Biochimica et Biophysica Acta, 662 (1981) 30–35
Elsevier/North-Holland Biomedical Press

BBA 69387

CHARACTERIZATION OF PANCREATIC ISLET CA^{2+}-ATPASE

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(Received March 4th, 1981)
(Revised manuscript received July 6th, 1981)

Key words: Ca^{2+}-ATPase; Insulin secretion; (Pancreatic islets)

Ca^{2+}-dependent ATPase (Ca^{2+}-dependent ATP phosphohydrolase, EC 3.6.1.3) present in a subcellular fraction derived from rat pancreatic islet homogenates was examined to determine kinetic parameters and responses to various substances with known effects upon insulin secretion. Experiments demonstrated the presence of a Ca^{2+}-ATPase with a K_m ATP of $7 \cdot 10^{-5}$ M and two K_m Ca of $1.3 \cdot 10^{-7}$ M and $5.7 \cdot 10^{-6}$ M. The enzyme had little activity in acidic media while retaining considerable activity in basic media. Optimal activity was obtained at pH 7.5. The enzyme was relatively temperature insensitive (Q_{10} = 1.49), since activity decreased less than 50% with a 15°C decrease in temperature. Studies on the stability of enzyme activity upon storage at −20°C indicated that for intact islets activity was stable for 3 weeks, while in homogenates activity was stable for only 1 week, after which activity rapidly declined in both cases. Certain substances known to either stimulate or inhibit insulin secretion were tested for their ability to alter enzyme activity. Potassium, glibenclamide and cyclic AMP had no effects upon activity. Mannoheptulose significantly suppressed enzyme activity while 2-deoxyglucose did not alter activity. These observations are consistent with the hypothesis that a Ca^{2+}-ATPase present in pancreatic islets may act as a modulator of pancreatic islet β cell activity.

Introduction

It is well established that Ca^{2+} and its movements are fundamental to normal insulin secretion [1,2]. Many investigators have described increases in Ca^{2+} uptake [3,4] or decreases in Ca^{2+} efflux [5] after exposure of islets to insulin-releasing concentrations of glucose. Furthermore, Hellman et al. [6] reported that changes in Ca^{2+} uptake by various modifiers of insulin release are in concordance with what would be predicted from their actions upon insulin. More recently, subcellular pools of calcium have been differentially linked with the two phases of release [7–9]. However, the systems responsible for regulating cellular calcium in islets have not been clearly delineated.

One of the major cellular systems believed to be responsible for regulating cytoplasmic calcium levels in most cells is Ca^{2+}-dependent ATPase. This enzyme has been found to be present in many types of cells [10–14]. Formby et al. [15] and Levin et al. [16] have previously reported the presence of this enzyme in pancreatic islets. In this study, we have characterized the basic kinetic properties of this enzyme and further examined the actions upon enzyme activity of substances which influence secretion.

Materials and Methods

Islet isolation. Islets were isolated from male Sprague-Dawley rats (300–500 g) by the method of Lacy and Kostianovsky [17]. After isolation, islets

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were collected in 0.3 M sucrose/10 mM Tris-HCl (pH 7.4), frozen on solid CO_2/acetone, and stored at −20°C for no longer than 3 weeks before use. Storage for longer periods of time resulted in loss of activity.

Enzyme preparation. Islets were thawed on ice and homogenized manually in a Ten Broeck tissue grinder (Kontes Glass Co., Vineland, NJ. Clearance 0.004–0.006 inches) with seven to eight vigorous passes of the plunger. Homogenates were then centrifuged at 600 × *g* for 10 min in a Sorvall RC2-B centrifuge. The supernatant was decanted, the pellet resuspended and then recentrifuged as above. This final pellet was found to contain most of the Ca^{2+}-ATPase present in the homogenates and was used in all studies described. After centrifugation, pellets were resuspended in 3 mM Tris-HCl (pH 7.4) (1 000 islets/2.6 ml) frozen and stored 2–5 days at −20°C before assaying for Ca^{2+}-ATPase.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase was assayed, in duplicate, in a final volume of 670 µl consisting of 50 mM Tris-HCl (pH 7.4), various concentrations of $CaCl_2$ and Tris-ATP as specified, 10^{-4} M ouabain, and any other additions as specified in Results. Assay tubes were first immersed in ice. To each tube, 0.5 ml Tris-HCl/$CaCl_2$ was added, followed by 0.01 ml ouabain and 0.1 ml tissue suspension containing 10–12 µg protein. 10 µl of appropriate test substance or distilled water was added as required. Tubes were then incubated at 37°C for 10 min. The reaction was initiated by the addition of 0.05 ml Tris-ATP, continued for 45 min and terminated by first immersing incubation tubes in ice and then by the addition of 0.05 ml 50% TCA. The incubation mixture was centrifuged at 1 100 × *g* for 10 min and assayed for inorganic phosphate by the method of Fiske and SubbaRow [18].

