REGULATION OF PANCREATIC ISLET-CELL PLASMA MEMBRANE Ca^{2+} + Mg^{2+}-ATPase BY CALMODULIN

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1. Introduction

A Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase (Ca^{2+} + Mg^{2+}-ATPase) activity exists in plasma membrane preparations from a variety of cells [1–7] including pancreatic islets [8]. In the erythrocyte plasma membrane, the Ca^{2+} + Mg^{2+}-ATPase is generally recognized as the enzymatic basis for active or ATP-dependent calcium extrusion. In this membrane, calmodulin has been shown to stimulate both ATP-dependent Ca^{2+} transport [9] and Ca^{2+} + Mg^{2+}-ATPase activity [1,10]. However, apart from the erythrocyte and synaptic plasma membranes of brain [4], it has not been possible to demonstrate a calmodulin-dependent regulation of plasma membrane Ca^{2+} + Mg^{2+}-ATPase activity in other tissues.

High levels of calmodulin have been demonstrated in islet cells [11,14]. Calmodulin has been indirectly implicated in stimulus–secretion coupling by phenothiazine-induced inhibition of insulin release from perifused islets [12], and directly by calcium-dependent activation of cAMP phosphodiesterase [13], adenylate cyclase [11], protein kinase [14], and ATP-dependent calcium transport in plasma membrane vesicles [8]. Here, we report that like ATP-dependent Ca^{2+} transport [8], the Ca^{2+} + Mg^{2+}-ATPase of the islet-cell plasma membrane is regulated by calmodulin. Furthermore, Mg^{2+} appears to be required for activation of the ATPase by calmodulin.

Abbreviations: CPZ, chlorpromazine; TFP, trifluoperazine; FLU, fluphenazine; SDS, sodium dodecyl sulfate; MES, 2(*N*-morpholino) ethane sulfonic acid; Pipes, piperazine-*N*,*N*'-bis (2-ethane sulfonic acid)

2. Materials and methods

Male Wistar strain rats (150–175 g) were obtained from Charles Rivers Labs (Wilmington MA). [γ-^{32}P]-ATP was purchased from ICN Corp. and Omnifluor from New England Nuclear (Boston MA). The phenothiazines, trifluoperazine and fluphenazine were gifts from Dr Harry Green (Smith, Kline and French) and chlorpromazine was obtained from Sigma Chemical Co. (St Louis MO). Calmodulin was prepared from rat brain utilizing fluphenazine agarose chromatography [15,16].

Pancreatic islets were isolated by collagenase digestion [17] and separated on dialyzed Ficoll gradients [18]. Plasma membranes were prepared and characterized as in [19] with the following modifications. Islets were homogenized in an isotonic buffer (50 mM MES, 1 mM EDTA, and 250 mM sucrose, pH 7.2) and nuclei and cell debris were removed by centrifugation at 600 × *g* for 5 min. A membrane particulate pellet obtained by centrifugation in the same buffer at 20 000 × *g* for 20 min, was rehomogenized in hypotonic medium (10 mM MES, 1 mM EGTA, pH 6), layered on a discontinuous sucrose gradient and centrifuged at 150 000 × *g* for 90 min. The plasma membrane-enriched fraction was collected from 1.14 and 1.16 density gradient layers. These fractions were characterized by 10-fold enrichment of 5'-nucleotidase specific activity and 11-fold enrichment of the binding of ^{125}I-labeled WGA compared to the homogenate. The plasma membrane fractions were resuspended and sonicated (Branson Sonifier 200) at 4°C for three 5 s intervals at 18 W, pelleted at 150 000 × *g* for 60 min, resuspended in hypotonic media and frozen

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at –40°C. Protein content was determined by a fluorometric method [20] with modifications as in [21].

