Inositol Cycling and Phosphoinositide Metabolism in Rat Pancreatic Islets

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Abstract. The effects of extracellular inositol and LiCl on intra-islet inositol cycling were investigated in isolated rat islets. Islets were cultured for 7 days in inositol-free RPMI 1640 containing 11.1 mM glucose and labeled with 3.7 MBq myo-[2-3H]inositol for the final 3 days. The labeled islets were then perifused under various conditions. There was a persistent increase in [3H] efflux from labeled islets stimulated with 16.7 mM glucose for 60 min. Addition of 5 mM inositol resulted in marked release of [3H] from islets and a decrease in radioactive inositol-lipid. When islets were perifused with 5 mM LiCl, the glucose-induced efflux of [3H] was greatly inhibited. The inhibitory effect of LiCl on [3H] efflux was partially corrected by the addition of 5 mM inositol. A prominent effect of LiCl was an increase in inositol monophosphate, indicating increased phospholipase C activity. This was detected within 5 min after glucose stimulation. The present data suggest that there is always very active intra-islet inositol cycling and that glucose can augment inositol-lipid metabolism. (Endocrinol Japon 38: 23-31, 1991)

IT HAS been suggested that phosphoinositide (PI) hydrolysis in pancreatic islets plays an important role in regulating glucose-induced insulin release [1-3]. Diacylglycerol activation of protein kinase C results from PI breakdown. Another important second-messenger molecule, inositol-1,4,5-trisphosphate, has been implicated in the mobilization of intracellular calcium [4, 5]

Up to now, very few studies have tested whether inositol cycling lasts as long as insulin secretion occurs. Previous studies [6-8] showed that [3H] inositol-labeled islets fail to continue efflux of [3H] while high concentrations of glucose are present in the perifusate. In this context, the observation described by Clements and Rhoten [6] that glucose-induced [3H] efflux is transient and disappears before the second phase of insulin secretion, suggests that their labeling technique may have failed to equilibrate inositol lipid in the islets.

Axen et al. [7] indicated that only 53% of the effluent radioactivity was free inositol and that the rest was present in some of the phospholipidated components. This finding is in agreement with earlier observations by Clements et al. Thus, their labeling method might be inappropriate for labeling the inositol pool in the islets. We therefore investigated the effect of chronic labeling of islets with radioactive inositol on glucose-induced [3H] efflux. To clarify intra-islet inositol cycling in the various conditions, the effect of LiCl (5 mM) and exogenous inositol (5 mM) was also studied, since Zawalich et al. [9] showed that the addition of LiCl inhibits insulin release and that such inhibition is relieved by the addition of exogenous inositol. The present data indicate that LiCl and inositol profoundly alter inositol cycling in rat pancreatic islets.

Materials and Methods

Prelabeling of islet phospholipids
Islets from fed female Sprague-Dawley rats were isolated by collagenase digestion [10]. Islets
were cultured in inositol-free RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 11.1 mM glucose, 20 mM HEPES, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The culture dishes were subsequently incubated at 37°C in a humidified atmosphere composed of 5% CO₂ in air. The islets were transferred to new culture dishes containing 6.0 ml fresh culture medium every day. At 96 h the medium was replaced with 5.0 ml of medium of the same composition but containing 740 KBq/ml myo-[2-³H] inositol (Specific Activity 625.3 GBq/mmol, Amersham, Arlington Heights, IL). The islets were then maintained in culture without changing the medium for an additional 72 h prior to the experiment.

Static incubation

Islets were labeled with 740 KBq/ml myo-[2-³H] inositol for 3 days as mentioned above. To stabilize the islets, they were incubated in the same culture medium for 60 min under 95% O₂-5% CO₂ at 37°C. The labeled islets were then washed five times with modified Krebs Ringer bicarbonate (KRB) buffer containing 0.5 mg/ml glucose and 0.2 mg/ml fatty acid-free albumin (Sigma, St. Louis, MO). Batches of 150 islets were placed into 13 × 100 mm tubes and washed five times with 1 ml of KRB containing 2.8 mM glucose without unlabeled inositol. The batches were then incubated for 10 min six times with 0.5 ml of KRB-2.8 mM glucose either containing no inositol or 5 mM inositol (Sigma). The incubation medium was harvested every 10 min and analyzed.

