Inhibition of Protein Kinase A–Induced Glucagon Synthesis and Secretion by Glucose

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The control of glucagon biosynthesis and secretion in the pancreatic islet was examined in response to protein kinase A stimulation at various glucose concentrations. Forskolin plus 3-isobutyl 1-methylxanthine (IBMX) stimulated both glucagon synthesis and secretion at a glucose concentration equivalent to hypoglycemia (0.5 g/L, $P < .001$), but not at higher glucose concentrations (1.0, 2.0, and 4.0 g/L, $P > .05$). Destruction of B cells with streptozotocin or inhibition of glycolysis with mannoheptulose did not reverse the inhibitory action of high glucose (4.0 g/L) on the response of glucagon to forskolin plus IBMX. In contrast, citrate but not EGTA treatment permitted forskolin plus IBMX to stimulate glucagon synthesis and secretion ($P < .05$ and $P < .001$, respectively) in the presence of high glucose. We conclude that citrate can block the inhibitory action of glucose on the response of A cells to the protein kinase A pathway, possibly through its effects on an intracellular metabolic pathway.

IN CONTRAST TO the understanding of the control of insulin production,[1] factors regulating glucagon synthesis and secretion are not well understood. Glucagon is synthesized in and secreted from the pancreatic A cell.[2] Glucagon is the most important hormone secreted in the postabsorptive state, and its secretion is normally inhibited by high glucose levels.[3] The recent detection of glucokinase in A cells[4] is consistent with a regulatory effect of high glucose on glucagon secretion. However, contradictory evidence has been obtained by Pipeleers et al,[5] who have shown a lack of effect of glucose alone on glucagon secretion from isolated pancreatic A cells, but an inhibitory effect of high versus low glucose concentrations in the presence of amino acids.

Glucagon secretion is affected by a number of secretagogues that affect the protein kinase A pathway, including epinephrine, vasoactive intestinal peptide, galanin, calcitonin gene–related peptide, and somatostatin.[6] Activation of the protein kinase A pathway is known to stimulate glucagon secretion from isolated pancreatic islets.[7,8] Cyclic adenosine monophosphate (cAMP)–protein kinase A pathway stimulation also increases proglucagon gene transcription in cell lines that have a fully functional protein kinase A system[7,9,10] and in rat pancreatic islets.[7] The effect of glucose on glucagon gene transcription has not been specifically studied, but it would appear that glucose concentrations of 0.5 and 2.0 g/L do not affect glucagon mRNA levels in isolated rat islets.[7]

The purpose of the present study was to examine the effects of glucose, as well as the possible interactions of glucose with the protein kinase A pathway, on glucagon synthesis and secretion using isolated rat pancreatic islets.

MATERIALS AND METHODS

Islet Isolation

The methods that were used have been described previously.[7,11] Adult male rats (350 to 375 g; Charles River, St Constant, Quebec, Canada) were anesthetized with Somnotol (MTC Pharmaceuticals, Cambridge, Ontario, Canada), and the pancreas was filled with 0.8 mg/mL collagenase (type IV; Sigma, St Louis, MO) in Hanks balanced salt solution (HBSS). The pancreas was carefully removed and digested for 20 minutes in a shaking (100 strokes/min) water bath (37°C) using sterile technique, and was then washed twice in ice-cold HBSS. The digest was then filtered through a nylon membrane (pore size, 500 μm; Thompson, Scarborough, Ontario, Canada) and washed, and the pellet was dispersed in 6 mL 25% (wt/vol) Ficoll (Pharmacia, Uppsala, Sweden) in HBSS, and then 4 mL each of 23%, 20%, and 11% (wt/vol) Ficoll was added to form a discontinuous gradient. The gradient was centrifuged at 1,500 × *g* for 10 minutes to separate the islets from the bulk of the acinar tissue, and islets were collected and washed twice in HBSS. The islets were viewed with a light microscope and were picked by hand into Dulbecco's minimum essential medium ([DMEM] GIBCO, Grand Island, NY) with 1 g/L glucose, gentamycin (GIBCO), 100 IU/mL penicillin (GIBCO), 100 μg/mL streptomycin (Flow Laboratories, McClean, VA), and 5% (vol/vol) fetal calf serum (GIBCO). The islets were incubated for 2 days in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. In streptozotocin and citrate experiments, islets were treated for 2 hours with either streptozotocin (8 mmol/L) or citrate (0.4 mmol/L) by adding a small volume of concentrated (100×) stock solution to the DMEM. The islets were washed twice and returned for 24 hours to media with 1.0 g/L glucose.

