Acute and chronic effects of glucose and carbachol on insulin secretion and phospholipase C activation: studies with diazoxide and atropine

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Yamazaki, Hanae, William Philbrick, Kathleen C. Zawalich, and Walter S. Zawalich. Acute and chronic effects of glucose and carbachol on insulin secretion and phospholipase C activation: studies with diazoxide and atropine. Am J Physiol Endocrinol Metab 290: E26–E33, 2006. First published August 16, 2005; doi:10.1152/ajpendo.00149.2005.—The acute and chronic effects of 20 mM glucose and 10 μM carbachol on β-cell responses were investigated. Acute exposure of rat islets to 20 mM glucose increased glucose usage rates and resulted in a large insulin-secretory response during a dynamic perfusion. The secretory, but not the metabolic, effect of 20 mM glucose was abolished by simultaneous exposure to 100 μM diazoxide. Glucose (20 mM) significantly increased inositol phosphate (IP) accumulation, an index of phospholipase C (PLC) activation, from [3H]inositol-prelabeled islets. Diazoxide, but not atropine, abolished this effect as well. Unlike 20 mM glucose, 10 μM carbachol (in the presence of 5 mM glucose) increased IP accumulation but had no effect on insulin secretion or glucose (5 mM) metabolism. The IP effect was abolished by 50 μM atropine but not by diazoxide. Chronic 3-h exposure of islets to 20 mM glucose or 10 μM carbachol profoundly reduced both the insulin-secretory and PLC responses to a subsequent 20 mM glucose stimulus. The adverse effects of chronic glucose exposure were abolished by diazoxide but not by atropine. In contrast, the adverse effects of carbachol were abolished by atropine but not by diazoxide. Prior 3 h of exposure to 20 mM glucose or carbachol had no inhibitory effect on glucose metabolism. Significant secretory responses could be evoked from 20 mM glucose- or carbachol-pretreated islets by the inclusion of forskolin. These findings support the concept that the early event in the evolution of β-cell desensitization is the impaired activation of islet PLC.

Hyperglycemia exerts a time-dependent, adverse effect on the secretory responsiveness of the pancreatic β-cell (9, 10, 22, 52), and a number of maneuvers have been used to interfere with this adverse action (11). Most recently, it has been suggested that diazoxide, a compound that abolishes insulin secretion by preventing closure of the ATP-sensitive potassium (KATP) channel, might prove to be useful in the therapy of hyperglycemia (10, 11). These compounds include the cholinergic agonist carbachol (53), the sulfonylurea tolbutamide (41), and the fuel secretagogue glycosamine (46), and the adenylate cyclase activator forskolin (44). What these compounds share in common is the ability, after chronic exposure to them, to impair glucose-induced activation of islet phospholipase C (PLC) (43, 49, 52). The activation of this enzyme generates a number of second messengers, including diacylglycerol and inositol phosphates (IP), thought to be intimately involved in the regulation of secretion (28).

Our interest in the role played by PLC in the acute regulation of secretion from control or desensitized islets prompted these studies. Because diazoxide has been advocated in the therapy of diabetes, used to provide a window of rest and recovery, emphasis was also placed on its impact on this signal transduction pathway. Our findings emphasize the significance of PLC in the time-dependent effects that both glucose and cholinergic stimulation exert on the β-cell.

Materials and Methods

The detailed methodologies employed to assess insulin output from collagenase-isolated islets have been previously described (48, 50). Male Sprague-Dawley rats (weighing 300–375 g) were purchased from Charles River and used in all studies. All animals were treated in a manner that complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the Yale University Animal Care and Use Committee approved all studies. The animals were fed ad libitum. After Nembutal (pentobarbital sodium, 50 mg/kg; Abbott, North Chicago, IL)-induced anesthesia, islets were isolated by collagenase digestion and handpicked, using a glass loop pipette, under a stereomicroscope into Krebs-Ringer bicarbonate (KRB) supplemented with 3 mM glucose. They were free of exocrine contamination.

Perfusion studies. Islets were loaded onto nylon filters (Sefar America, Kansas City, MO). Some groups were immediately perfused after the isolation. The islets were perfused in a KRB buffer at a flow rate of 1 ml/min for 30 min in the presence of 5 mM glucose to establish basal and stable insulin secretory rates. After this 30-min stabilization period they were then perfused with the appropriate agonist or agonist combinations, as indicated in the figure legends and in Results. Perifusate solutions were gassed with 95% O2–5% CO2 and maintained at 37°C. Insulin released into the medium was measured by radioimmunoassay (RIA) (1).