Perfusion experiment

To measure the efflux of radioactive water-soluble inositol and inositol metabolites, islets prelabeled with [³H] inositol were divided after washing with KRB containing 2.8 mM glucose. 70–100 islets were placed on 8.0 µm millipore filters in perifusion chambers. Islets were perfused simultaneously at 37°C with continuously gassed (95% O₂-5% CO₂) KRB of the same composition as had been used for washing. Glucose was present in a concentration of 2.8 mM during the first 60 min of perifusion (equilibration period or basal perifusion) and was increased to 16.7 mM by turning the stopcock after 60 min to initiate stimulation. The effluent was collected over 5-min intervals for the initial 55 min and at 1 min intervals for the final 5 min. Perifusates were delivered at the rate of 1.0 ml/min with a peristaltic pump (Gilson). In presenting the data on effluents, no corrections have been made for the lag of approximately 3 min due to “dead-space” contributed by the 2.7 ml volume of the perifusion chamber and associated tubing. 5 mM inositol was present either during the equilibration period or the stimulation period or both as indicated in the tables and figures. The radioactivity of the effluent was determined in a 0.4 ml aliquot plus 10 ml scintillation fluid and counted in a liquid scintillation spectrometer. Values for efflux rates of water-soluble radioactivity in individual channels were expressed as % of mean values observed during the five one-min collections between the 56th and 60th min.

Extraction and separation of islet phospholipids and water-soluble inositol metabolites

After a 60 min equilibration period (i.e., t₀) and at the end of perifusion, the millipore filters were immediately removed from the chamber and introduced into 3 ml chloroform/methanol (1/1, v/v) containing carrier lipid (25 µg lipid phosphorus). Extracts were centrifuged, washed with 3.0 ml chloroform/methanol (1/1) and the supernatant combined with the original extract. The extract was taken to a volume of 10 ml with 3 ml chloroform and 1 ml methanol. Islet residue was washed with two 1.0 ml aliquots of water, and the washings were combined with the original extract and mixed. The phases were separated by centrifugation and the upper phase recovered as the islet water soluble fraction. The lower phase was then washed two times with half its volume of theoretical upper phase (0.9% NaCl/methanol/chloroform, 49/47/31, by volume). The washed lower phase was evaporated to dryness under a stream of N₂ and the lipid residue was taken up into a small volume of chloroform/methanol (1/1, v/v). An aliquot was dried and counted for total lipid.

The water-soluble extracts were dried, resuspended with water and applied to columns containing 1.0 ml of Dowex-1 (X8: formate form; Bio Rad). The phosphate esters were eluted by the stepwise addition of solutions containing increasing levels of formate as described by Berridge et al. [11]. The radioactivity of 0.5 ml fractions eluted from the columns was counted after the addition
of 10 ml scintillation fluid. Recovery of radioactivity was 87.7±2.5% of the total applied to the column.

Statistics

Statistical significance was determined by Student's t test for unpaired data or analysis of variance. P<0.05 was considered significant.

Results

To assess whether three days' labeling equilibrates inositol lipid in the islets in the culture condition, islets were incubated for only 2 h with 925 KBq myo-[2-3H] inositol and then perifused for 90 min; i.e., 30 min for equilibration and 60 min for stimulation, i.e., exactly the same method as described by Axen et al. [7]. As shown in Fig. 1, we confirmed their findings showing that [3H] efflux peaks at approximately 40 min and then gradually decreases. On the other hand, islets which had been cultured with 3.7 MBq myo-[2-3H] inositol for 3 days showed a persistent increase in efflux of [3H] until 60 min after stimulation with 16.7 mM glucose. This difference was not due to labeled radioactivity, since Axen et al. labeled islets with 925 KBq myo-[2-3H] inositol in 200 μl media and we labeled them with 740 KBq/ml, so that the total difference in radiation exposure was about 6-fold i.e., 4625 vs 740 KBq per ml. Therefore, it should be pointed out that basal perifusion removes most of the excess water-soluble material and that when the islets are stimulated for prolonged periods the lipid origin of [3H] efflux may