Phosphatase assays. *p*-Nitrophenyl phosphatase activity was assessed by measuring *p*-nitrophenol release from *p*-nitrophenyl phosphate under alkaline (pH 9.0, EC 3.1.3.1) or acid (pH 5.0, EC 3.1.3.2) conditions [19]. The following was added to iced assay tubes: 0.5 ml 50 mM Tris-HCl (pH 9.0) or Na-acetate/acetate (pH 5.0) with 5 mM $MgCl_2$ and 0.1 ml tissue suspension. After a 10 min incubation at 37°C, 0.05 ml of 65 mM *p*-nitrophenyl phosphate was added to initiate the reaction. After an additional 30 min, the reaction was terminated by immersing tubes in ice and adding 0.05 ml 2 N NaOH. Samples were then read directly at 420 mµ and the amount of product formed was computed according to the Beer-Lambert law ($\epsilon = 1.32 \cdot 10^4$).

Definitions and measurements. Initially, four types of controls (five experiments each) were compared: (1) omission of tissue, (2) omission of Ca^{2+}, (3) omission of ATP until after completion of the reaction and (4) addition of TCA before the incubation. No significant differences were noted among these controls (less than 5% variation). Ca^{2+}-ATPase activity was consequently defined as the difference in ATP hydrolysis between samples containing tissue plus other additions and those without tissue but with all other additions. Less than 4% of the ATP present in the incubation media was hydrolyzed during incubations. Enzymatic hydrolysis exceeded non-enzymatic by 20–400% depending upon the Ca^{2+} concentration. Protein was measured according to the method of Lowry et al. [20]. Free calcium was measured with an ionized Ca^{2+}-sensitive electrode (Radiometer, Copenhagen, Denmark). Mg^{2+} and Ca^{2+} concentrations were verified by atomic absorption spectroscopy. By this method, a trace amount of Mg^{2+} (0.13% contamination) was found in the Ca^{2+}-ATPase media. Statistical comparisons were done by a paired Student's *t* test.

Results

Course of enzyme activity. The rate of ATP hydrolysis as a function of length of incubation is shown in Fig. 1. In these experiments, the concentration of free calcium was 45 µM and total ATP was 3 mM. Activity of the enzyme was linear from 1 to 60 min under our conditions. The reaction had achieved steady-state (specific activity had stabilized) by 15 min, well before the end of the 45 min incubation period used in further experiments.

Michaelis constant for Ca^{2+}. We have previously reported that kinetic analysis for ATP yielded a linear Eadie-Hofstee plot with an apparent K_m ATP of $7 \cdot 10^{-5}$ M [16]. A similar plot for calcium, depicted in Fig. 2, is visibly biphasic. Calcium concentrations ranging from $5 \cdot 10^{-5}$ M to $1 \cdot 10^{-7}$ M were used in these experiments. ATP was fixed at 3 mM. Kinetic parameters were determined by computer analysis of the data from three separate experiments [21]. The higher affinity component showed a K_m Ca^{2+} of

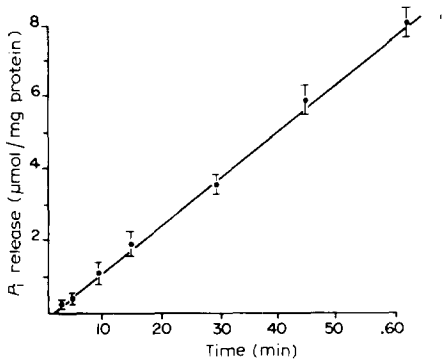

Fig. 1. ATP hydrolysis over time by pancreatic islet Ca^{2+}-ATPase. ATP hydrolysis was assessed by measuring phosphate release from incubates containing 45 μM calcium, 3 mM ATP and enzyme. Each point represents the mean ± S.E. for three experiments.