Other groups of islets, after being loaded onto nylon filters, were incubated for 3 h. The filter, with islets attached, was placed in a small glass vial. KRB solution (240 μl), supplemented with the compounds listed in Results, was then added. The vials were gently aerated for 10 s with 95% O2–5% CO2 and maintained at 37°C. After 90 min they were aerated again for 10 s. After 3 h they were then perfused as described above.

Islet labeling for IP studies. Groups of 18–26 islets were loaded onto nylon filters and incubated for 1.5–3 h in a myo-2-[3H]inositol-containing KRB solution that was made up as follows: 10 μCi of...
myo-2-[3H]inositol (specific activity 16–23 Ci/mmol) were placed in a 10 × 75-mm culture tube. To this aliquot of tracer, 255 μl of warmed (to 37°C) and oxygenated KRB medium supplemented with 5 mM glucose ± 10 μM carbachol or 20 mM glucose were added. In additional studies, this incubation medium was supplemented with 100 μM diazoxide or 50 μM atropine as indicated. After mixing, 240 μl of this solution were gently added to the vial with islets. The vial was capped with a rubber stopper, gassed for 10 s with 95% O2–5% CO2, and placed in a metabolic shaker at 37°C. After 90 min, the vials were again gently oxygenated. After the labeling period, the islets were washed with 5 ml of fresh KRB and used for IP measurements.

IP studies. After a washing with 5 ml of fresh KRB to remove free labeled inositol, the islets on nylon filters were placed in small glass vials. Added gently to the vial were 400 μl of warmed (to 37°C) KRB supplemented with 10 mM LiCl to prevent IP degradation and the appropriate agonists as indicated. The vials were capped, and after 30 min the generation of IPs was stopped by adding 400 μl of 20% perchloric acid. Total IPs formed were then measured using Dowex columns, as described previously (6, 38).

Efflux studies. Islets were labeled as described above for the IP studies. However, after the 3-h incubation, these islets were subsequently perfused. Samples (200 μl) were analyzed every 2 min for [3H]inositol radioactivity from minutes 28 to 70 of the perfusion. Fractional efflux rates were calculated as described previously (12, 37, 39).

Glucose utilization rates. The usage of glucose was measured by determining the rate of [3H2O] formation from [5-3H]glucose. After isolation, groups of 15–20 islets were incubated in 125 μl of 5–20 mM glucose supplemented with tracer [5-3H]glucose. In some experiments, diazoxide or carbachol was also included where indicated. The [3H2O] formed during the subsequent 1-h incubation was separated from the unused [3H]glucose as described previously (36). In additional studies, islets were first incubated for 3 h with 5 mM glucose alone, 20 mM glucose alone, or 10 μM atropine plus 5 mM glucose before usage of 20 mM glucose was measured.

Total islet insulin. After the perfusion, the islets still on filters were retrieved and placed in small glass vials. Hanks’ balanced salt solution was used for the islet isolation. The perfusion medium consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM CaCl2, 1 mM MgCl2, 5 mM NaHCO3, and 0.17 g/dl bovine serum albumin. The [125I]-labeled insulin for the insulin assay, [3H]inositol, [5-3H]glucose, and [3H2O] for the glucose usage studies were purchased from PerkinElmer Life Sciences (Boston, MA). Bovine serum albumin (RIA grade), glucose, carbachol, atropine, diazoxide, and the salts used to make the Hanks’ balanced salt solution and perfusion medium were purchased from Sigma (St. Louis, MO). Forskolin was purchased from Calbiochem (La Jolla, CA). Rat insulin standard (lot no. 615-ZS-157) was the generous gift of Dr. Gerald Gold (Eli Lilly, Indianapolis, IN). Collagenase (type P) was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Statistics. Statistical significance was determined using Student’s t-test for unpaired data or analysis of variance. A P value of ≤0.05 was taken as significant. Values presented in the figures and in RESULTS represent means ± SE of at least three observations.