On the day of the experiment, islets were picked into groups of 20 or 25 per tube in 1 mL DMEM. An effort was made to distribute the same number of small, medium, and large islets to each tube. Islets were centrifuged for 4 minutes at 1,500 × *g*, and then 0.9 mL medium was carefully removed and 0.5 mL DMEM containing various concentrations of glucose, experimental treatments, and 0.5% (vol/vol) fetal calf serum was added for 24 hours. Islets were incubated with or without forskolin (10 μmol/L; Sigma) plus 3-isobutyl 1-methylxanthine ([IBMX] 10 μmol/L; Sigma) to increase adenylyl cyclase and inhibit phosphodiesterase activity, respectively.[12,13] We have previously demonstrated that treatment with forskolin plus IBMX in the presence of 10 mmol/L glucose increases islet cAMP biosynthesis to 700% of control values.[14] Islets were also incubated with forskolin or IBMX in the presence of 2 mmol/L EGTA (Sigma) or 20 mmol/L mannoheptulose

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(Sigma). Three to seven replicates were used for each experimental treatment, and experiments were repeated three to 12 times.

Reversed-Phase Extraction of Peptides

At the end of the 24-hour incubation, the islets were centrifuged at 1,500 × *g* for 4 minutes, the medium (0.4 mL) was removed and placed in 1 mL 0.1% (vol/vol) trifluoroacetic acid (TFA), and the islets were homogenized in 1 mL 1N HCL containing formic acid (5% vol/vol), TFA (1% vol/vol), and NaCl (1% wt/vol) using a hand-held homogenizer. The methods used have been described previously.[7,9,11,15-18] Islet homogenates and media were separately passed twice through one C-18 Sep-Pak (Waters Associates, Milford, MA). The cartridges were eluted with 4 mL 80% (vol/vol) isopropyl alcohol in 0.1% (vol/vol) TFA, and the eluates were stored at −20°C until radioimmunoassay.

Radioimmunoassays

Aliquots of Sep-Pak eluates were evaporated under reduced pressure in a rotary evaporator (SpeedVac; Emerston Instruments, Scarborough, Ontario, Canada). Immunoreactive glucagon (IRG) levels were measured with the C-terminally directed O4A antiserum (Dr R. Unger, Dallas, TX) as described previously.[7,9,11,16-18] Control and experimental groups were both analyzed in the same assay. The range of values for these radioimmunoassays was 4 to 400 pg. Insulin level was measured with Wright's antibody (School of Medicine, University of Indiana, Bloomington, IN), and the range for the insulin assay was 20 to 800 pg.

Analysis and Statistics

All experiments included paired controls. Synthesis of hormone is expressed as the mean total content (islets + media) of glucagon ± SEM. Secretion of glucagon is expressed as the mean concentration of hormone in the media ± SEM. The data were analyzed by ANOVA with the SAS program for IBM computers (Statistical Analysis System, Cary, NC). In some cases, logarithmic transformation of the data was performed to normalize variances.

RESULTS

Basal glucagon content and secretion were not significantly affected by the various concentrations of glucose ($P > 0.05$; Fig 1). Forskolin (10 μmol/L) plus IBMX (10 μmol/L) significantly increased glucagon content and secretion at 0.5 g/L glucose ($P < .001$ and $P < .001$, respectively; Fig 1); however, higher concentrations of glucose (1.0, 2.0, and 4.0 g/L) abolished the stimulatory action of forskolin plus IBMX on glucagon content and secretion. To understand the mechanisms underlying the inhibitory action of glucose on the response of glucagon to forskolin plus IBMX, all further experiments were performed with 4.0 g/L glucose.

Streptozotocin treatment of islets reduced basal insulin content by 25% and significantly reduced secretion by 75% ($P < .01$). Islet glucagon content was also reduced, but did not reach statistical significance (Fig 2). Despite the decrease in insulin, basal glucagon secretion was not increased in streptozotocin-treated islets. Furthermore, forskolin plus IBMX treatment did not significantly stimulate glucagon synthesis or secretion in either control or streptozotocin-treated islets.

Treatment of islets with mannoheptulose to block glycolysis[19-21] inhibited basal secretion of insulin to less than 50% of control levels ($P < .05$). However, glucagon release was not affected in mannoheptulose-treated islets (Fig 3). Furthermore, forskolin plus IBMX did not significantly alter glucagon synthesis or secretion ($P > .05$) in mannoheptulose-treated islets.