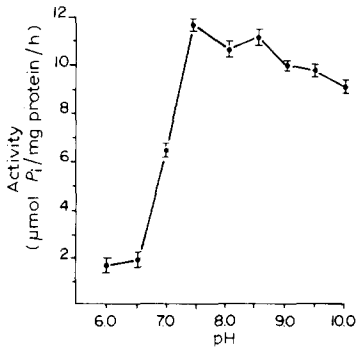

Fig. 3. Effects of pH upon Ca^{2+}-ATPase. pH was adjusted by addition of 1 N HCl to Tris base (50 mM). Each point is the mean ± S.E. of three experiments.

$1.3 \cdot 10^{-7}$ M while the lower affinity component showed a considerably lower K_m of $5.7 \cdot 10^{-6}$ M. The V values were approx. 3.2 and 4.0 μmol P_i/mg protein per h, respectively, for the high and low affinity components. The total V agreed well with that found from kinetic analysis for ATP.

pH effects. The pH dependence of enzyme activity is depicted in Fig. 3. The pH of the incubation medium was adjusted by the addition of 1 N HCl to 50 mM Tris-HCl base (pH 10.4) until the desired pH was obtained. In acidic media (pH < 6.5) enzyme activity was quite low. Activity rose sharply between pH 6.5 and 7.5, peaked at 7.5, then very gradually decreased as the pH approached 10.0.

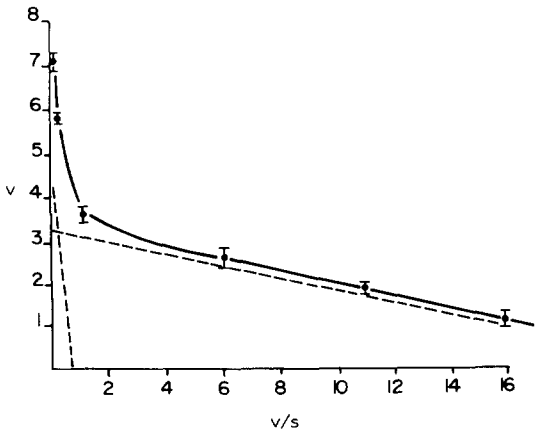

Fig. 2. Eadie-Hofstee plot of Ca^{2+}-ATPase with calcium as the varied substrate. V is μmol P_i/mg protein per h. S is μM. Each point represents the mean ± S.E. for three experiments.

p-Nitrophenyl phosphatase. This phosphatase, present in rat pancreatic islets, has been described previously by Lernmark et al. [22]. In view of our results from Ca^{2+}-ATPase pH dependence, we were interested in measuring and characterizing phosphatase activity at alkaline pH in our preparation. We were able to detect a phosphatase with a specific activity of 2.8 μmol *p*-nitrophenol/mg protein per h. Similar to the findings of Lernmark, we noted this activity was dependent upon the presence of magnesium, activated by low concentrations of potassium (1 mM potassium increased specific activity to 3.1), and unaffected by 1 mM calcium. We were also able to detect phosphatase activity at pH 5.0. This activity was 3-fold higher than that found at alkaline pH, however no attempts were made to further characterize this activity.

Temperature effects. The temperature dependence of the enzyme was subsequently examined. The reaction was run at three different temperatures: 37, 27 and 22°C. The results show activity was linearly related to incubation temperature, however, the enzyme was found to be fairly temperature insensitive (Q_{10} = 1.49). At 37°C, specific activity was 7.35 μmol P_i/mg protein per h. Decreasing the incubation temperature to 27°C resulted in a 31% decrease in specific activity to 5.04 and further decreasing the temperature to 22°C decreased activity to 3.98 (46%↓).

TABLE I

EFFECT OF AGING ON ENZYME SPECIFIC ACTIVITY

Ca^{2+}-ATPase was assayed with 3 mM ATP and 45 μM Ca^{2+}. Islets were stored for indicated time periods as whole, unhomogenized tissue, then homogenized and assayed 2 days later or as total homogenates which were assayed at the indicated times. Specific activities given are in μmol P_i/mg protein per h.

Length of storage	Fresh	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks
Type							
Frozen whole islets Ca^{2+}-ATPase	8.02	7.96	7.76	7.90	6.32	4.98	2.30
Frozen homogenate Ca^{2+}-ATPase	8.25	7.76	5.82	4.46	2.89	–	–

Enzyme aging. One feature of Ca^{2+}-ATPase noted upon storage at –20°C for any length of time was a gradual loss of activity. This phenomenon was noted in both intact islets and tissue homogenates, although activity decreased more rapidly in homogenates than in intact islets. Two experiments were conducted to examine this phenomenon. In the first experiment, a group of 1 000 islets was homogenized and centrifuged, and aliquots were stored for various time periods. In the second experiment, groups of 200 islets were stored for various periods, homogenized, and assayed for activity. In islets stored as intact tissue (Expt. 1), activity remained fairly constant for up to 4 weeks of storage, after which activity gradually declined (Table I). However, in islets stored as homogenized tissue (Expt. 2), activity remained high only throughout the first week and thereafter declined rapidly.