RESULTS

Acute effects of 20 mM glucose and 10 μM carbachol on insulin secretion, IP accumulation, and glucose usage. In the initial series of experiments, the impact of stimulating islets with 20 mM glucose was compared with the impact of stimulating islets with 10 μM carbachol (in the presence of 5 mM glucose). As shown in Fig. 1, 20 mM glucose stimulation resulted in a dramatic augmentation of insulin release from perfused islets. Compared with prestimulatory rates of ~30 pg·islet−1·min−1, insulin secretion increased to >600 pg·islet−1·min−1 during the second phase response. The inclusion of 100 μM diazoxide completely abolished insulin secretion in response to glucose, whereas the muscarinic antagonist atropine (50 μM) had no effect (Fig. 1). In the presence of 5 mM glucose, the addition of 10 μM carbachol to the perfusion medium failed to increase the secretion of insulin.

In additional studies, the acute impact of glucose or carbachol on IP accumulation was determined in [3H]inositol-prelabeled islets. Levels of labeled IP averaged ~5,100 cpm/40 islets after 30 min of incubation (Table 1, line 1). Both 10 μM carbachol and 20 mM glucose resulted in significant increments in IP accumulation (Table 1, lines 2 and 3). Similarly to its inhibitory effect on glucose-induced insulin secretion, the addition of diazoxide abolished the stimulatory effect of 20 mM glucose on IP accumulation (Table 1, line 4).

In additional studies, the usage of glucose in the presence of diazoxide or carbachol was determined. After isolation, groups of islets were incubated for 1 h with [5-3H]glucose in the presence of different glucose levels. In the presence of 5 mM glucose alone, usage rates averaged 65.7 ± 2 pmol·islet−1·h−1 (n = 4). Carbachol (10 μM), despite increasing IP accumulation, had no effect on glucose usage rates that now averaged 65.2 ± 4 pmol·islet−1·h−1 (n = 5). In the presence of 20 mM glucose alone, usage rates averaged 220 ± 1 pmol·islet−1·h−1 (n = 6). Despite its marked inhibitory effect on secretion, the addition of 100 μM diazoxide did not adversely impact glucose usage. It now averaged 203 ± 9 pmol·islet−1·h−1 (n = 6).

Chronic effects of 20 mM glucose and 10 μM carbachol on insulin secretion, IP accumulation, and glucose usage. The primary goal of the present studies was to determine the
Table 1. **Inositol phosphate accumulation**

<table>
<thead>
<tr>
<th>Line</th>
<th>Labeling Protocol (3 h)</th>
<th>Stimulation (30 min)</th>
<th>IP Accumulation (cpm/30 min -1/40 islets -1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G5</td>
<td>G5</td>
<td>5,126 ± 478 (n=9)</td>
</tr>
<tr>
<td>2</td>
<td>G5</td>
<td>G5 + carbachol 10 μM</td>
<td>11,696 ± 565 (n=10)</td>
</tr>
<tr>
<td>3</td>
<td>G5</td>
<td>G20</td>
<td>17,076 ± 885 (n=8)</td>
</tr>
<tr>
<td>4</td>
<td>G5</td>
<td>G20 + diaz. 100 μM</td>
<td>6,478 ± 979 (n=5)</td>
</tr>
<tr>
<td>5</td>
<td>G5 + carbachol 10 μM</td>
<td>G5</td>
<td>5,439 ± 348 (n=5)</td>
</tr>
<tr>
<td>6</td>
<td>G5 + carbachol 10 μM</td>
<td>G20</td>
<td>11,005 ± 618 (n=6)</td>
</tr>
<tr>
<td>7</td>
<td>G5 + carbachol 10 μM</td>
<td>G20 + diaz. 100 μM</td>
<td>6,645 ± 295 (n=3)</td>
</tr>
<tr>
<td>8</td>
<td>G5 + carbachol 10 μM</td>
<td>G20</td>
<td>11,005 ± 968 (n=4)</td>
</tr>
<tr>
<td>9</td>
<td>G5</td>
<td>G5</td>
<td>6,205 ± 470 (n=3)</td>
</tr>
<tr>
<td>10</td>
<td>G20</td>
<td>G20</td>
<td>8,037 ± 812 (n=3)</td>
</tr>
<tr>
<td>11</td>
<td>G20 + atropine 50 μM</td>
<td>G20</td>
<td>9,512 ± 613 (n=3)</td>
</tr>
<tr>
<td>12</td>
<td>G20 + diaz. 100 μM</td>
<td>G20</td>
<td>10,658 ± 814 (n=4)</td>
</tr>
</tbody>
</table>

