aggregative cells we measured 1.9 ± 0.6 and 14.8 ± 5.6 nmol P_i generated per min per mg protein (n = 4) at 1.5 μM and 250 μM Ca^{2+}, respectively.

Both La^{3+} and Cd^{2+} stimulated ATPase activity and appeared to compete with Ca^{2+} for enzyme activity, as deduced from the result that ATPase activities were not additive in the presence of Ca^{2+} and competitor (Table I). 300 μM sodium azide did not significantly alter the high-affinity Ca^{2+}-ATPase activity (92 ± 1% of control, n = 2).

TABLE I

Ca^{2+}-ATPase ACTIVITY

La^{3+} and Cd^{2+} compete with Ca^{2+} for Ca^{2+}-ATPase activity. Enzyme activity of plasma membranes is expressed in nmol P_i per min per mg protein. Total concentrations are given. 10 μM total calcium corresponds to 1.5 μM free Ca^{2+}.

Compound	Concentration (μM)	Activity ± S.D.	n
Ca^{2+}	10	2.15 ± 0.55	4
La^{3+}	300	3.34 ± 1.56	4
$Ca^{2+} + La^{3+}$	10 + 300	2.44 ± 0.85	4
Ca^{2+}	10	1.70 ± 0.30	2
Cd^{2+}	100	4.20 ± 1.02	2
$Ca^{2+} + Cd^{2+}$	10 + 100	3.90 ± 1.55	2

Mg^{2+}-ATPase activity

The Ca^{2+}-pumping ATPase from human erythrocyte membranes requires both Ca^{2+} and Mg^{2+} [27]. Ca^{2+}-ATPase activity in *D. discoideum* occurred in the absence of added Mg^{2+}. In the presence of Mg^{2+} a major ATPase activity with an apparent $K_{0.5}$ value of 8 μM for Mg^{2+} was determined (Fig. 4).

Fig. 5 investigates the question whether Mg^{2+}-ATPase activity is stimulated in the presence of Ca^{2+}. We found a slight activation at low Ca^{2+} concentrations and an inhibition at higher Ca^{2+} concentrations (Fig. 5), in confirmation of an earlier report [28]. Measurement of Ca^{2+}-ATPase activity in the same plasma membrane preparation showed that the increase in ATPase activity, in the presence of Ca^{2+} and Mg^{2+}, can be explained by the activation of the Ca^{2+}-ATPase (Fig. 5). In addition, there may be an elevation of ATPase activity due to the increase in the free Mg^{2+} concentration which results when EGTA is omitted (see legend to Fig. 5). In any case, no net stimulation of Mg^{2+}-ATPase activity by Ca^{2+} is occurring and the extent of inhibition by Ca^{2+} is even greater when the Ca^{2+}-ATPase activity is taken into account (Fig. 5). The data also indicate that Ca^{2+}-ATPase activity is not stimulated by Mg^{2+} since the activity in the presence of both ions does not exceed the sum of either activity alone.

Calmodulin over the range 0.05–5 μg/ml did not alter the extent of inhibition caused by Ca^{2+} (not shown).