Effects of agents which influence insulin release. We have previously reported the effects of certain insulin secretagogues and inhibitors of insulin secretion upon ATPase activity in islets [16]. Here we have investigated other compounds with known effects upon insulin release. Two inhibitors of insulin release known to interfere with glucose metabolism are 2-deoxyglucose and mannoheptulose. When tested in our system, 2-deoxyglucose (300 mg%) was without effect upon enzyme activity while mannoheptulose (300 mg%) inhibited enzyme activity 10% (Table II). On the other hand, glibenclamide (0.5 mM) and cyclic AMP (10^{-5} M), both stimuli for insulin release, had no effect upon enzyme activity. Potassium (1 mM) also had no effect upon this enzyme.

TABLE II

ACTIONS UPON ISLET Ca^{2+}-ATPase OF SUBSTANCES WHICH ALTER GLUCOSE-INDUCED INSULIN RELEASE

Substance	*n*	Change in activity from control	*P*
14 mM Mannoheptulose	5	−0.59 ± 0.19	<0.02
18 mM 2-deoxyglucose	5	+0.15 ± 0.37	n.s.
0.5 mM Glibenclamide	5	−0.09 ± 0.29	n.s.
1 mM KCl	5	−0.62 ± 0.42	n.s.
0.01 mM cyclic AMP	8	−0.31 ± 0.30	n.s.

All incubations were run as duplicate samples paired with controls. Activity values are reported as the mean difference (±S.E.) in specific activity obtained in the presence and absence of the substance. Values of activity are in μmol P_i/mg protein per h. n.s. indicates not significant at $P < 0.05$.

Discussion

In these studies we have identified some of the more important kinetic characteristics of rat pancreatic islet Ca^{2+}-dependent ATPase. The Eadie-Hofstee plots indicate that two K_m values for Ca^{2+} were present in our subcellular fraction. Formby et al. [15] previously reported this same finding in islets, obtaining values of $6.9 \cdot 10^{-8}$ M and $4.2 \cdot 10^{-6}$ M. Our studies produced very similar results with values of $1.3 \cdot 10^{-7}$ M and $5.7 \cdot 10^{-6}$ M. These findings signify either a single enzyme with two apparent K_m values for Ca^{2+} or two enzymes with distinct K_m Ca^{2+} values. Although our studies do not differentiate these two possibilities, the latter is

considered more likely since other investigators [23] have identified the presence of two distinct Ca^{2+}-ATPases in renal cells, one in endoplasmic reticulum and the other in plasma membrane, each with a distinct K_m Ca^{2+}.

A previously reported study from our laboratory [16] determined that the ATP concentrations required for half-maximal stimulation of islet Ca^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, and Mg^{2+}-ATPase were $7 \cdot 10^{-5}$ M, $5.4 \cdot 10^{-4}$ M, and $7.1 \cdot 10^{-5}$ M, respectively. Since normal intracellular levels of ATP are about $5 \cdot 10^{-3}$ M [24] in vivo, all of these enzymes should be fully saturated with ATP. Consequently, regulation of activity should be independent of fluctuations in ATP levels normally occurring in cells.

The calcium concentrations which we found to be required for half-maximal activation of Ca^{2+}-ATPase by calcium are close to levels found intracellularly [25]. Since normal intracellular ATP concentrations are too high to exert regulatory influences on enzyme activity (about 40-times K_m), calcium levels more likely control activity. Because half-maximal ATPase activity is obtained at calcium levels close to those found intracellularly, as calcium levels in the cell rise above the K_m for calcium enzyme activity would be expected to increase rapidly. As calcium levels drop, enzyme activity very likely drops. Thus, calcium could regulate pump activity and, reciprocally, pump activity may regulate intracellular calcium. This idea has previously been proposed by other investigators for other cell types [10,23] and data obtained in the present study provide support for the hypothesis that this mechanism is operative in islets as well.

