Phosphatidylinositol 3-kinase inhibitors reveal a unique mechanism of enhancing insulin secretion in 832/13 rat insulinoma cells

J. Jason Collier a,1,2, Steven M. White a,1, Gregory M. Dick b, Donald K. Scott a,*

a Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, United States
b Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, United States

Received 13 September 2004

Abstract

Hyperinsulinemia exists before the onset of overt type 2 diabetes mellitus. This response is at least partly due to enhanced insulin release from pancreatic β-cells. Increased insulin secretion can be mimicked in vitro by acute culture of 832/13 rat insulinoma cells with phosphatidylinositol 3-kinase (PI-3K) inhibitors, a treatment that would theoretically simulate insulin resistance. We demonstrate in this study that while the PI-3K inhibitors Wortmannin and LY294002 both block Akt phosphorylation, only LY29002 significantly augments insulin secretion. LY294002 treatment potentiates insulin secretion over both basal and stimulatory glucose concentrations. This effect correlates with a significant increase in action potential duration. There was no change in resting or peak membrane potential under any of the treatment conditions, demonstrating that the cells remain healthy under the acute treatments used in this study. By contrast, Wortmannin has no effect on action potential duration. A partial explanation for these findings is that LY294002 potently inhibits voltage-dependent potassium channels, but does not affect voltage-gated calcium currents. We conclude that while PI-3K may play a role in regulating insulin secretion, there are diverse effects of the established inhibitors of this enzyme on β-cell insulin secretory responses.

Keywords: Pancreatic β-cells; Insulin secretion; Insulin resistance; PI3 kinase inhibitor; Wortmannin; LY294002; Potassium current; Action potential duration; Patch clamp

Pancreatic insulin secretion is regulated by a multitude of signaling events, controlled by various protein and lipid kinases. The exact roles of these signaling molecules are not completely understood [8,15]. Phosphatidylinositol 3-kinase (PI-3K), a component of the insulin signaling pathway, contributes to β-cell function by catalyzing the phosphorylation of several phosphatidylinositol compounds [1,3]. These lipids regulate the β-cell ion channels [17,19] and control the exocytosis of insulin [3,18,22]. Diminished PI-3K activity, which is associated with insulin resistance in muscle, fat, and liver, increases glucose-stimulated insulin secretion from islet β-cells by an unknown mechanism [4,6,29]. Islets isolated from insulin resistant rodents display left-shifted insulin response curves when compared with their control counterparts and inhibition of PI-3K is unable to further augment insulin release [28,29]. Taken together, these studies are consistent with a role for PI-3K in regulating the secretion of insulin. However, the biochemical and electrophysiological mechanisms underlying these effects on ion channel activity and insulin secretion are largely unexplored.

The biochemical events that stimulate insulin secretion by fuel metabolism include: (1) closure of ATP-sensitive
potassium channels, leading to a buildup of positive ions inside the cell; (2) opening of voltage-gated calcium channels, which provides an increase in intracellular calcium that is a signal for insulin release; (3) opening of voltage-dependent potassium channels, which serve to repolarize the β-cell membrane. The intracellular signaling molecules that contribute to the appropriate pattern of ion channel regulation and insulin secretion are just starting to become apparent [19,24,26]. It has been proposed that the β-cell recognizes how much insulin is currently present in its immediate surroundings by signals generated through the PI-3K portion of the insulin signaling pathway [23]. By this view, an insulin resistant animal would have defective PI-3K activity and would underestimate the insulin present leading to hypersecretion of insulin. We attempted to partially recreate a state of insulin resistance in vitro using acute pharmacologic inhibition of PI-3K in the INS-1-derived 832/13 rat insulinoma cell line. Insulin secretion assays were coupled to electrophysiological measurements under various concentrations of glucose and PI-3K inhibitors. Here we report that the PI-3K inhibitor LY294002, but not Wortmannin, inhibits voltage-dependent potassium channels, extends action potential duration, and thus enhances insulin release.

Materials and methods

Materials. LY294002 (a PI-3K inhibitor) was from Promega. All other reagents were from Sigma Chemical unless otherwise noted.

Cell culture and measurement of insulin secretion. The isolation, characterization, and culture of the INS-1-derived 832/13 cell line have been described [13]. Insulin output after secretagogue treatment in this study was measured by radioimmunoassay (RIA) using the ImmunoChem Coated Tube RIA kit (ICN Pharmaceuticals, Costa Mesa, CA) as described previously [13]. This assay detects both rat and human insulin. All RIA data are shown as absolute insulin output (μIU/mL/μg total protein).