The Ca^{2+} + Mg^{2+}-ATPase activity was assayed by monitoring the release of ^{32}P from [γ-^{32}P]ATP in 0.5–1.0 ml reaction vol. The assay medium contained 2 μg protein, 10 mM Tris–Pipes (pH 7.5), 20 mM NaN_3, 2 mM EGTA and 0.25 mM [γ-^{32}P]ATP (1.0 μCi/tube). The assays were run at pH 7.2 for 30 min at 37°C and the reaction was terminated by the addition of 500 μl 3% SDS. Calcium when present was 1.6 or 1.9 mM (equivalent to 0.2 and 1.1 μM free calcium, respectively). The calcium stocks were standardized by atomic absorption spectroscopy and the free calcium levels were determined as in [8] with stability constant for Ca^{2+}-EGTA at pH 7.2 being $10^{7.24}$. Calcium-stimulated ATPase activity was determined by subtracting values obtained with 2 mM EGTA alone (Mg^{2+}-ATPase) from those obtained in the presence of chelator and calcium. Although there is a requirement for μM levels of Mg^{2+} for the expression of Ca^{2+}-stimulated ATPase activity [8] a further increase in Mg^{2+} increases the Mg^{2+}-ATPase activity and produces an apparent reduction in the calcium-stimulated activity. Thus, most studies employed endogenous levels of Mg^{2+} (8.5–9 μM). To establish the Mg^{2+} requirement for calmodulin, ATPase assays were also performed in the presence of 200 μM Mg^{2+} + 1.5 mM ATP. Calmodulin was 2.8–24 μg/ml [168–1440 nM] at 1.1 μM free Ca^{2+}.

A modification of the procedures in [22] was used to aid in removing avidly bound endogenous calmodulin from islet cell plasma membranes. Frozen and thawed membranes were incubated for 30 min at 4°C in the presence of 10 mM Tris–Pipes (pH 7.5), 2 mM EGTA, 1.9 mM $CaCl_2$ and 100 μM TFP. This preparation was then centrifuged at 150 000 × *g* for 60 min, resuspended in 10 mM MES + 1 mM EGTA and used in the Ca^{2+} + Mg^{2+}-ATPase assay.

3. Results

The specific activity of the Mg^{2+}-ATPase determined in the presence of endogenous Mg^{2+} was 10.6 ± 1.6 nm P_i . mg $protein^{-1}$. min^{-1} and the Ca^{2+}-stimulated activity was 106 ± 15 nm P_i . mg^{-1} . min^{-1} in the presence of 0.2 μM free Ca^{2+} (N = 4). The effect of 3 different phenothiazines was evaluated on the Ca^{2+} + Mg^{2+}-ATPase obtained from islet cell plasma membranes. As indicated in fig.1, half-maximal inhibition of the Ca^{2+}-stimulated activity occurred at 75 μM TFP, with maximal inhibition (86%) being observed in the presence of 200 μM TFP. The addition of FLU to the incubation medium also produced a dose-dependent inhibition of the Ca^{2+}-stimulated activity with a similar degree of potency, whereas CPZ was the least effective of the phenothiazines in inhibiting the Ca^{2+} + Mg^{2+}-ATPase activity. Neither TFP, FLU nor CPZ affected the Mg^{2+}-ATPase activity over the range of concentrations evaluated in the absence of exogenous Mg^{2+}.

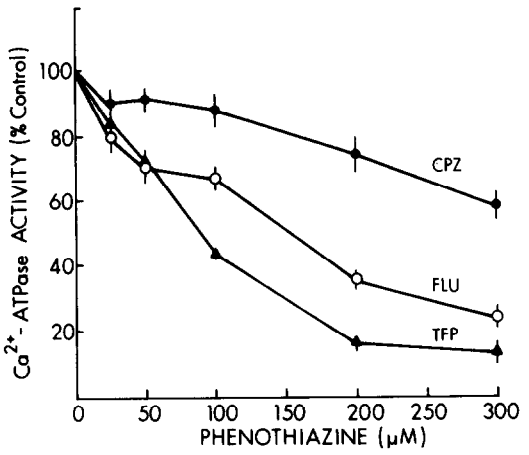

Fig.1. Phenothiazine-induced inhibition of Ca^{2+} + Mg^{2+}-ATPase. Three separate islet-cell plasma membrane fractions were evaluated without added Mg^{2+} for Ca^{2+} + Mg^{2+}-ATPase activity. Incubations (30 min, 37°C) contained chlorpromazine (CPZ, •), fluphenazine (FLU, ○), or trifluoperazine (TFP, ▲) with 0.2 μM free Ca^{2+}. The data represent the mean ± SEM percent of Ca^{2+}-stimulated ATPase in the absence of phenothiazine (146 ± 34 nm P_i . mg $protein^{-1}$. min^{-1}). Assuming maximal inhibition at 300 μM phenothiazine, half-maximal inhibition occurred at 163 μM CPZ, 119 μM FLU and 75 μM TFP.

