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REPORT

A new approach for determination of Na,K-ATPase activity: application to intact pancreatic β-cells

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Abstract It has been postulated that a decrease in Na,K-ATPase-mediated ion gradients may be a contributing mechanism to insulin secretion. However, the precise role of the Na,K-ATPase in pancreatic β-cell membrane depolarization and insulin secretion signalling have been difficult to evaluate, mostly because data reporting changes in enzymatic activity have been obtained in cell homogenates or membrane preparations, lacking intact intracellular signalling pathways. The aim of this work was to develop a method to characterize Na,K-ATPase activity in intact pancreatic β-cells that will allow the investigation of putative Na,K-ATPase activity regulation by glucose and its possible role in insulin secretion signalling. This work demonstrates for the first time that it is possible to determine Na,K-ATPase activity in intact pancreatic β-cells and that this is a suitable method for the study of the mechanisms involved in the Na,K-ATPase regulation and eventually its relevance for insulin secretion signalling.

Keywords Pancreatic β-cell · Na,K-ATPase · Enzymatic activity · Insulin secretion

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The current pancreatic β-cell stimulus-secretion dogma states that glucose metabolism leads to an increase of ATP/ADP ratio, closure of ATP-sensitive K^+ channels, membrane depolarization, and opening of the voltage-dependent Ca^{2+} channels and Ca^{2+} influx, which triggers insulin exocytose. Na,K-ATPase is the principal system contributing to restore the equilibrium of Na^+ and K^+ ions across the plasma membrane. Since this system carries an outward current, Na,K-ATPase contributes for resting membrane potential in pancreatic β-cell. To perform Na^+ and K^+ transport against their concentration gradients, Na,K-ATPase consumes a considerable amount of ATP. It was estimated that in pancreatic β-cell, this pump consumes as much as 75% of the basal energy production (Malaisse et al. 1978). Taking this observation into account, several investigators have suggested that Na,K-ATPase inhibition may play a relevant role in insulin secretion regulation cascade (Owada et al. 1999; Elmi et al. 2000, 2001a; Dufer et al. 2009). Therefore, the development of methods where intact intracellular signalling pathways are conserved are most relevant, since it will allow the investigation of the mechanisms underlying the regulation of Na,K-ATPase activity and its possible contribution to insulin release.

The method here described enables the study of Na,K-ATPase activity in cultured intact pancreatic β-cells preserving insulin secretion signaling pathways, responding to physiological stimuli. This method presents the advantage of allowing simultaneous or sequential measurements of other parameters such as insulin secretion or ionic transport.

Islets of Langerhans were isolated from Wistar rat pancreata by liberase digestion, an enzymatic method developed in accordance to Salgado et al. (1996). Briefly, rats were euthanized by cervical dislocation. After exposing the abdominal cavity, the distal outlet of the common bile

duct was collapsed. The duct was then cannulated close to the junction with the cholecystic and hepatic ducts, and the pancreas was distended by injecting approximately 8 mL of an ice-cold salt solution supplemented with approximately 0.25 mg/mL liberase (type RI, Roche). This solution had the following composition: 115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.56 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25 mM HEPES, 8.4 mM glucose, pH 7.4 after saturation with 95% O_2/5% CO_2. The pancreas was then dissected, transferred into a 50-mL polypropylene centrifuge tube, and incubated at 37°C under gentle manual shaking for 8–12 min. Liberase digestion was stopped by adding a large excess of ice-cold solution supplemented with 2% bovine serum albumin (fraction V, protease free, Sigma). The tube was shaken vigorously to tissue disruption. The islets and pieces of exocrine tissue were let to sediment, the supernatant was removed, and a large excess of the solution was added again. This washing step was repeated twice to free the solution from liberase. Intact islets were then handpicked at room temperature using the tip of an automatic pipette, a process that was repeated twice to separate the islets from any contaminating pieces of exocrine tissue. In the last transference, islets were picked to a polypropylene microtube.

Isolated pancreatic β-cells were obtained by mechanic disruption of the islets in a Ca^{2+}-free medium. Three cycles of centrifugation were performed to completely change from the islet isolation medium to zero-Ca^{2+} medium, followed by homogenation with an automatic pipette. When the solution turned turbid and without visible particles, cells were centrifuged, the supernatant was discarded, and sterile culture medium (RPMI 1640 containing 8.4 mM glucose, 10% heat-inactivated fetal calf serum, 25 mM $NaHCO_3$, and antibiotics—100 μg/mL streptomycin and 100 U/mL penicillin—pH adjusted to 7.2 to give 7.4 in the incubator; all products from Sigma) was added. Cell suspension was homogenized, and three washing steps were performed to sterilization (Salgado et al. 1996).