Protein isolation and immunoblots. 832/13 cells were treated as described in the figure legends. At the appropriate time point, these cells were washed 2× with ice-cold PBS and then pelleted in PBS by centrifugation at 600g for 10 min. The resulting pellet was lysed in 1× Passive Lysis Buffer (Promega) with the following components added: Complete Protease Inhibitor Cocktail (Roche), 1 mM sodium orthovanadate (Sigma), 10 mM sodium pyrophosphate (Mallinkrodt), 10 μM cantharidic acid (Sigma), and 100 nM calyculin A (Cell Signaling). The proteins were quantitated using the BCA assay (Pierce Chemical Company) and separated on 10% polyacrylamide gels (Gradiopore), followed by transfer to PVDF membranes (Pierce Chemical). Blot immunolabeling was performed essentially as described [10], except that primary antibody incubations were for three hours and secondary antibody incubations were for 2 h, respectively. The antibodies used were Akt (Cell Signaling, catalog # 9272), PO4-Akt-S473 (Cell Signaling, catalog # 9271), and Tubulin (Sigma, catalog # T3559). Detection of tubulin served as a control for protein loading.

Electrophysiological recordings and data analysis. 832/13 pancreatic β-cells were cultured for 2 days on 12 mm glass coverslips in 35 mm Petri dishes in RPMI medium prior to electrophysiological recordings. After being passaged, cells were grown in the presence of 11 mM glucose and then switched to medium containing 5 mM glucose for 24 h prior to performing recordings (to maintain consistency with the insulin secretion measurements). For electrophysiological recordings, coverslips were transferred to a recording chamber mounted on an inverted microscope (Nikon Diaphot-TMD) and superfused with extracellular recording solution. All experiments were conducted at room temperature (22–25°C). Whole-cell voltage-clamp and current-clamp experiments were carried out using the standard Giga-seal patch-clamp method [9]. Recording electrodes were fabricated from 1.5 mm thin-walled borosilicate glass tubes (Garner Glass, Clermont, CA), using a Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA) and heat-polished (MF-83, Narishige Scientific Instruments, Tokyo, Japan) before use. Each of the pipettes used had a tip resistance of approximately 3 MΩ when filled with the internal solutions. Recordings were performed using Axoclamp 2B patch-clamp amplifier and Clampex8 software (Axon Instruments, Union City, CA). Data were filtered at 2 kHz, and data were acquired using Clampfit8 software (Axon Instruments).

Voltage-dependent Ca2+ currents were elicited in the continuous single-electrode voltage-clamp (eSEVC) mode using a protocol in which cells were held at −80 mV for 50 ms followed by 10 nS steps (100 ms) to 60 mV and returned to −80 mV (30 ms) after each step. For whole-cell voltage-clamp Ca2+ current recordings, the extracellular bath solution contained: 10 mM CaCl2, 110 mM tetraethylammonium–Cl (TEA–Cl), 10 mM CsCl, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes), and 0.5 mM 3,4-diaminopyridine, at pH 7.3. Depending on the experiment, glucose was present in the extracellular solution at concentrations of 2, 4, or 20 mM. The intracellular pipette solution contained: 130 mM N-methyl-d-glucamine, 20 mM EGTA (free acid), 5 mM bis2-aminophenoxyethane-N,N’,N,N’-tetraacetate (BAPTA), 10 mM Hepes, 6 mM MgCl2, and 4 mM CaCl2, with pH adjusted to 7.3 using KOH. Action potentials were elicited in the BRIDGE (current-clamp) mode by stimulating the cells with a 10 ms, 200 pA square-wave current. Recordings were made 1 min following establishment of the whole-cell configuration. For current-clamp recordings, the extracellular bath solution contained: 150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes at pH 7.3. Depending on the experiment, glucose was present in the extracellular solution at concentrations of 2, 4, or 20 mM. The intracellular pipette solution contained: 140 mM KCl, 10 mM NaCl, 2 mM MgCl2, and 5 mM Hepes at pH 7.4. Action potential duration was calculated as the difference in the time from initiation of the action potential until the time that the membrane potential returned to within 10 mV of the resting membrane potential. Data are expressed as averages ± SEM.

Statistical analysis. SPSS version 11.5 was used for a two-way ANOVA of the insulin secretion data. If significant differences were observed, a Student–Newman–Keuls test was performed post hoc to determine which means were different. GraphPad version 5.0 was used for a one-way ANOVA of the electrophysiological measurements data, with a Tukey’s multiple comparison test performed post hoc after indications of significance. Significance was assumed when p was <0.05.