Phenothiazines also inhibited the Ca^{2+} + Mg^{2+}-ATPase in a dose-dependent manner when assayed in the presence of 200 μM Mg^{2+}. All 3 phenothiazines tested produced a greater % inhibition of Ca^{2+} + Mg^{2+}-ATPase in the presence of 200 μM Mg^{2+} as compared to the inhibition obtained in the absence of added Mg^{2+} (table 1). It was also observed that in the presence of 200 μM Mg^{2+}, the phenothiazines inhibited the Mg^{2+}-ATPase activity at ⩾100 μM (not shown).

Even though the inhibition of the Ca^{2+} + Mg^{2+}-ATPase activity by phenothiazines suggested that the membranes contain endogenously bound calmodulin, the Ca^{2+} + Mg^{2+}-stimulated ATPase could be stimulated

Table 1
Effect of Mg^{2+} on phenothiazine-induced inhibition of Ca^{2+} + Mg^{2+}-ATPase activity

Agent	Ca^{2+} + Mg^{2+}-ATPase (% control)	
	Endogenous Mg^{2+}	200 μM Mg^{2+}
CPZ	91.7 ± 3.5	76.0 ± 8.9
TFP	72.8 ± 3.9	31.7 ± 9.2
FLU	70.9 ± 4.4	55.0 ± 10.5

Incubations were performed (30 min, 37°C) with or without added Mg^{2+} and at 50 μM phenothiazine. The data represent the percent of Ca^{2+}-stimulated ATPase activity determined in the absence of phenothiazine (mean ± SEM, N = 3–4). Control Ca^{2+} + Mg^{2+}-ATPase was 140 ± 33 and 52 ± 8 nm P_i . mg^{-1} . min^{-1} at endogenous and 200 μM Mg^{2+}, respectively

Table 2
Effect of calmodulin on Ca^{2+} + Mg^{2+}-ATPase activity in control and TFP pretreated plasma membranes without added Mg^{2+}

Calmodulin (μg/ml)	(–) TFP Treatment (nmol P_i . mg protein^{-1} . min^{-1})	(+) TFP Treatment
0	147 ± 29	83 ± 11
2.8	146 ± 28	90 ± 8
6.0	145 ± 27	91 ± 10
12.0	148 ± 25	91 ± 9
24.0	141 ± 25	88 ± 7

In each assay (N = 4–5), one-half of the plasma membrane fraction was pretreated (30 min, 40°C) with 100 μM TFP. Both control and TFP pretreated membranes were assayed without added Mg^{2+} at 1.1 μM free Ca^{2+} (mean ± SEM)

further by the addition of calmodulin (fig.2, lower curve). In this assay system, 200 μM Mg^{2+} was included in the incubation medium and the degree of enhancement of this enzyme activity depended both on [Ca^{2+}] as well as [calmodulin]. Preliminary studies determined free Ca^{2+} at 1.1 μM as optimal for obtaining calmodulin stimulation of Ca^{2+} + Mg^{2+}-ATPase activity in this system. Calmodulin concentrations

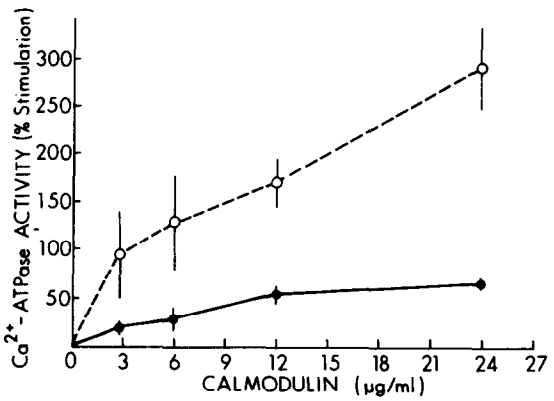

Fig.2. Effect of calmodulin on Ca^{2+} + Mg^{2+}-ATPase activity in control and TFP-pretreated plasma membranes with 200 μM Mg^{2+}. The plasma membrane fractions were separated, one-half pretreated (30 min, 4°C) with 100 μM TFP. Both fractions were assayed in the presence of 200 μM Mg^{2+} at 1.1 μM free Ca^{2+}. The data shows the percent stimulation of Ca^{2+} + Mg^{2+}-ATPase activity in control (•) and TFP-pretreated (○) plasma membrane in response to exogenously added calmodulin (mean ± SEM, N = 3). Ca^{2+} + Mg^{2+}-ATPase activity in the absence of any added calmodulin was 133 ± 33 nm P_i . mg^{-1} . min^{-1} and 15 ± 3 nm P_i . mg^{-1} . min^{-1} for control and TFP pretreated membranes, respectively.

