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Effect of different insulin secretagogues and blocking agents on islet cell Ca^{2+}-ATPase activity

Claudio M. Gronda [a], Juan P. Rossi [b] and Juan J. Gagliardino [a]

[a] *CENEXA - Centro de Endocrinología Experimental y Aplicada (UNLP-CONICET), Facultad de Ciencias Médicas, La Plata, and* [b] *IQUIFIB - Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Buenos Aires (Argentina)*

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Plasma membrane Ca^{2+}-ATPase activity was measured in rat islet homogenates. The enzyme was inhibited, in a dose-dependent manner, when the islets were preincubated for 5 min with different concentrations of glucose (2 to 16 mM). This inhibition disappeared almost entirely after 15 min incubation, regardless of the glucose concentration in the medium. Simultaneous measurement of insulin in the medium revealed a stimulatory effect of glucose upon insulin secretion. The Ca^{2+}-ATPase activity was also inhibited when the islets were preincubated for 3 min with other stimulators of insulin secretion such as gliclazide (76 μM), tolbutamide (1.5 mM), glucagon (1.4 μM) + theophylline (10 mM) and ketoisocaproic acid (15 mM). Conversely, the activity of the enzyme was significantly enhanced when the islets were preincubated briefly with the insulin secretion blocker, somatostatin (1.4 μM). Neither glucose nor any of the other substances tested when added directly to the enzyme assay medium modified significantly the Ca^{2+}-ATPase activity measured in the islet homogenates. These results would suggest that the activity of the islet plasma membrane is modulated by one or more of the intracellular metabolites produced when the islets are challenged by the insulin stimulator or blocking agents.

Introduction

The relationship between calcium transmembrane fluxes and its distribution within B cells with the release of insulin has been well-documented [1–7]. The presence of Ca^{2+}-ATPase, the enzyme responsible for the active extrusion of calcium from the cytoplasm, has also been demonstrated in several islet cell organelles [8–10]. However, conflicting results have been reported regarding the effect of several insulin-secretagogue agents upon the activity of this enzyme. While several authors have described an inhibitory effect of glucose and other stimulators of insulin release upon the Ca^{2+}-ATPase activity of different islet cell fractions [8,9,11], others were unable to demonstrate any direct effect of these agents upon the enzyme activity measured in the islet cell plasma membrane [12]. Some of the controversies could be resolved on the basis of differences in the preparations used (homogenates or cell fractions), their degree of purity, the experimental models of choice, and the reliability of the methods employed.

In an attempt to elucidate this problem, we have studied the Ca^{2+}-ATPase activity of islet cell

Abbreviation: KIC, ketoisocaproic acid.

Correspondence: J.J. Gagliardino, CENEXA (UNLP-CONICET), Facultad de Ciencias Médicas, Calles 60 y 120, 1900 La Plata, Argentina.

membranes in crude homogenates obtained from islets previously incubated with different stimulators or inhibitors of insulin release.

Materials and Methods

Materials

Collagenase was obtained from Serva, Heidelberg; Trasylol® (100000 KIU) was kindly provided by Bayer Argentina. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [13] except that unlabelled orthophosphate was not added to the incubation mixture. ^{32}P-labelled orthophosphate was provided by Comisión Nacional de Energía Atómica (Argentina). Tolbutamide and gliclazide were kindly provided by Hoechst and Les Laboratories Servier, respectively. ATP, enzymes and cofactors used for the synthesis of [γ-^{32}P]ATP and other reagents were obtained from Sigma, U.S.A. Somatostatin (UCR Peptide Department, Brussels, Belgium), was kindly provided by Dr. J.C. Basabe and glucagon by Dr. Lise Heding from Novo, Denmark.

Methods

Animals and preparation of islets. Pancreases from fed male rats (approx. 200 g body weight) were used to obtain isolated islets after initial collagenase digestion [14].