Subsequently, cells were counted in the presence of trypan blue to evaluate the viability rate and the cells concentration (Butler et al. 1992). Cells were then seeded in a 96-well microplate (10^4 cells/microwell) and incubated overnight at 37°C under a 5% CO_2/95% air-humidified atmosphere. The culture medium was renovated 24 h later, and the experiments were performed within 48 h from isolation.

Na,K-ATPase activity was determined directly in adherent cells. The enzymatic assay was developed in two parallel steps: one for the evaluation of total ATPase activity (incubation medium: 2 mM ATP, 100 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 50 mM Tris–HCl pH 7.4) and another for the evaluation of ouabain-insensitive ATPase activity by selectively inhibiting Na,K-ATPase (incubation medium: 2 mM ATP, 1 mM Ouabain, 15 mM NaCl, 10 mM $MgCl_2$, 90 mM *N*-methyl-D-glucamine chloride, 1 mM EGTA, 50 mM Tris–HCl pH 7.4). The last medium allowed the total inhibition of Na,K-ATPase, maintaining osmolarity. The ATPase activity was calculated by colorimetric quantification of inorganic phosphate (Pi) formed in 30 min, and Na,K-ATPase activity was determined by the difference between the results obtained using the experimental conditions previously described, i.e., in the absence and presence of ouabain.

To determine Na,K-ATPase activity, culture medium was removed, and the cells were washed with the respective incubation media. After washing, 100 μL of enzymatic assay media were added to each well, and cells were incubated for 30 min in a CO_2 incubator at 37°C. After this time period, 60 μL of supernatant was transferred to another microplate containing the same volume of trichloroacetic acid (TCA) 11.5%, where Pi was determined by a colorimetric method (Taussky and Shorr 1953). Briefly, a calibration curve was prepared with KH_2PO_4 in TCA 11.5% (5–320 μM range), and 80 μL of sulfomolybdic reagent was added to each well.

At the end of the experiment, trypan blue dye and glucose-induced insulin secretion were monitored to assess cell viability and physiological control, respectively, throughout the assay. For the viability test, batches of cells were washed, and the trypan blue dye exclusion was evaluated. The culture media were collected, and insulin concentration was measured using a competitive ELISA method. Finally, cells were lysed with 0.05% triton-X for subsequent protein quantification by BCA method (Calbiochem kit), for normalization purposes.

The linear range of the enzymatic method to determine Na,K-ATPase activity was assessed by performing the experiments using incubation periods between 7 and 60 min. The enzymatic reaction was found linear at least during 60 min, for both ouabain-sensitive and total ATPase activities (Fig. 1). A 30-min time period was chosen for the routine enzymatic assay since, after this time period, the amount of Pi released was easily quantifiable and the experiments were amenable to perform.

The kinetic parameters observed in adherent culture cells were also determined. The K_M^{app} for ATP (measured with 115 mM NaCl and 5 mM KCl) was 0.32 mM. This value is in a good agreement with the kinetic parameters published for islet homogenates (Elmi et al. 2000), suggesting that the characteristic of the enzymatic system was maintained using this method. The IC_{50} and IC_{80} for ouabain were 0.37 and 0.44 mM, respectively. Taking these results into account, a concentration of 1 mM ouabain was chosen for total inhibition of Na,K-ATPase.

To evaluate Na,K-ATPase sensitivity to glucose, the cells were preincubated first with 2.1 mM glucose for 30 min to

reach basal metabolic level. Subsequently, a 30-min incubation period, with (2.1 and 8.4 mM) or without glucose, was performed. Incubation media were then collected for insulin quantification. Cells were washed, and enzymatic reaction media were added. Na,K-ATPase activity was assessed as described previously.

The results obtained are shown in Fig. 2. In the absence of glucose, the Na,K-ATPase activity was 0.124± 0.006 μmol/min/mg Pi. Raising glucose concentration to 2.1 mM induced a 32% increase in Na,K-ATPase activity (0.183±0.009 μmol/min/mg Pi; $p<0.05$). Challenging the cells with 8.4 mM glucose evoked a 57% reduction on Na, K-ATPase activity, when compared to the effect of 2.1 mM glucose ($p<0.05$).

The cell viability was assessed by the Trypan Blue exclusion test, performed in randomly chosen wells, before and after the experiments were carried out. In all analytical conditions, it was observed that cell viability was always higher than 98% throughout the assay. Furthermore, basal insulin release observed in response to 2.1 mM glucose was below 0.23 ng/10^6 cells/min whereas 8.4 mM glucose stimulated insulin secretion by at least 4-fold (0.83 ng/10^6 cells/min), showing that the β-cells were normally responding to physiological stimuli.