ranging from 2.8–24 μg/ml were evaluated and as indicated in fig.2, Ca^{2}l-stimulated activity was enhanced by calmodulin in a dose-dependent manner with maximal stimulation (67%) occurring at 24 μg/ml.

Pretreatment of the plasma membrane preparation with TFP in an attempt to dissociate endogenous calmodulin increased the sensitivity of the Ca^{2+} + Mg^{2+}-ATPase to exogenously added calmodulin. Under these conditions the maximum response of the ATPase to calmodulin (24 μg/ml) was increased 400% over the non-pretreated tissue (fig.2 upper curve). Mg^{2+}-ATPase activity was not stimulated at any of the calmodulin concentrations.

To determine the Mg^{2+} dependency of the stimulation of the Ca^{2+} + Mg^{2+}-ATPase activity by calmodulin, similar studies were performed in the absence of exogenously added Mg^{2+} to the incubation medium. Under these conditions (table 2), calmodulin exerted no detectable effect on the Ca^{2+}-stimulated ATPase either with or without TFP pretreatment. These results indicate a requirement of Mg^{2+} for calmodulin enhancement of the Ca^{2+} + Mg^{2+}-ATPase activity in this tissue preparation.

4. Discussion

Our previous studies indicated that calmodulin activates Ca^{2+} uptake by islet-cell plasma membrane vesicles [8]. We now demonstrate calmodulin activation of Ca^{2+}-stimulated ATPase activity which is thought to mediate this transport process. Inhibition of the Ca^{2+} + Mg^{2+}-ATPase activity by TFP, FLU,

and CPZ provides indirect evidence for a requirement of calmodulin for this enzyme activity. The relative degree of inhibition produced by these agents (TFP ≥ FLU > CPZ) is consistent with their effects on erythrocyte plasma membrane Ca^{2+} + Mg^{2+}-ATPase activity and indicates that endogenous calmodulin is required for full enzyme activity [1,10,23,24]. In the erythrocyte plasma membrane, inhibition of the Ca^{2+} + Mg^{2+}-ATPase by phenothiazines may be a reflection of endogenously bound calmodulin [1]. This may be explained, in part, by the high levels of calmodulin present in islets [11,14] as well as by the apparent high affinity of calmodulin for the plasma membrane ATPase.

Calmodulin directly enhanced the Ca^{2+} + Mg^{2+}-ATPase activity by 67% when 200 μM Mg^{2+} was present in the incubation medium. Since inhibition of the activity by phenothiazines indicated the presence of significant levels of endogenous calmodulin, procedures were used to more effectively remove bound calmodulin from this membrane preparation. In this approach, membranes were pretreated with TFP in the presence of Ca^{2+} to dissociate endogenous calmodulin. TFP pretreatment reduced the Ca^{2+}-stimulated ATPase activity and resulted in a 4-fold enhancement of the calmodulin activation of the Ca^{2+} + Mg^{2+}-ATPase. This enhancing effect by calmodulin is most likely explained by replacement of calmodulin depleted by the pretreatment procedure. Although the effect of residual TFP from the pretreatment conditions is a consideration in this experimental design, it would be <1 μM in the assay medium.

In [8], calmodulin stimulation of ATP-dependent Ca^{2+} uptake in islet-cell plasma membrane vesicles was determined in the presence of 2 mM Mg^{2+}. The presence of Mg^{2+} in the transport assay is thought to be necessary for coupling ATP hydrolysis to Ca^{2+} transport in this system. However, the failure of calmodulin to activate Ca^{2+} + Mg^{2+}-ATPase in the absence of exogenously added Mg^{2+} as well as the enhancement of phenothiazine-induced inhibition by Mg^{2+} indicates a requirement for this cation for calmodulin activation of plasma membrane Ca^{2+} + Mg^{2+}-ATPase.

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