![Graph showing efflux of [3H] in islets](image)

**Fig. 1.** Islets were labeled with either 925 KBq for 120 min or 3.7 MBq myo-[2-3H] inositol for 3 days. Washed islets were perifused with KRB containing 2.8 mM glucose (G2.8) for 30 min and the medium was then switched to KRB containing 16.7 mM glucose (G16.7) for 60 min. Sixty μM inositol was present throughout the perifusion. At the end of perifusion, islets were extracted as described in “Materials and Methods”. Open circles indicate [3H] efflux from islets labeled for 3 days. Closed circles indicate [3H] from islets labeled for 120 min. Basal levels of [3H] efflux were 18.6 ± 6.1 and 51.9 ± 3.7 dpm/50 islets/min in short vs chronic labeling method. [3H] efflux at 40 min was 100.8 ± 25.8 and 159.8 ± 9.6 dpm/50 islet/min in short vs chronic labeling method. Results represent means for 4 separate observations.
Table 1. Tissue partition on [3H] from [3H] inositol labeled islets (DMP/150 islets)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>0</th>
<th>+inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>103700</td>
<td>104230</td>
</tr>
<tr>
<td>Water-Soluble</td>
<td>15930</td>
<td>16105</td>
</tr>
<tr>
<td>[3H] efflux 25 min</td>
<td>0</td>
<td>12245</td>
</tr>
<tr>
<td>Total count</td>
<td>119630</td>
<td>132580</td>
</tr>
</tbody>
</table>

The islets were labeled with 740 KBq/ml myo-[2-3H] inositol for 3 days and then 150 islets each were incubated in the same culture medium for 60 min under 95% O2-5% CO2. Islets were washed 5 times with 1 ml KRB containing 2.8 mM glucose, and 3 ml chloroform/methanol (1/1, v/v) was added regarded as being at to. Another batch of islets was incubated in 0.5 ml KRB and with 5 mM inositol for 10 min. Five additional incubations were repeated at 37°C under 95% O2-5% CO2. At the end of incubation, islets were extracted as described in "Materials and Methods". The data represent the mean of 2 separate observation.

The next set of experiments was carried out to determine how to process the islets prior to stimulation. For this purpose, static experiments were performed with serial washings with or without 5 mM inositol, as described in "Materials and Methods" (Table 1). The data show some reduction in lipid [3H] inositol in the presence of inositol carrier vis-a-vis non-inositol carrier. The efflux values indicate that the presence of inositol in the medium resulted in some outflow of [3H] and concomitant with a reduction in the labeled lipid inositol, suggesting the lipid hydrolysis is occurring. Cumulative [3H] effluxes during each 10 min incubation period were plotted (Fig. 2). The [3H] efflux was much greater in the presence of inositol than in its absence. The difference was apparent even during the first 10 min incubation and became greater during subsequent incubation.

These results were confirmed when labeled islets were perifused with KRB containing 2.8 mM glucose with or without inositol for 60 min (Table 2). The perifusion experiment provided a better steady state situation in which to characterize the effects of stimulation. At the end of basal perifusion for 60 min, islets were regarded as being at zero time (i.e., 0) and tissue partition of radioactivity was examined (Table 2). The fraction of islet radioactivity present in the form of a water-soluble fraction was substantially smaller, i.e., 14.5%, when prior perifusion was inositol-free than when inositol was present, in which case the water-soluble fraction averaged 23.3% of total radioactivity (p<0.01). When the two basal conditions are compared, it is suggested that there is some inositol recycling even under basal conditions. Analysis of the water-soluble fraction showed that the major products are free-inositol, glycerophosphoinositol (GPI) and inositol monophosphate.