Current theories of ion transport by ATPases propose that paired ion countertransport mechanisms are operative in these enzymes and, further, that Mg^{2+} is apparently the ion countertransported with Ca^{2+} by Ca^{2+}-ATPase. If this were so, one would expect to observe Mg^{2+} dependence of Ca^{2+}-ATPase activity. However, such dependence is not always seen. Some investigators have reported a Mg^{2+} requirement for Ca^{2+}-ATPase in certain cell types [10,14]. In other cell types, enzyme activity is obtained without added Mg^{2+} [11–13]. It appears that in islets the Mg^{2+} requirement for activity is small, if it exists at all, since no Mg^{2+} was added to our assay media. Because trace Mg^{2+} was detected in our calcium solutions, a Mg^{2+} requirement cannot be ruled out.

Islet Ca^{2+}-ATPase appears unique in that, unlike Ca^{2+}-ATPases from other tissues [12,26], activity is quite high over a broad range of neutral and basic pH values. One possible explanation for this phenomenon is that another phosphatase is responsible for the majority of activity in the basic regions of the pH curve. However, Ca^{2+}-ATPase activity in islets was found to possess a number of characteristics distinguishing it from other nonspecific phosphatases. The latter activity had a requirement for Mg^{2+}, and could be activated by low concentrations of potassium, and calcium was found to have little effect upon specific activity. Ca^{2+}-ATPase, on the other hand, did not depend upon added magnesium and was unaffected by potassium. Furthermore, the activity of our nonspecific phosphatase seemed to low to account for the considerable activity of Ca^{2+}-ATPase seen at basic pH. Thus, it seems unlikely that activity at basic pH was produced by a nonspecific phosphatase and it seems more likely that this activity was Ca^{2+}-ATPase.

We have previously suggested that insulin secretagogues elicit complex interaction patterns among $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase and adenylate cyclase to influence the ionic environment of the β cell [16]. Each of the various secretory stimuli or inhibitors previously reported elicited unique patterns of activation or suppression of the three cation-dependent ATPases. The results of this present study further support this hypothesis.

It is very likely that a variety of secretagogues act upon islet cation 'pump' enzymes in different patterns. For example, we previously found that glucose inhibited Ca^{2+}-ATPase in islets [16], yet the sulfonylurea glibenclamide, also a good stimulus for insulin release [27], failed to inhibit islet Ca^{2+}-ATPase. However, Kawazu et al. [28] recently suggested that sulfonylureas might act through cation 'pump' enzymes since glibenclamide was found to inhibit $(Na^+ + K^+)$-ATPase. We also previously found that arginine inhibited $(Na^+ + K^+)$-ATPase but not Ca^{2+}-ATPase [16]. Cyclic AMP, known to be involved in glucose-induced insulin release [29], also failed to alter Ca^{2+}-ATPase activity in our system, although Formby et al. [15] reported suppression of the high affinity component of this enzyme by cyclic AMP at 10^{-5} M.

Mannoheptulose, an inhibitor of glucose-induced insulin release, suppressed Ca^{2+}-ATPase activity while

another secretory inhibitor, 2-deoxyglucose, had no effect on Ca^{2+}-ATPase. Both of these glucose analogs are known to interfere with glucose metabolism, but some important structural differences exist between them. The number two hydroxyl group, not present in 2-deoxyglucose, might play an important role in recognition of molecules by receptor sites. Molecules in which the number two hydroxyl is altered (i.e., streptozotocin and glucosamine) impair glucose-induced insulin release [30]. Yet, analogs in which the number two hydroxyl is intact (i.e., 3-*O*-methyl glucose and phlordizin) have no effect upon glucose-induced insulin release [30]. Mannoheptulose, which contains a hydroxyl at a position equivalent to the number two site of glucose, may thus interact with receptor sites, whereas 2-deoxyglucose may not. Mannoheptulose has further been suggested to act as a competitive inhibitor of insulin release [30]. Our results are consistent with the possibility that mannoheptulose and glucose may compete for sites on Ca^{2+}-ATPase since both molecules seem to interact with this enzyme.

In this study we have characterized some of the basic biochemical parameters of rat pancreatic islet Ca^{2+}-ATPase and found most of them to be consistent with those reported for other Ca^{2+}-ATPases. We have also presented additional data indicating the complexities of secretagogue interactions with membrane enzymes. We feel that this data adds further support to the hypothesis that an enzymatic calcium-regulatory system may act as a modulator of endocrine pancreatic function.

Acknowledgements

This work was supported by grants from the Veterans Administration (5218-01), National Institutes of Health (AM 21031-03), American Diabetes Association, Kroc Foundation and Upjohn Laboratories.

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