Islet incubation. Groups of 100 islets were placed in glass tubes with 1.1 ml of Krebs-Ringer bicarbonate buffer at pH 7.4, containing 1% bovine serum albumin, 400 U/ml Trasylol and glucose (2 to 16.6 mM), ketoisocaproic acid (KIC, 15 mM), tolbutamide (1.5 mM) or gliclazide (76 μM), glucagon (1.4 μM) and theophylline (10 mM), insulin (15 nM) or somatostatin 1.4 μM), and incubated for different time periods as stated in the text and tables. The buffer was previously gassed with a mixture of 95% O_2/5% CO_2. The incubations were stopped either by immersing the tubes in an ice/water bath or by adding to the tubes 8.9 ml of cold 225 μM EGTA-Tris at pH 7.2.

Enzyme preparation. After the incubation period, the tubes containing the medium plus the islets were centrifuged for 1 min at 1700 × *g* (4°C). The supernatant was discarded and the precipitated islets were resuspended in 300 mM sucrose/10 mM Tris-HCl (pH 7.24) at 4°C. The islet suspension was then transferred to a microhomogenizer (tissue grinder, Potter-Elvehjem, Teflon, size 18, rod o.d. 1/8 inch, 0.5 ml capacity, from Kontes Scientific Glassware Instruments), washed twice at 4°C and homogenized in 0.5 ml of the same buffer by 200 excursions of the plunger.

Ca^{2+}-ATPase assay. ATPase activity was measured in 0.5 ml of 50 mM Tris-HCl (pH 7.24 at 37°C), 0.1 mM ouabain, 1 mM [γ-^{32}P]ATP, 1 mM EGTA and sufficient $CaCl_2$ to obtain 1.1–1.8 μM of free calcium and other additions as shown in Results. Of the tissue homogenate, 0.05 ml containing 5–6 μg of protein (equivalent to 10 islets) was added to each tube.

Ca^{2+}-ATPase activity represents the difference between the activity measured in the above medium and that measured in the same medium without calcium. After 45 min of incubation at 37°C, the tubes were transferred to an ice/water bath; and after 1 min, first 0.75 ml of 0.5% (w/v) ammonium molybdate in 5% (v/v) perchloric acid, and then 0.6 ml of isobutanol was added to each tube. After 15 s, the mixture was vigorously stirred for 20 s and then spun down for 3 min at 1700 × *g* (4°C). The radioactivity was measured in an aliquot of the organic phase by liquid scintillation and from this value was calculated the amount of inorganic phosphate liberated from ATP. All determinations were performed in triplicate.

Under these experimental conditions, no more than 4% of the ATP in the reaction mixture underwent enzymatic hydrolysis and the rate of appearance of ^{32}P remained constant for up to 90 min of incubation time.

Free calcium was measured with a Ca^{2+}-sensitive electrode [15]. Protein was measured according to the method of Lowry et al. [16].

Insulin secretion. Aliquots of the medium incubation of the islets were separated and kept at −20°C until insulin concentration was determined by radioimmunoassay [17]. Statistical analysis of the data was performed using the Student's *t*-test. Theoretical equations were adjusted to the experimental results by least-squares non-linear regression by the procedure of Gauss-Newton. The program was run on a microcomputer with 14

digit precision (Rossi, R.C. and Garrahan, P.J., unpublished data).

Results

Table I shows that KIC, gliclazide, tolbutamide, glucagon, somatostatin, insulin and different concentrations of glucose, when added directly to the Ca^{2+}-ATPase assay medium, did not modify the plasma membrane Ca^{2+}-ATPase activity measured in homogenates of fresh non-incubated islets. These results agree with those previously reported by Kotagal et al. [12].