Fig. 2. Cumulative efflux of [3H] during 60 min static incubation. Batches of 150 islets were incubated as described in Table 1. The radioactivity of an aliquot of the medium was counted and totaled. Darkly shaded columns represent cumulative efflux of [3H] in the absence of inositol. Open columns represent cumulative efflux of [3H] in the presence of 5 mM inositol. Each column represents the mean for 2 separate experiments.

Table 2. Tissue partition of [3H] from [3H] inositol labeled islets (DMP/10 islets)

<table>
<thead>
<tr>
<th>Perfusion Condition</th>
<th>Total Lipid</th>
<th>Water-Soluble Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2.8–INS → to (9)</td>
<td>85330 ± 1959</td>
<td>14528 ± 1958</td>
</tr>
<tr>
<td>G2.8+INS → to (7)</td>
<td>76699 ± 1645*</td>
<td>23308 ± 1646*</td>
</tr>
</tbody>
</table>

The islets were perifused with KRB containing 2.8 mM glucose (G2.8) with or without inositol (INS) for 60 min. At the end of perifusion, the islets were regarded as being at to and extracted as in "Materials and Methods". Results are provided in the form of mean ± SE of the number of observations given in parentheses. The total counts have been adjusted for 10×10^4 DPM/10 islets. +P<0.01.
Fig. 3. [3H] release from islets stimulated with glucose. Islets were cultured and labeled with 740 KBq/ml myo-[2-3H] inositol for 3 days, washed by perfusion with buffers containing 2.8 mM glucose for 60 min and then perfused with either 2.8 mM (G2.8) or 16.7 mM (G16.7) glucose. 5 mM inositol was present throughout perfusion. Results represent the mean ± SE for the number of observations given in parentheses.

In an earlier perfusion study, 5 mM inositol was employed throughout perfusion. As shown in Fig. 3, [3H] efflux remained low during perfusion when 2.8 mM glucose was present in the medium. When glucose concentrations were increased to 16.7 mM, [3H] release gradually increased and continued throughout the period of stimulation. When islets were challenged with 16.7 mM glucose and 5 mM inositol after basal perfusion without inositol, a significant rise in [3H] release occurred (Fig. 4). The magnitude of [3H] release was much greater in the presence of inositol than that in the absence of inositol during the stimulation period.

The total $[^{3}H]$ efflux during the 40 min stimulation period was expressed as $\sum_{i=0}^{40}$ efflux (Table 3). Efflux data indicated again that there is a much greater output of $[^{3}H]$ in the presence of 5 mM inositol than in inositol-free media. When islets were stimulated with 16.7 mM glucose under inositol-free conditions, total inositol lipid was reduced by 14.7% of $t_0$. On the other hand, a 33.3% reduction in PI occurred in the presence of inositol, and this reduction in inositol-phospholipid accounts for the increase in outflow of $[^{3}H]$, since the water-soluble fraction under the perfusion conditions examined did not change significantly. When inositol was present throughout perfusion, loss of $[^{3}H]$ from the inositol lipid fraction was less (26.5% of $t_0$) than when inositol was added only during the stimulation period (33.3% of $t_0$). This seems to agree with the finding that glucose-induced efflux of $[^{3}H]$ is enhanced more drastically in the presence of inositol only during the stimulation period and not throughout perfusion. Glucose stimulation did not have any significant effect on water-soluble products when islets were perfused in inositol-free medium (Table 4).

The next set of perfusions was performed in order to determine the effect of LiCl on glucose-induced $[^{3}H]$ efflux. As shown in Fig. 4, $[^{3}H]$ release was reduced in the presence of 5 mM LiCl and total 40-min $[^{3}H]$ efflux decreased to 49.3% (Table 3). In contrast, when 5 mM inositol was present in the perfusion medium, LiCl was less effective in inhibiting $[^{3}H]$ release. The most significant effect of LiCl was a 61.4% increase in the water-soluble fraction when compared with 16.7 mM glucose alone. This increase in the water-soluble fraction was accounted for by the increase in IMP as summarized in Table 4. As
expected on the basis of the results of $[^3]$H efflux, free inositol was profoundly decreased in the presence of 5 mM LiCl, especially in inositol-free medium, while IMP increased to the extent of 63.3±1.3% of the total count. LiCl's effect on IMP formation remained significant in the presence of inositol ($p<0.001$).