Values are means ± SE. G5, 5 mM glucose; diaz, diazoxide; G20, 20 mM glucose. Groups of islets were incubated for 3 h with [3H]inositol to label their phosphoinositide pools. Also included during the labeling period were the compounds indicated in the 2nd column. Inositol phosphate (IP) accumulation (column 3) was then assessed. Statistical analyses: line 1 vs. lines 2 and 3; line 3 vs. line 4; line 3 vs. lines 6, 10, 11, and 12 all significant.

conssequences of prolonged exposure to glucose or carbachol on islet physiology. With the control data described above serving as the backdrop, additional studies were conducted. In these experiments, islets were isolated and before the perfusion were incubated for 3 h with 5 mM glucose (control), 5 mM glucose plus 10 μM carbachol, or 20 mM glucose. To disrupt the activation of PLC during this time, atropine or diazoxide was included in some studies.

Control islets incubated for 3 h with 5 mM glucose and subsequently perfused with 20 mM glucose responded, as anticipated, with brisk increment in insulin secretion (Fig. 2). Peak second-phase release rates averaged 375–400 pg·islet -1·h -1 (n = 20). When 20 mM glucose was included during the 3 h incubation, the subsequent secretory response to 20 mM glucose was dramatically deranged. For example, although control islets released insulin at peak rates approaching 400 pg·islet -1·h -1, this value now averaged only 80–90 pg·islet -1·h -1 (n = 10). A similar lesion in the secretory response to 20 mM glucose was also induced by chronic 3 h exposure to 10 μM carbachol as well. Peak release rates now averaged 120–130 pg·islet -1·h -1 (n = 14; Fig. 2).

Attempts were made to block the desensitizing impact of prior 20 mM glucose or carbachol exposure on the insulin secretory process. Diazoxide (100 μM) inclusion, together with 20 mM glucose for the 3-h period before the perfusion, completely abolished the adverse effect of chronic hyperglycemia on islet responsiveness (Fig. 3). In sharp contrast, 50 μM atropine exerted no protective effect against chronic hyperglycemia.

When carbachol was employed to desensitize islets, atropine (50 μM, n = 7), but not diazoxide (100 μM, n = 10; results not shown), significantly altered the adverse impact of sustained cholinergic stimulation on subsequent islet responses to 20 mM glucose (Fig. 4). Most dramatic was atropine’s protective effect on sustained second-phase secretion rates. Peak second phase release rates to 20 mM glucose measured during the subsequent perfusion now averaged >400 pg·islet -1·h -1, a response similar to that evoked from control islets incubated for 3 h with 5 mM glucose alone.

![Fig. 2. Insulin secretion from islets incubated for 3 h with 20 mM glucose or carbachol. Groups of islets were isolated and incubated for 3 h with 5 mM glucose alone (G5, ○, control), 20 mM glucose (G20, ▲), or 5 mM glucose + 10 μM carbachol (G5 + C, ◦), as indicated in MATERIALS AND METHODS and RESULTS. After that, islets were perfused for 30 min with 5 mM glucose before stimulation with 20 mM glucose for 40 min (onset of stimulation indicated by vertical line). Values are given as means ± SE. *Significant (P < 0.05) difference between experimental groups and control release values at this time.](http://ajpendo.physiology.org/)

![Fig. 3. Effect of diazoxide on 20 mM glucose-induced desensitization of insulin secretion. Two groups of islets were studied. One group (▲, n = 10; same data as in Fig. 2) was incubated for 3 h with 20 mM glucose before perfusion; the other group (○, n = 6) was incubated for 3 h with 20 mM glucose + 100 μM diazoxide before stimulation with 20 mM glucose during subsequent perfusion. *Significant (P < 0.05) difference between experimental groups at these time points.](http://ajpendo.physiology.org/)
Additional studies were then conducted to explore the effects of chronic hyperglycemia and cholinergic stimulation on IP accumulation in islets. In these studies, control islets were incubated for 3 h with 5 mM glucose plus [3H]inositol. In addition to the label, various compounds listed in Table 1 were also included during the incubation. After a washing, IP levels in response to various stimulants were determined. Compared with islets labeled in the presence of 5 mM glucose, inclusion of 10 μM carbachol or 20 mM glucose during this period significantly reduced IP responses to a subsequent 20 mM glucose stimulus (Table 1, line 3 vs. lines 6 and 10). Atropine protected islet IP responses against the adverse impact of carbachol but had no such beneficial action against 20 mM glucose (Table 7, lines 6, 7, and 11). Diazoxide had no effect on carbachol-induced desensitization of IP responses (Table 1, line 6 vs. line 8).