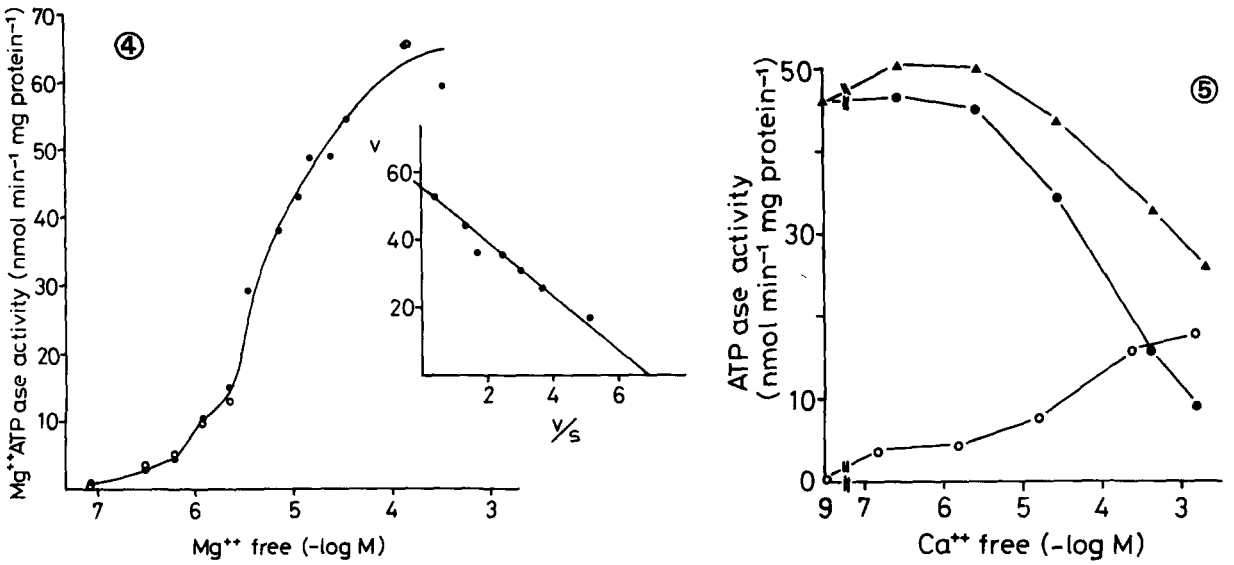

Fig. 4. Dependence of plasma membrane Mg^{2+}-ATPase activity on Mg^{2+} concentration. Mg^{2+}-EDTA buffers were used up to 3 μM free Mg^{2+} [18,19]. In order to calculate V_{max} and $K_{0.5}$ of the major Mg^{2+}-ATPase the activity of the minor component, 12.6 nmol per min per mg protein, was deducted. In the Eadie-Hofstee plot V is expressed in nmol P_i per min per mg protein; S, the concentration of Mg^{2+}, is expressed in μM ($S = 3.3$ to 130 μM). Plasma membranes were prepared from vegetative cells according to Condeelis and Taylor [16] (closed circles), or as described in Materials and Methods (open circles).

Fig. 5. Effect of Ca^{2+} on the activity of plasma membrane Mg^{2+}-ATPase. Plasma membranes were prepared from vegetative cells as described in Materials and Methods. ATPase activity (▲) was assayed in the presence of 1 mM $MgCl_2$ and 0.3 mM EGTA or the indicated Ca^{2+} concentrations. Ca^{2+} ATPase activity (○) was assayed in the presence of the indicated Ca^{2+} concentrations. The closed circles represent ATPase activity in the presence of Mg^{2+} and Ca^{2+} minus Ca^{2+}-ATPase activity. ATPase activity is expressed in nmol P_i generated per min per mg protein. One out of three independent experiments is shown.

Discussion

The cAMP-induced uptake and release of Ca^{2+} seem to proceed by different mechanisms. Ca^{2+} uptake is inhibited by organic Ca^{2+} channel antagonists and is likely to occur via Ca^{2+} channels (Bumann, J., et al., unpublished data). Ca^{2+} release, however, is carried out by a plasma-membrane Ca^{2+}-ATPase. This interpretation is supported by the following results. (1) Ca^{2+} uptake and release were differentially sensitive to azide and La^{3+} (Figs. 1, 2). Azide does not act by significantly reducing the ATP concentration. However, since 0.5 mM azide inhibits the Mg^{2+}-ATPase activity of mitochondria [28], it seems likely that the transfer of high-energy phosphates to the plasma membrane is transiently blocked by azide treatment [29]. (2) La^{3+} inhibited Ca^{2+} release and competed with Ca^{2+} for stimulation of the Ca^{2+}-ATPase activity (Fig. 2, Table I). (3) The activity of the high-affinity Ca^{2+}-ATPase generated 3 nmol P_i per min per mg protein at 1 μM Ca^{2+} (Fig. 3). Considering a 30–40-fold purification of the membranes, this activity would correspond to a transport of $(4.5–6) \cdot 10^6$ calcium ions per cell per min if a ratio of ATP/Ca^{2+} of 1:1 is assumed. The rate of Ca^{2+} release measured was $(3–8) \cdot 10^6$ calcium ions per cell per min (Figs. 1, 2). This close correlation of ATPase activity and Ca^{2+} release rate as well as the high affinity for Ca^{2+} of the ATPase suggest a role for it in regulating the free Ca^{2+} concentration in the cell.

(4) It is known from work on other systems that Ca^{2+} efflux can be mediated either by Na^+/Ca^{2+} exchange [9] or by a specific Ca^{2+}-ATPase [10]. In *D. discoideum*, the reported internal Na^+ concentration is about 5 mM [30,31]. Our measurements are at external Na^+ levels of 0.3–5 mM, much lower than might be expected for a counterion to Ca^{2+}. Also, no reduction in the rate

of Ca^{2+} release was observed, even when the external Na^+ was completely replaced by K^+ (not shown).

All of this suggests that an Na^+/Ca^{2+} exchange, even if it exists, is unlikely to be important under our experimental conditions; rather, Ca^{2+} release seems to depend essentially on the activity of the high-affinity Ca^{2+}-ATPase. In addition to the high-affinity Ca^{2+}-ATPase, the membranes contain a low-affinity Ca^{2+}-ATPase activity (Fig. 3). This enzyme activity could serve in cases where higher loads of Ca^{2+} have to be removed or – as has been suggested for a low-affinity Ca^{2+}-ATPase from erythrocytes [32] – in the regulation of cell shape.

Kinetic data revealed Mg^{2+}-ATPase activity in plasma membranes of *D. discoideum* with high and low affinity (Fig. 4). In other studies, plasma membranes of *D. discoideum* were reported to contain Mg^{2+}-ATPase activity [28,33,34] as well as an ecto-Mg^{2+}-ATPase [35]; however, $K_{0.5}$ values with respect to Mg^{2+} were not determined. Also, an Mg^{2+}-ATPase has been partially purified and shown to pump protons [33]. The ecto-Mg^{2+}-ATPase was suggested to participate in cAMP-independent uptake of Ca^{2+} [35]. We found no significant stimulation of Mg^{2+}-ATPase activity by Ca^{2+} if Ca^{2+}-ATPase activity was subtracted (Fig. 5), and elevated concentrations of Ca^{2+} proved to be inhibitory [28,34].

Thus, in *D. discoideum*, Ca^{2+}-ATPases seem to function in the absence of elevated Mg^{2+} concentrations.

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