To test the time course of the effect of LiCl on inositol cycling, $[^3]$H inositol-labeled islets were perifused for 0, 5, 8, 15 and 40 min after basal perifusion. At the end of perifusion, lipids were extracted as described above. Although total lipid radioactivity decreased only gradually, the effect of LiCl was apparent within 5 min after stimulation (Fig. 5). Analysis of water-soluble components clearly demonstrated an effect of LiCl, indicating a decrease in free inositol and a marked increase in IMP.

**Discussion**

These studies demonstrate that there is very active intra-islet inositol cycling. The efflux of $[^3]$H from islets was studied both in the presence and
Table 3. Tissue partition of \(^{3}H\) from \(^{3}H\) inositol labeled islets (DMP/10 islets)

<table>
<thead>
<tr>
<th>Perfusion Condition</th>
<th>Total Lipid</th>
<th>Water-soluble Fraction</th>
<th>Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2.8–INS → G16.7–INS</td>
<td>(6) 72972±2705</td>
<td>19207±1395</td>
<td>7821±1666</td>
</tr>
<tr>
<td>G2.8–INS → G16.7+INS</td>
<td>(4) 57090±5839</td>
<td>22642±2576</td>
<td>20269±6834</td>
</tr>
<tr>
<td>G2.8–INS → G16.7–INS+Li</td>
<td>(4) 69082±2945</td>
<td>30997±3881</td>
<td>3125±115</td>
</tr>
<tr>
<td>G2.8+INS→ G16.7+INS</td>
<td>(7) 56392±3326</td>
<td>22844±2310</td>
<td>20764±1970</td>
</tr>
<tr>
<td>G2.8+INS→ G16.7+INS+Li</td>
<td>(3) 55690±837</td>
<td>27664±1818</td>
<td>15646±1632</td>
</tr>
</tbody>
</table>

Islets were perifused with KRB containing 2.8 mM glucose (G2.8) in the presence or absence of 5 mM inositol (INS) for 60 min. Perifusion media were then switched and islets were perifused for 40 min. Tissue partition of \(^{3}H\) radioactivity was analyzed as described in “Materials and Methods”. The results represent means ± SE for the number of observations given in parentheses. a: P<0.02, b: P<0.01, c: P<0.001 vs. G2.8–INS → G16.7–INS. The total counts have been adjusted for 10×10^4 DPM/10 islets.

Table 4. Analysis of water-soluble fractions

<table>
<thead>
<tr>
<th>Perfusion Condition</th>
<th>Inositol</th>
<th>IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) G2.8–INS → to (6)</td>
<td>54.7 ± 2.2</td>
<td>8.6 ± 2.1</td>
</tr>
<tr>
<td>2) G2.8–INS → G16.7–INS (6)</td>
<td>57.9 ± 6.4</td>
<td>6.0 ± 1.6</td>
</tr>
<tr>
<td>3) G2.8–INS → G16.7–INS+Li (4)</td>
<td>20.6 ± 5.1</td>
<td>63.3 ± 1.3</td>
</tr>
<tr>
<td>4) G2.8–INS → G16.7+INS (4)</td>
<td>70.8 ± 3.1</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>5) G2.8+INS → to (6)</td>
<td>66.3 ± 3.9</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>6) G2.8+INS → G16.7+INS (6)</td>
<td>76.4 ± 3.1</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>7) G2.8+INS → G16.7+INS+Li (5)</td>
<td>58.8 ± 5.9</td>
<td>28.0 ± 4.2</td>
</tr>
</tbody>
</table>

P values

1) vs 5) P < 0.05
2) vs 3) P < 0.01
2) vs 4) N.S.
3) vs 7) P < 0.01
6) vs 7) P < 0.05

IMP: inositol monophosphate. N.S. = not significant.