In our studies exploring the potential protective effect of diazoxide on 20 mM glucose-induced desensitization of IP accumulation, little protective effect was noted (Table 1, line 12). We reasoned that the cellular accumulation of diazoxide during the 3-h incubation and our failure to completely remove it during the brief washing period was in all likelihood responsible for this observation. Thus its persistence during the subsequent 30-min stimulation period with 20 mM glucose may have masked any potential protective effect of diazoxide against 20 mM glucose-induced desensitization of PLC. To address this issue, islets were labeled with [3H]inositol in the presence of 20 mM glucose ± 100 μM diazoxide. After this 3-h period, islets were perfused for 30 min with 5 mM glucose alone and then stimulated for 40 min with 20 mM glucose. We previously observed that this type of protocol allowed us to establish that diazoxide completely protected the secretory process from the adverse impact of chronic, 20-mM glucose exposure (see Fig. 3). The efflux results are presented in Fig. 5. By use of this approach to measure PLC activation, 100 μM diazoxide provided complete protection against the adverse effects of 20 mM glucose on the subsequent PLC response.

In studies where the effects of prior carbachol or 20 mM glucose exposure on glucose utilization were determined, we found that despite profound reductions in PLC activation, neither compound had any adverse effect on glucose usage rates. Islets exposed to 5 mM glucose for 3 h and then 20 mM glucose for 1 h utilized glucose at the rate of 175 ± 26 (n = 5) pmol·islet⁻¹·h⁻¹. Prior exposure to 10 μM carbachol or 20 mM glucose for 3 h resulted in 20 mM glucose usage rates of 171 ± 22 (n = 3) or 182 ± 15 (n = 6) pmol·islet⁻¹·h⁻¹, respectively.

Effects of chronic 20 mM glucose and carbachol exposure on insulin secretion: effects of forskolin. As shown in Table 2,

### Table 2. Insulin content in isolated islets

<table>
<thead>
<tr>
<th>Line</th>
<th>3-h Incubation</th>
<th>Perfusion</th>
<th>Total Insulin (μg/islet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G5</td>
<td></td>
<td>161 ± 11</td>
</tr>
<tr>
<td>2</td>
<td>G20</td>
<td></td>
<td>81 ± 13*</td>
</tr>
<tr>
<td>3</td>
<td>G20 + diazoxide</td>
<td>G5 &gt; G20</td>
<td>136 ± 17</td>
</tr>
<tr>
<td>4</td>
<td>G5 + carbachol</td>
<td>G5 &gt; G20</td>
<td>129 ± 12</td>
</tr>
<tr>
<td>5</td>
<td>G5 + carbachol + diazoxide</td>
<td>G5 &gt; G20</td>
<td>132 ± 10</td>
</tr>
<tr>
<td>6</td>
<td>G20 + atropine</td>
<td>G5 &gt; G20</td>
<td>75 ± 9*</td>
</tr>
<tr>
<td>7</td>
<td>G5 + carbachol + atropine</td>
<td>G5 &gt; G20</td>
<td>142 ± 18</td>
</tr>
<tr>
<td>8</td>
<td>G20</td>
<td>G5 &gt; G20 + forskolin</td>
<td>66 ± 10*</td>
</tr>
<tr>
<td>9</td>
<td>G5 + carbachol</td>
<td>G5 &gt; G20 + forskolin</td>
<td>129 ± 10</td>
</tr>
</tbody>
</table>