Results and discussion

LY294002 and Wortmannin have distinct effects on insulin secretion

Islets isolated from insulin resistant rodents display left-shifted glucose-stimulated insulin secretion curves when compared to their control counterparts [28,29]. In addition, both Wortmannin and LY294002, inhibitors of PI-3K, have previously been shown to enhance...
insulin release from islets isolated from lean, but not obese, rodents [28,29]. Therefore, it is possible that rodent models of insulin resistance display maximal PI-3K inhibition. In an attempt to mimic this condition in vitro, we treated the robustly glucose-responsive INS-1-derived cell line 832/13 with pharmacologic inhibitors of PI-3K. We found that 50 μM LY294002 and 50 nM Wortmannin were sufficient to block the insulin-stimulated phosphorylation of Akt in these cells (Fig. 1A). Akt is a kinase that is downstream of PI-3K in the insulin signaling pathway [7]. Furthermore, we found that 50 μM LY294002, a quercetin-derived PI-3K inhibitor [27], increased the amount of insulin released (i.e., a left shift in the response curve) from 832/13 cells over a range of glucose concentrations from 2 to 16 mM (Fig. 1B). A particularly large enhancement occurred at 4 mM glucose, a concentration that does not normally evoke maximum amounts of insulin secretion [13], but one that does provide increased insulin secretion from β-cells isolated from animals in the prediabetic state [14]. Wortmannin, which is a structurally distinct inhibitor of the PI-3K family of enzymes, regulated insulin secretion differently than LY294002 (Fig. 1B). While Wortmannin appeared to increase glucose-stimulated insulin secretion at 4 mM glucose, but did not reach statistical significance (2-fold over vehicle control, *p < 0.09), it had no effect at stimulatory glucose concentrations. Overall, these observations agree with previous studies where rodent islets perfused with an intermediate glucose concentration in the presence of PI-3K inhibitors displayed augmented insulin secretion [28,29].

**LY294002, but not Wortmannin, produces prolonged action potential durations at submaximal glucose concentrations**

Membrane depolarization and repolarization is necessary for regulated insulin secretion [11,12,21]. In pancreatic β-cells, depolarization can result from either Na⁺ or Ca²⁺ influx, decreased K⁺ efflux, or release of Ca²⁺ from intracellular stores [2]. In an effort to determine how LY294002 augments β-cell insulin release, total cellular electrical activity (depolarization and repolarization) was examined using electrically induced action potentials. Increasing the glucose concentration from 4 to 20 mM lengthened the APD from 21 ± 4 to 62 ± 5 ms (*p < 0.001) without altering the
with LY294002 we measured whole-cell Ca\(^{2+}\) and K\(^{+}\) currents, which represent two of the major depolarizing and repolarizing currents in the MIN6 mouse insulinoma cell line. Specifically, we examined voltage-dependent calcium currents and voltage-dependent potassium currents occurred at +60 mV. To control for differences in cell size, data were normalized by dividing the measured current (pA) by cellular capacitance (pF). Data are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Resting (mV)</th>
<th>Peak (mV)</th>
<th>Action potential duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM glucose</td>
<td>−59 ± 13</td>
<td>40 ± 9</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>4 mM glucose + DMSO</td>
<td>−58 ± 14</td>
<td>48 ± 8</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>4 mM glucose + 50 nM Wortmannin</td>
<td>−58 ± 12</td>
<td>43 ± 9</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>4 mM glucose + 50 μM LY294002</td>
<td>−55 ± 10</td>
<td>43 ± 11</td>
<td>52 ± 4*</td>
</tr>
<tr>
<td>20 mM glucose</td>
<td>−59 ± 16</td>
<td>44 ± 10</td>
<td>62 ± 5*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

LY294002 does not increase currents through voltage-dependent Ca\(^{2+}\) channels

LY294002 treatment consistently enhanced insulin secretion in 832/13 cells throughout the concentration curve (Fig. 1A) and generated prolonged action potential durations (Fig. 2 and Table 1). In an attempt to explain the extended action potential durations observed with LY294002 we measured whole-cell Ca\(^{2+}\) and K\(^{+}\) currents in the 832/13 cells. Specifically, we examined voltage-dependent calcium currents and voltage-dependent potassium currents, which represent two of the major depolarizing and repolarizing currents in the β-cell [11,20,21]. The voltage-dependent Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) density in cells exposed to 4 mM glucose for 2 h measured 10 pA/pF ± 3 pA/pF (see Table 2), which did not differ from the I\(_{\text{Ca}}\) density in 832/13 cells following a 2-h incubation in the presence of 4 mM glucose plus LY294002 (12 pA/pF ± 4 pA/pF). Also, there was no difference in the I\(_{\text{Ca}}\) density when cells were exposed to DMSO (the vehicle control) in the presence of 4 mM glucose (10 pA/pF ± 2 pA/pF, data not shown).