Although a number of Krebs cycle intermediates, includ-

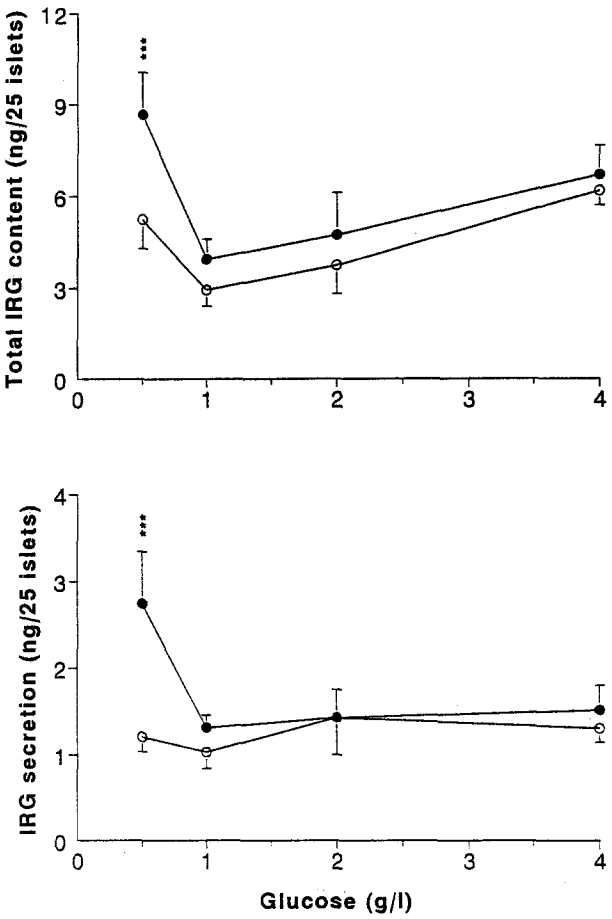

Fig 1. Effect of forskolin plus IBMX on IRG total content and secretion at various glucose concentrations (n = 3 to 12). (○) Mean values from control untreated islets; (●) mean values from forskolin plus IBMX–treated islets. ***$P < .001$, a significant effect of forskolin plus IBMX *v* controls at the same glucose concentration.

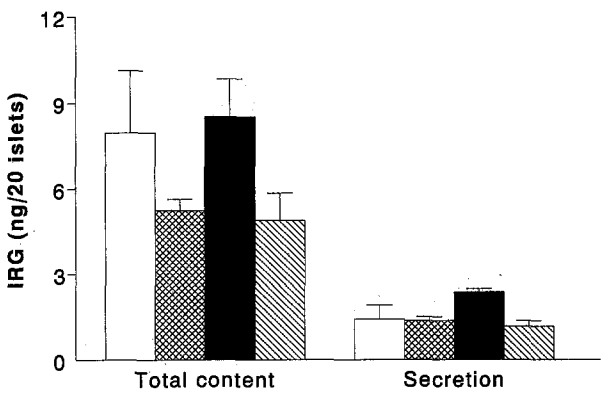

Fig 2. Effect of forskolin plus IBMX treatment on IRG content and secretion at 4.0 g/L glucose in streptozotocin-treated islets. (□) Control media; (▨) streptozotocin; (■) forskolin plus IBMX; (▧) streptozotocin plus forskolin plus IBMX (n = 3).

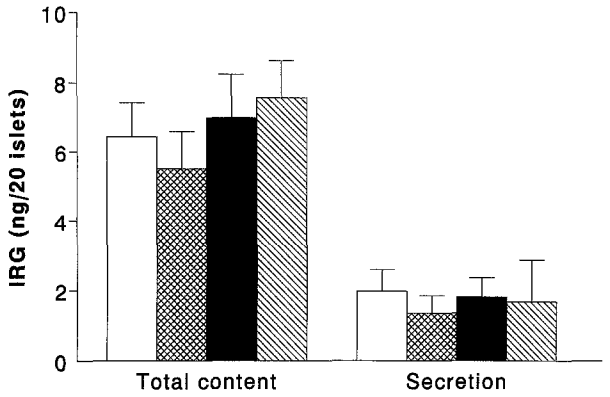

Fig 3. Effect of forskolin plus IBMX treatment on IRG content and secretion at 4.0 g/L glucose in mannoheptulose-treated islets (□) Control media; (▨) mannoheptulose, (■) forskolin plus IBMX; (▧) mannoheptulose plus forskolin plus IBMX (n = 6).