The Ca^{2+}-ATPase activity measured in homogenates of islets previously incubated with glucose varied according to the glucose concentration in the medium and the length of the incubation period employed (Fig. 1A). 3.3 mM glucose produced a constant but insignificant decrease in the enzyme activity throughout the entire incubation period studied. Conversely, in the islets incubated with 16.6 mM glucose, a significant decrease in the Ca^{2+}-ATPase activity was observed by 3 min, followed by a recovery towards control values

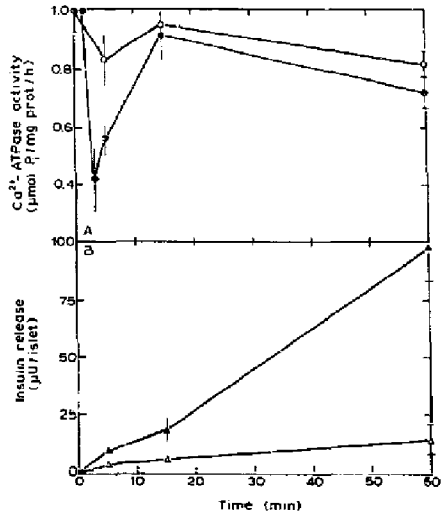

Fig. 1. Ca^{2+}-ATPase activity measured in homogenates of islets incubated in the presence of glucose for different time periods. Each value represents the average of three independent experiments performed in triplicate (A). Insulin released by these islets is shown in B, where each value represents the average ± S.E. of three experiments.

TABLE I

CHANGES IN THE ISLET CELL MEMBRANE Ca^{2+}-ATPase ACTIVITY PRODUCED BY DIFFERENT SUBSTANCES ADDED TO THE ENZYME ASSAY MEDIUM

Each value represents the average of three cases ± S.E. The enzyme activity was measured in homogenates of fresh non-incubated islets without (control) or with the addition of the different substances tested (see Materials and Methods). None of these substances significantly modified the Ca^{2+}-ATPase activity measured in the control sample.

Substance	Ca^{2+}-ATPase activity (μmol P_i/mg protein per h)
Control	1.149 ± 0.159
Glucose (2 mM)	1.304 ± 0.087
Glucose (4 mM)	1.398 ± 0.016
Glucose (8 mM)	1.417 ± 0.075
KIC (15 mM)	1.040 ± 0.093
Gliclazide (76 μM)	1.327 ± 0.076
Tolbutamide (1.5 mM)	1.126 ± 0.103
Glucagon (1.4 μM)	1.237 ± 0.022
Somatostatin (1.4 μM)	1.085 ± 0.021
Insulin (15 nM) [a]	1.030 ± 0.023

[a] This concentration corresponds to that measured in the incubation medium in Fig. 1.

thereafter. The enzyme activity measured at 60 min, however, was still below the control value, even though this difference was not significant. The insulin released by the islets during the incubation period is shown in Fig. 1B. As is well known, a low glucose concentration slightly stimulates the release of insulin, while 16.6 mM glucose elicits a large secretory response. Although the largest inhibition by high glucose of Ca^{2+}-ATPase activity was obtained after 3 min of incubation (Fig. 1A), the scatter of the data was larger at this time than after 5 min, probably because of the sharp decrease in the enzyme activity induced by glucose.

No significant differences in the Ca^{2+}-ATPase activity were observed in islets incubated for 3 min at 37 °C in Krebs-Ringer bicarbonate buffer without glucose with respect to nonincubated islets (0.90 ± 0.02 [11] and 1.10 ± 0.12 [8], respectively).

Fig. 2 shows the Ca^{2+}-ATPase activity measured after a 5 min preincubation of whole islets

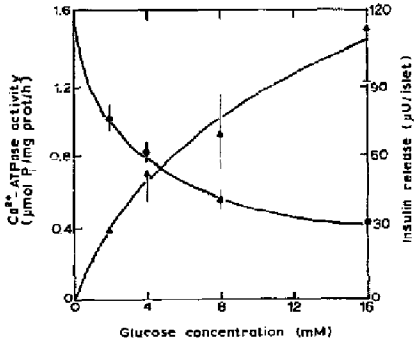

Fig. 2. Plasma membrane Ca^{2+}-ATPase activity (●) and insulin release (▲) of islets incubated with different glucose concentrations. Each value represents the average ± S.E. of three different experiments performed in triplicate.

with 2–16.6 mM glucose and insulin release measured simultaneously.