Groups of rat islets were isolated and incubated for 3 h as indicated. They were then perfused for 30 min with 5 mM glucose (G5) and for an additional 40 min with 20 mM glucose (G20) ± the indicated additions. After this, total islet insulin content was determined. *Significant difference between this and line 1.
compared with islets exposed to 5 mM glucose, prior 3-h exposure to 20 mM glucose, but not carbachol, significantly reduced insulin stores when measured after the perifusion (Table 1, line 1 vs. lines 3 and 4). Similarly to its protective effect on both secretion and PLC activation, diazoxide, but not atropine, prevented this effect (line 2). It might be reasonably argued that the underlying secretory lesion induced by chronic hyperglycemia is simply a result of lowered insulin stores in the islet. To test this hypothesis, islets were exposed to 20 mM glucose or 10 μM carbachol for 3 h and then stimulated during the dynamic perifusion with the combination of 20 mM glucose plus 500 nM forskolin (32). Forskolin exerted a significant restorative effect on these islets, an effect that was significant, regardless of the protocol used to desensitize them (Fig. 6). For example, islets desensitized by prior exposure to 20 mM glucose or 10 μM carbachol for 3 h released insulin at rates of ~100 pg·islet⁻¹·h⁻¹ during peak second phase secretion (Fig. 6). After 3 h of exposure to 20 mM glucose, the addition of 500 nM forskolin during the subsequent perifusion with 20 mM glucose increased the release rate to 300–400 pg·islet⁻¹·min⁻¹ (n = 6; Fig. 6A). Even more dramatic was the impact of forskolin on carbachol-desensitized islets that were stimulated with 20 mM glucose (Fig. 6B). Peak second-phase release rates of 700–800 pg·islet⁻¹·min⁻¹ (n = 7) were noted.

It should be emphasized, however, that, although 500 nM forskolin exerted a strong stimulatory effect on 20 mM glucose-desensitized islets, it did not normalize secretion from them. For example, control islets maintained for 3 h with 5 mM glucose and subsequently stimulated with the combination of 20 mM glucose plus 500 nM forskolin displayed greater secretory responses than those evoked from desensitized islets. During the final 20 min of stimulation, release rates to the combination of glucose plus forskolin averaged 1.002 ± 0.05 pg·islet⁻¹·min⁻¹ (n = 4; results not shown) from these islets.

Time dependence of 20 mM glucose-induced desensitization of PLC activation and insulin secretion. In the final series of experiments, we explored the impact of reducing the exposure compared with islets exposed to 5 mM glucose, prior 3-h exposure to 20 mM glucose, but not carbachol, significantly reduced insulin stores when measured after the perifusion (Table 1, line 1 vs. lines 3 and 4). Similarly to its protective effect on both secretion and PLC activation, diazoxide, but not atropine, prevented this effect (line 2). It might be reasonably argued that the underlying secretory lesion induced by chronic hyperglycemia is simply a result of lowered insulin stores in the islet. To test this hypothesis, islets were exposed to 20 mM glucose or 10 μM carbachol for 3 h and then stimulated during the dynamic perifusion with the combination of 20 mM glucose plus 500 nM forskolin (32). Forskolin exerted a significant restorative effect on these islets, an effect that was significant, regardless of the protocol used to desensitize them (Fig. 6). For example, islets desensitized by prior exposure to 20 mM glucose or 10 μM carbachol for 3 h released insulin at rates of ~100 pg·islet⁻¹·h⁻¹ during peak second phase secretion (Fig. 6). After 3 h of exposure to 20 mM glucose, the addition of 500 nM forskolin during the subsequent perifusion with 20 mM glucose increased the release rate to 300–400 pg·islet⁻¹·min⁻¹ (n = 6; Fig. 6A). Even more dramatic was the impact of forskolin on carbachol-desensitized islets that were stimulated with 20 mM glucose (Fig. 6B). Peak second-phase release rates of 700–800 pg·islet⁻¹·min⁻¹ (n = 7) were noted.

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Time dependence of 20 mM glucose-induced desensitization of PLC activation and insulin secretion. In the final series of experiments, we explored the impact of reducing the exposure
period to 20 mM glucose from 3 h to 1.5 h. The results are given in Fig. 7. A prior 90-min exposure period to 20 mM glucose resulted in significant reductions in the ability of a subsequent 20 mM glucose stimulus to activate PLC, monitored by fractional efflux rates, and stimulate insulin secretion.