The results shown in Fig. 2 are in agreement with data previously obtained in our laboratory using islet homogenates, where a 66% reduction of Na,K-ATPase activity was observed in response to a glucose rise from 2.1 to 8.4 mM ($p<0.05$). Moreover, regarding the profile of Na, K-ATPase activity response to glucose, similar results were also reported by others using rat islet homogenates

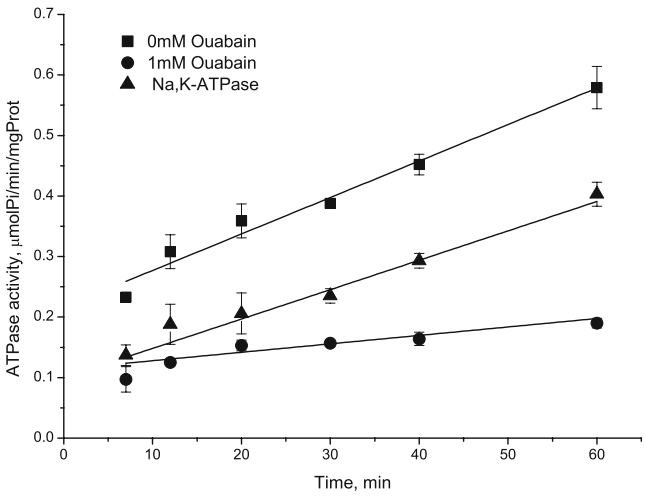

Figure 1. ATPase-induced ATP hydrolysis over time; cells were incubated with the ATPase activity reactional media for 7 to 60 min. Each point represents the mean ± SEM of three parallel experiments performed independently. Linear regression analysis data: $y=0.012x+0.458$, $r=0.979$, $p=0.0013$; $y=0.0026+0.224$, $r=0.780$, $p=0.0470$; $y=0.0099x+0.238$, $r=0.989$, $p=0.0006$; for total, ouabain-insensitive and ouabain-sensitive ATPase activity, respectively.

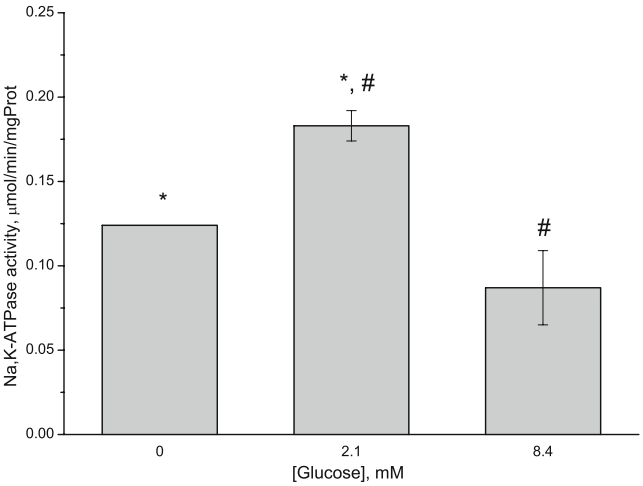

Figure 2. Effect of glucose concentration on Na,K-ATPase activity in pancreatic β-cell. Na,K-ATPase activity was determined in batches of cells previously incubated for 30 min with (2.1 and 8.4 mM) or without glucose for 30 min. Each *column* represents the mean ± SEM of four to seven determinations performed independently in cells batches pooled from islets obtained from three pancreata. Statistical significance was accessed by ANOVA (*$p<0.05$ or #$p<0.05$).

(Levin et al. 1978), heat shock permeabilized mouse pancreatic β-cells (Owada et al. 1999), and freshly isolated mouse islets (Elmi et al. 2001b).

Additionally, the absolute values of the enzymatic activity obtained with our intact cell methodology were similar to the previously reported for rat pancreatic islets homogenates (Levin et al. 1978), indicating that this is a feasible method for Na,K-ATPase activity determination.

However, in experiments using islet homogenates, including ours and those reported in the literature (Owada et al. 1999; Elmi et al. 2001a, b), the cells were stimulated with glucose while intact but the Na,K-ATPase activity was determined after the cells or islets were disrupted thus turning the biological material unusable for further measurements. The method we have developed has the advantage of overcoming this inconvenience, since the cells were maintained viable throughout the assay creating the possibility of simultaneous or sequential experiments using the same cells. Moreover, this method also allowed the use of lower amounts of biological material per experiment rendering the studies easier to perform.

This work demonstrates for the first time that it is possible to determine Na,K-ATPase activity in intact pancreatic β-cells. Moreover, it allows the investigation of the mechanisms underlying glucose-induced putative regulation of Na,K-ATPase activity, and more importantly, it may be used to investigate the physiological role of this pump in insulin secretion signalling.

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