The curve that fits the experimental points of Ca^{2+}-ATPase vs. glucose concentration corresponded to the following equation:

$$V = V_0/(1 + [\text{glucose}]/K_{0.5}) + V_{\text{residual}} \quad (1)$$

where V_0 is the Ca^{2+}-ATPase activity measured in the absence of glucose, $K_{0.5}$ the glucose concentration producing half-maximal inhibition of enzyme activity and V_{residual} the Ca^{2+}-ATPase activity remaining in the presence of the largest concentration of glucose tested. The experimental values for these parameters were: $V_0 = 1.35 \pm 0.28$ μmol P_i/mg protein per h; $K_{0.5} = 3.16 \pm 0.22$ mM glucose and $V_{\text{residual}} = 0.193 \pm 0.099$ μmol P_i/h. An attempt to adjust the experimental points to an equation similar to Eqn. 1 but without the term V_{residual} produces a bias in the fitting of the experimental points corresponding to all the different concentrations of glucose tested. In this situation, a clear dose-dependent inhibition was obtained. The curve that fits the experimental points of insulin release vs. glucose concentration corresponded to the equation:

$$I = T/1 + K_{0.5}[\text{glucose}] \quad (2)$$

where T is the maximum amount of insulin released, and $K_{0.5}$ the concentration of glucose inducing half-maximal release of insulin. In our conditions, the experimental values obtained for these parameters were $T = 170.0 \pm 25.2$ μU/islet and $K_{0.5} = 9.36 \pm 1.88$ mM.

The effect of agents stimulating insulin release by different mechanisms upon Ca^{2+}-ATPase activity are shown in Table II. These compounds

TABLE II

Ca^{2+}-ATPase ACTIVITY MEASURED IN ISLETS INCUBATED FOR 3 MIN WITH DIFFERENT SUBSTANCES

Each value represents the mean ± S.E. Number of cases in parentheses.

Substance	Ca^{2+}-ATPase activity (μmol P_i/mg protein per h)
Glucose (3.3 mM)	0.997 ± 0.022 (3)
+ insulin (15 nM)	1.123 ± 0.122 (6)
+ gliclazide (76 μM)	0.277 ± 0.009 (3) **
+ tolbutamide (1.5 mM)	0.562 ± 0.040 (6) **
+ glucagon (1.4 μM) and theophylline (10 mM)	0.830 ± 0.018 (3) *
KIC (15 mM)	0.402 ± 0.036 (6) **

* $P < 0.005$.
** $P < 0.001$.

TABLE III

EFFECT OF SOMATOSTATIN UPON Ca^{2+}-ATPase ACTIVITY

Each value represents the mean ± S.E. Number of cases in parentheses. The islets were incubated for 3 min (as described in Materials and Methods) in the presence of the above-mentioned substances.

Glucose (3.3 mM)	Glucose (3.3 mM) + somatostatin (1.4 μM)	Glucose (8 mM)	Glucose (8 mM) + somatostatin (1.4 μM)
0.762 ± 0.034 (2)	1.215 ± 0.110 (3) ($P < 0.05$)	0.569 ± 0.050 (5) *	1.245 ± 0.052 (5) ($P < 0.001$)

* vs. glucose (3.3 mM), $P < 0.025$.

include fuel molecules (KIC), oral hypoglycemic agents (gliclazide and tolbutamide) and agents which affect mainly islet cAMP content (glucagon and theophylline). After a 3 min incubation with every one of these agents the activity of the enzyme measured in homogenates of the incubated islets decreased significantly with respect to the corresponding controls.