DISCUSSION

The β-cell odyssey from being exquisitely glucose sensitive to glucose insensitive is a complex journey, the steps of which are only slowly being identified. Their elucidation, however, is of paramount importance to our understanding of how and why the β-cell fails or decompensates in type 2 diabetes. Considering their potential pathophysiological and therapeutic potential, we focused on identifying the earliest biochemical anomalies in desensitized islets. The approach utilized in the present studies was to use a highly responsive and sensitive (to glucose stimulation) system, the collagenase-isolated rat islet, and subject it to a number of manipulations. Because glucose exerts multiple effects on the β-cell, from increases in glucose metabolites to second messengers to gene transcription, we wanted to compare the effects of glucose with those induced by a compound, carbachol, whose only established effect is the activation of β-cell PLC (14, 18, 55). This cholinergically activated process requires the M1 muscarinic receptor type and generates a number of important second messengers, including IPs and diacylglycerol (7, 28, 30). One central question was to determine the role of PLC activation in determining islet sensitivity to glucose and its vulnerability to sustained hyperglycemia.

In the initial series of experiments, the acute effects of 20 mM glucose or carbachol on several parameters of β-cell activation were assessed. Not surprisingly, 20 mM glucose stimulation resulted in a dramatic augmentation of insulin secretion, a response that paralleled in magnitude findings made using the perfused pancreas or human islets studied in vivo using the hyperglycemic clamp technique (15–17, 20, 26, 34). The secretory response to glucose was abolished by diazoxide but was unaffected by the cholinergic antagonist atropine. In sharp contrast to 20 mM glucose, carbachol (in the presence of 5 mM glucose) did not alter insulin secretion rates. The exquisite glucose dependence of cholinergic stimulation of insulin secretion is well established (18, 23).

By use of labeled IP accumulation to monitor its activity, 20 mM glucose significantly increased PLC activation in parallel with secretion. The stimulatory effect of glucose was abolished by diazoxide but was left intact by atropine. In contrast to its ineffectiveness on the secretory process, carbachol also significantly increased PLC activation.

An analysis of glucose usage rates under these different experimental conditions revealed the following. First, rates of glucose metabolism in the presence of 5 mM glucose averaged 60–70 pmol·islet·1–1·h·1. Carbachol did not affect this process. Second, raising the glucose level to 20 mM increased usage about threefold. Third, diazoxide, despite its profound inhibitory effect on 20 mM glucose-induced insulin secretion and PLC activation, exerted no inhibitory effect on glucose usage rates by the islet. The failure of diazoxide to alter glucose metabolism has been noted previously (3, 5).

After first establishing some of the characteristics of acute β-cell activation by 20 mM glucose or carbachol, we focused our attention on how chronic exposure to these compounds affects this cell. In both instances, chronic, sustained 3-h exposure to high glucose or carbachol profoundly altered the insulin-secretory process. Peak second-phase secretion rates in response to 20 mM glucose measured during a subsequent dynamic perfusion, after basal release was taken into account, were reduced by >75%. The inhibitory effect of prior 20 mM glucose was paralleled by a significant reduction in PLC activation but no untoward effect on metabolism. Of particular interest in terms of understanding the biochemical lesion involved and its potential as a target for therapeutic intervention, diazoxide protected both PLC and the secretory process from 20 mM glucose-induced desensitization. This protective action occurred despite the inability of diazoxide to alter glucose metabolism. This suggests that, at least with this model of β-cell inactivation, altered PLC activation may be critically involved and is the potential Achilles’ heel of the secretory process.

It should also be noted that it proved possible to induce significant defects in secretion and PLC activation after only 1.5 h of exposure to 20 mM glucose. A fertile future area of investigation, perhaps, is an analysis of the impact of moderate (6–10 mM) hyperglycemia on these two parameters of β-cell activation.

Our findings made with carbachol further support the importance of PLC activation as an integral component in the induction of desensitization. Unlike glucose, a nutrient that exerts multiple effects on the β-cell, cholinergic stimuli exert one critically important physiological effect, the activation of PLC (18). They do not stimulate insulin secretion or lower insulin stores of the islet after chronic exposure to them. Thus their negative effect, as reported here, cannot be due to excessive stimulation of the insulin-secretory machinery or to lowered insulin content of the β-cell. They have no effect on glucose usage rates, thus excluding excessive accumulation of metabolic products, toxic oxygen radicals, or oxidative stress as underlying its adverse effect on the secretory process. Most importantly, as demonstrated herein, carbachol’s negative effects on the subsequent activation of both PLC and secretion are effectively thwarted by atropine. Interestingly, atropine did not protect the β-cell from 20 mM glucose, attesting to its specificity and supporting further the role of PLC.