In order to demonstrate that voltage-gated Ca\(^{2+}\) currents increase in response to a physiological stimulus, 832/13 cells were exposed to 20 mM glucose, an amount that can elicit maximal amounts of insulin secretion [13]. This treatment resulted in an I\(_{\text{Ca}}\) density of 42 pA/pF ± 8 pA/pF (Table 2), indicating that these cells behave similarly to isolated islets [16,25]. These results demonstrate that LY294002 treatment at submaximal glucose concentrations does not cause an increase in whole-cell voltage-dependent Ca\(^{2+}\) currents (Table 2). Therefore, the depolarizing effects of LY294002 must be due to the activity of another ion current.

LY294002 inhibits voltage-dependent potassium currents

Prolonged action potential durations can result from either increased depolarizing currents (e.g., voltage-dependent Ca\(^{2+}\) currents) or decreased repolarizing currents (e.g., voltage-gated K\(^{+}\) currents). Because LY294002 is a potent inhibitor of voltage-dependent K\(^{+}\) currents in the MIN6 mouse insulinoma cell line...
we next investigated whether the same effect was true of the INS-1-derived 832/13 rat insulinoma cells. This was important due to potential phenotypic differences that have been documented regarding secretory responses of rat and mouse islets [30]. Therefore, using the same amount of LY294002 (50 \mu M) that inhibits Akt and robustly stimulates insulin secretion (Fig. 1), we determined whether this concentration was also a potent inhibitor of voltage-dependent K^+ currents in 832/13 INS-1 cells. Treating 832/13 cells with 50 \mu M LY294002 in 4 mM glucose significantly (p < 0.001) reduces the peak voltage-dependent K^+ current density from 102 ± 35 to 23 ± 7 pA/pF (Fig. 3 and Table 2).

The peak voltage-dependent K^+ current density in cells treated with 20 mM glucose (77 ± 20 pA/pF) was not significantly different from the peak current density measured in cells treated with 4 mM glucose alone. These results are consistent with the prolonged action potential durations (Table 1) and demonstrate the ability of LY294002 to inhibit voltage-dependent potassium K^+ channels in INS-1 832/13 cells. A similar conclusion was reached in MIN6 cells wherein LY294002 and a non-PI-3K-inhibiting structural analog were found to block Kv currents, while Wortmannin did not recapitulate these effects [5]. Thus, the ability of LY294002 and structural derivatives to inhibit K^+ channels may represent a novel mechanism to enhance insulin secretion irrespective of fuel metabolism.

Summary

A key finding here is that the increased APD generated by treatment with LY294002 in the presence of 4 mM glucose closely mimics the APD produced by 20 mM glucose alone (Table 1). The most likely explanation for the prolonged APD seen with LY294002 treatment is inhibition of voltage-dependent K^+ channels (Fig. 3 and Table 2). Because cellular electrical activity is closely coupled with insulin secretion, we examined action potentials recorded from 832/13 cells. At low glucose concentrations, LY294002 caused prolongation of action potentials to the same degree as high (stimulatory) glucose concentrations. Wortmannin, a structurally distinct PI-3K inhibitor, did not reproduce these effects, thus suggesting that acute pharmacologic PI-3K inhibition alone is insufficient to produce changes in \( \beta \)-cell electrical activity in rat insulinoma cells. These results are in agreement with those of Wheeler and co-workers [5]. Together, these findings suggest that caution must be used when interpreting the results of PI-3K inhibitors, particularly in electrically excitable tissues, such as \( \beta \)-cells from the pancreatic islets.

Acknowledgments

We thank Dr. Christopher B. Newgard for 832/13 cells, Dr. T. Bruce Ferguson for use of his laboratory during a portion of these studies, Dr. Ming Li for helpful technical discussions, and Dr. Anna Brzezinska for electrophysiological resources. The authors thank Rebecca Shea for excellent technical assistance. We also thank Drs. William C. Claycomb and Christopher B. Newgard for critical comments on the manuscript. D.K.S. is supported by a Career Development Award from the American Diabetes Association.

Fig. 3. LY294002 blocks voltage-gated potassium (Kv) Currents. 832/13 cells were cultured on glass coverslips for 16 hours in medium containing 4 mM glucose prior to experiments. Using the whole-cell configuration of the patch-clamp technique in voltage-clamp mode, Kv currents were measured by applying voltage steps (500 ms) from –80 to +60 mV in 10 mV increments from a holding potential of ~80 mV. Representative current traces are shown for cells cultured for 10 min. (A) 4 mM glucose, (B) 20 mM glucose, (C) 4 mM glucose + 50 \mu M LY294002, or (D) 20 mM glucose + 50 mM LY294002. Current density (pA/pF) was used to normalize for cell size. Data collected from 9 or more individual experiments are tabulated in Table 2.
References