The Ca^{2+}-ATPase activity remained unchanged in islets preincubated for 3 min with 3.3 mM glucose plus insulin in a concentration similar to that measured in the medium of islets incubated for the same period with 16.6 mM glucose.

The effect of somatostatin, a potent and physiologic inhibitor of insulin secretion upon Ca^{2+}-ATPase activity, was also tested. When previously incubated with a somatostatin concentration sufficient to block the glucose-induced insulin secretion, the activity of the enzyme measured in islet homogenates was significantly enhanced respect to control values (Table III).

Discussion

We have studied the effect of different insulin secretagogues, insulin and an insulin blocker upon the plasma membrane Ca^{2+}-ATPase activity measured in rat islet homogenates. The specificity of the method employed to measure plasma membrane Ca^{2+}-ATPase activity and its accuracy have been demonstrated and extensively discussed in previous reports [18,19].

Confirming the results previously reported by Kotagal et al. [12], we found that, within the range of concentrations employed, glucose and several other simulators of insulin release exerted no direct effect upon the plasma membrane Ca^{2+}-ATPase activity. Insulin also failed to affect such activity when directly added to the assay medium. Conversely, the activity of the enzyme assayed in homogenates of islets preincubated for up to 60 min with 16.6 mM glucose appeared significantly inhibited throughout the first 5 min of incubation. Thereafter, the values obtained were not significantly different from those measured in the preincubated control islets. The largest inhibition by glucose was observed after a 3 min incubation. However, the inhibition by glucose was dose-dependent only after 5 min of incubation. The sharpness of the drop in the enzyme activity at a high concentration of glucose in earlier periods, together with the greater experimental error encountered at this short time, could explain the dispersion of the data observed in Fig. 1.

Islets incubated for 3 min with other insulin secretagogues, such as KIC, gliclazide, tolbutamide, and glucagon plus theophylline, also showed a significant decrease in plasma membrane Ca^{2+}-ATPase activity. No significant changes were detected in the activity of the enzyme measured in homogenates of islets preincubated with insulin.

On the other hand, when assayed in homogenates of islets previously incubated with the insulin-release blocker, somatostatin, the activity of the enzyme was significantly enhanced.

According to a previous report [12], none of the agents tested exerts direct effects upon the enzyme activity. Conversely, the Ca^{2+}-ATPase activity was significantly altered in homogenates of islets previously incubated with all of the compounds tested, except insulin. Together, these results would suggest that the activity of the islet plasma membrane ATPase is modulated by one or more of the intracellular products resulting from the incubation of the islets with agents that block or stimulate insulin secretion. Insulin released by these agents would not be involved in such effect. It has been demonstrated that glucose 6-phosphate inhibits the plasma membrane Ca^{2+}-ATPase activity [12]. Hence, the increment in the intracellular concentration of this compound could explain the early inhibition of the enzyme activity, detected in islets incubated with high glucose. However, such mechanism cannot explain the inhibitory effect of KIC, tolbutamide and gliclazide, compounds which do not alter the intracellular levels of glucose 6-phosphate. Consequently, the nature of the intracellular signal(s) responsible for rapid drop in plasma membrane Ca^{2+}-ATPase activity under these conditions remains unclear.

Glucose stimulates the phospholipid turnover in islet membranes and the acidic fraction of these compounds can stimulate the Ca^{2+}-ATPase activity [22,23]. Consequently, this effect could, at least in part, explain the recovery of the Ca^{2+}-ATPase activity found in the islets after the 5 min incubation with glucose. A progressive increment of calcium deposits was observed previously in the B-cell plasma membrane of pancreases perfused

with high glucose [3]. This sequestering of the divalent cation might also provide a stimulus of the enzyme that contributed to the observed recovery of Ca^{2+}-ATPase activity.