We considered the possibility that, at least in the case of glucose, the diminished availability of releasable insulin might contribute to alterations in hormone secretion. Indeed, compared with carbachol-pretreated islets, chronic exposure to 20 mM glucose lowered insulin content, at least when measured after the perfusion. However, as demonstrated when forskolin was included during the perfusion with 20 mM glucose, despite the decline in total insulin stores, these islets are still quite responsive, and release rates comparable with those achieved from control 5 mM glucose-pretreated islets were observed. However, compared with the response of carbachol-pretreated islets, there was an observable difference suggesting that insulin stores might contribute in some small way to the secretory lesion. The restorative effect of forskolin on the secretory process is reminiscent of the effect of the gut hormone glucagon-like peptide-1 on secretion from desensitized islets (51).

When taken in its entirety, the following sequence of events seems reasonable in light of what has been previously reported.
(4, 9–11, 33, 52, 54), combined with the present findings. Acutely, both glucose and carbachol activate islet PLC. This event alone, however, is ineffective in activating secretion to any appreciable extent. Additional fail-safe substrates provided for by glucose supplement the generation of PLC-activated second messengers, culminating in a brisk biphasic insulin secretory response. The signals include not only metabolites and cofactors but also other second messengers such as cAMP (13, 24, 25, 45). However, the events associated with prolonged stimulation by glucose, despite initially activating the secretory process, plant the seeds for eventual β-cell failure or desensitization. Because chronic carbachol exposure faithfully reproduces the negative impact of chronic 20 mM glucose on the secretory process, and does so without increasing glucose metabolism or insulin secretion, a reasonable working hypothesis is that excessive stimulation of PLC underlies aberrant secretion that is irrelevant to the agonist used to activate the enzyme. The primary common denominator between carbachol- and glucose-induced desensitization is PLC.

The regulation of PLC has been studied in detail by Rhee and colleagues (29, 30) and Ryu et al. (31), and their findings may be particularly relevant to the β-cell and the biochemical events described herein. In their comprehensive studies, they found that PLC was susceptible to negative feedback regulation by either PKC or PKA. Thus compounds that activate PKC or elevate cAMP have the potential to impair activation of PLC. We previously observed (44) that, similarly to the effects reported here and elsewhere with sustained glucose and carbachol exposure (51), prolonged exposure to forskolin or IBMX also impairs glucose-induced PLC activation and results in defective insulin secretion as well. This suggests that, regardless of the compounds used to induce desensitization, their ability to impair secretion might be dependent upon their ability to inactivate PLC. Our working hypothesis, and one supported by a number of observations (28, 40, 46, 47), is that although short-term exposure to compounds that activate PLC positively affect secretion, playing an important role in both biphasic secretion and the induction of time-dependent sensitization or memory, prolonged activation of the enzyme sets into motion a series of events that inhibit its activation.

Most recently, it has been suggested that an imposed period of β-cell rest might help to restore the secretory responsiveness of the cell. It has been reported that diazoxide, which inhibits secretion, protects against chronic hyperglycemia (2, 35). These studies both confirm and expand the concept put forth almost 30 yr ago that diazoxide might be useful in the therapy of diabetes (19). The protective action of diazoxide against sustained hyperglycemia is thought to be due to a direct β-cell effect (27) and has been confirmed in vitro as well (8). In the present report, we confirmed the protective action of the K<sub>ATP</sub> channel opener against hyperglycemia-induced impaired secretion. This protective effect was not due to the ability of diazoxide to reduce the usage of glucose. Diazoxide did not affect glucose metabolism, as shown here and elsewhere (3, 5); its protection appeared to be due, at least in part, to its ability to interfere with glucose-induced PLC activation. In a similar fashion, the muscarinic antagonist atropine also blocked carbachol-induced desensitization, presumably by interfering with PLC activation. Finally, the observations made with this endocrine system reinforce what has been noted with other systems as well; the sustained stimulation of those pathways that so elegantly control hormone secretion can, if their stimulation is unrestrained, impair target cell performance. In the β-cell, this adverse process culminates in diabetes, and efforts should now be made to utilize this information therapeutically.

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**REFERENCES**