Water-soluble fractions were applied to resin columns as in “Materials and Methods”. Each fraction was counted and results expressed as % of total count. Results are shown as mean ± SE of the number of observations given in parentheses.

Absence of inositol. The efflux pattern in response to glucose stimulation was neither monophasic nor biphasic, whether or not high concentrations of inositol were added to the perfusion medium. When the glucose concentration was increased to 16.7 mM, release of \(^{3}H\) gradually increased and continued throughout the period of stimulation. This is in sharp contrast to the results observed by Clements et al. [6] and Axen et al. [7] who found a fall-off in the outflow of radioactivity. We observed the same results when islets were pre-labeled for only 2 h and perfused. The difference in the labeling technique may be very important, since water-soluble components must be derived from equivalently labeled pools of intracellular lipid. Conceivably, if equilibrium labeling had not occurred, there could be a breakdown of PI which had not been labeled as well as some PI which had been labeled more intensively. It might also be important to show whether there is slowly exchanging pool of PI that becomes important in the prolonged second phase of insulin secretion. It remains unclear, however, whether the chronic labeling method brings all of the inositol in the islets to a specific activity equilibrium, i.e. brings the specific activity of every inositol containing compound in the islets to the same specific activity as that of the medium. Proof will depend on establishing that the specific activity of PI is the same as that of the free inositol in the medium.

Even under steady state conditions, some out-
flow of $[^3H]$ in the absence of extracellular inositol was observed (Fig. 2). When 5 mM inositol was added during the incubation period, $[^3H]$ efflux was markedly enhanced, and this is attended by a reduction in radioactive lipid inositol. Since all of this occurred under 2.8 mM glucose conditions, it is likely that there is a very active turnover of lipid inositol going on all the time, as much as approximately 10% per h (Tables 1 and 2).

When islets were stimulated with glucose under inositol free condition, in other words, in inositol starvation, the efflux was very small and reduction of inositol lipid was only 14.7% of $t_0$ (Tables 2 and 3). These observations indicate that changing patterns in the outflow of radioactivity from prelabeled islets is an indication of the magnitude of the recycling process.

The next set of experiments was performed to see what pathway would be activated when PI turnover was stimulated. At this point, we used LiCl as a tool to prevent inositol recycling, since Berridge et al. [12] clearly demonstrated such an effect in the salivary glands. As shown in Fig. 4, 5 mM LiCl reduced the glucose-stimulated efflux of $[^3H]$ in the absence of inositol. This effect of LiCl was reversible, because when the perifusion medium was switched to glucose alone, the efflux of $[^3H]$ slowly recovered (data not shown). This inhibitory effect on efflux is consistent with the hypothesis that LiCl acts by blocking the inositol monophosphatase that converts IMP into free inositol. Another consequence of inhibiting this enzyme is that IMP would accumulate in the cell. Analysis of the water-soluble fraction clearly showed that LiCl reduces free-inositol and increases IMP (Table 4). Thus, it is possible to unequivocally demonstrate the presence of phospholipase C activity in the islets. However, the degradation product is usually cleaved so rapidly that the pool is very small except when blocked by the pharmacological technique which LiCl provides.

As shown in Figure 5, IMP increased dramatically in the presence of LiCl within 5 min after stimulation. This was associated with the peak of the first phase of glucose-induced insulin release (data not shown). Since the dead space of our perifusion system is 2.7 ml, significant hydrolysis of PI already occurred within approximately 3 min. These data provide unassailable evidence that some breakdown had occurred. It may well be the most sensitive index for determining the magnitude of IMP production reflecting the hydrolysis of PI. In other words, IMP can be used as index of inositol lipid hydrolysis.

We were unable to document any precise functional role of intra-islet inositol cycling in regulating insulin release. Recent data from many laboratories, however, indicate a high association of lipid inositol with B cell recognition and processing sites as well as possible recycling of this inositol during glucose-stimulation [13]. Further work is obviously necessary to resolve the mechanism underlying PI metabolism in the pancreatic islets.

Acknowledgments

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References