Maximal inhibition of Ca^{2+}-ATPase activity was obtained after 3 min of preincubation of the islets with glucose (Fig. 1). Hence, the enzyme activity ($V_{residual}$ in Eqn. 1) measured after 5 min of preincubation (Fig. 2) could represent a partial recovery of the enzyme function from the inhibition induced earlier by glucose. For this reason, it is possible that other $K_{0.5}$ values would be obtained for Ca^{2+}-ATPase if the activity of the enzyme were measured either before or after a 5 min preincubation. Otherwise, we cannot discount the possibility that there exists a Ca^{2+}-ATPase fraction that is insensitive to inhibition by glucose.

The $K_{0.5}$ value obtained in Eqn. 1 for Ca^{2+}-ATPase activity measured as a function of medium-glucose concentration (3–16 mM glucose) would suggest that, under basal conditions, the enzyme is already partially inhibited. Otherwise, the curve represented in Fig. 2 would suggest that the largest inhibition of the enzyme is obtained when the glucose concentration is increased from 2 to 8 mM.

The Ca^{2+}-ATPase $K_{0.5}$ value was generally lower than the corresponding value for insulin secretion. This fact would suggest that the effect upon Ca^{2+}-ATPase activity of raising the glucose concentration in the medium precedes the one exerted on insulin secretion. When $K_{0.5}$ for insulin secretion, as a function of glucose concentration in the medium, was calculated from the data of other authors [24], a nearly identical result was obtained. Furthermore, among these data, the $K_{0.5}$ value for glucose oxidation and for B-cell electrical activity were also generally lower than the $K_{0.5}$ corresponding to insulin release. Together, these results might indicate that the inhibition of Ca^{2+}-ATPase participates in the mechanism of the glucose-induced insulin secretion.

Henquin et al. [25] found that nutrient insulin secretagogues decrease $^{45}Ca^{2+}$ efflux from islet cells by a mechanism other than the inhibition of sodium–calcium countertransport. Our results could provide an alternative explanation for such a mechanism.

The early inhibition of the Ca^{2+}-ATPase activity might correspond temporally to the first peak of insulin secretion [26]. This peak is not significantly affected by blocking the influx of calcium from the extracellular space [27]. Under such circumstances, the release of calcium from the pool within the endoplasmic reticulum might be sufficient to increase the cytosolic concentration of the free divalent cation and thereby trigger the release of insulin [2,28,29]. An increase in the IP_3 concentration might be the signal involved in these movements of the intracellular calcium [2,28–31]. A simultaneous inhibition of plasma membrane Ca^{2+}-ATPase activity, being responsible for an outward flow of calcium, might favor the increment in cytosolic Ca^{2+} concentration induced by the cation released from the endoplasmic reticulum. The subsequent recovery of the enzyme activity, together with the participation of other intracellular calcium pools, would avoid a further increment in the concentration of free cytosolic calcium. Hence, these effects would not only keep the cytosolic calcium concentration within a suitable range to ensure an adequate release of insulin, but would also protect the cell against a possible deleterious effect of a large increment in intracellular calcium.

Although in our experiments we focused all our attention on the plasma membrane Ca^{2+}-ATPase activity, the possible participation in intact cells of a similar enzyme located in other subcellular structures might also be considered. In that situation, by sharing with the plasma membrane ATPase the role of controlling cytosolic calcium concentration, these ATPase would improve the efficiency of such a control.

The inhibitory effect of somatostatin upon insulin secretion has been ascribed to its action upon the sequestering of B cell calcium levels [1]. Moreover, it has been recently demonstrated that somatostatin inhibits the glucose-induced increased in cytosolic free Ca^{2+} of islet cells [32]. The enhancement of the ATPase activity described here could be, at least in part, responsible for this latter effect.

Although more experimental evidence is necessary to test all these assumptions, our data would suggest that the plasma membrane Ca^{2+}-ATPase participates in some fashion in the regulation of the release of insulin.

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