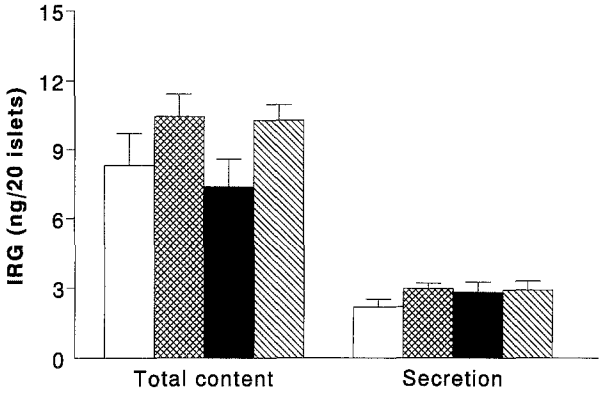

Fig 5. Effect of forskolin plus IBMX treatment on IRG content and secretion at 4.0 g/L glucose in EGTA-treated islets. (□) Control media; (▨) EGTA; (■) forskolin plus IBMX; (▧) EGTA plus forskolin plus IBMX (n = 4).

ing citrate, are known to affect insulin secretion,[22-25] treatment of islets with citrate did not alter basal glucagon content or secretion. However, forskolin and IBMX treatment of citrate-treated islets was found to stimulate both glucagon synthesis and secretion ($P < .05$ and $P < .001$, respectively; Fig 4). Therefore, it appears that citrate can block the inhibitory action of high glucose on the response of glucagon to forskolin plus IBMX. In contrast, forskolin plus IBMX had no significant effect on glucagon content or secretion by EGTA-treated islets ($P > .05$; Fig 5). We conclude that citrate does not exert its action by binding calcium.

DISCUSSION

Some of the factors regulating insulin synthesis and secretion have been elucidated, but the control of the A cell is not understood. In the present study, forskolin plus IBMX treatment stimulated glucagon synthesis at low (0.5 g/L) but not at high (4.0 g/L) concentrations of glucose. The effects of forskolin plus IBMX on glucagon biosynthesis at low glucose concentrations can be partially explained by effects on transcription. The proglucagon gene contains a cAMP response element that confers transcriptional

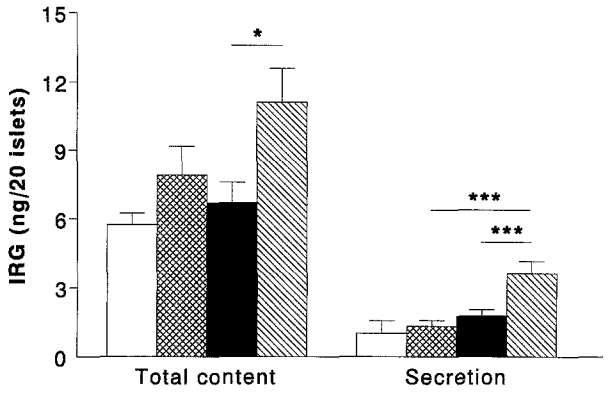

Fig 4. Effect of forskolin plus IBMX treatment on IRG content and secretion at 4.0 g/L glucose in citrate-treated islets (□) Control media; (▨) citrate; (■) forskolin plus IBMX; (▧) citrate plus forskolin plus IBMX (n = 10). *$P < .05$, ***$P < .001$.

sensitivity to protein kinase A pathway activation.[7,9,10,17,18] Forskolin also stimulates proglucagon mRNA accumulation in rat islets incubated at a glucose concentration of either 0.5 or 2.0 g/L.[7] The present study is the first to show that glucagon biosynthesis is increased by forskolin at 0.5 g/L but not at higher concentrations of glucose. This discrepancy could be explained if glucose inhibits glucagon synthesis at the level of protein translation. It is known that glucose can modulate insulin translation.[26] Alternatively, glucose may increase intracellular degradation of glucagon. Further studies will be required to determine the mechanism whereby forskolin plus IBMX affects proglucagon mRNA and glucagon biosynthesis differentially at different glucose concentrations.

Forskolin plus IBMX also increased glucagon secretion at a low glucose concentration (0.5 g/L); however, glucose concentrations equivalent to normoglycemia or hyperglycemia inhibited glucagon secretion in response to forskolin plus IBMX. Pipeleers et al[5] have reported that glucose inhibits the glucagon secretory response to dibutyryl cAMP in the presence of amino acids. Hii and Howell[8] have also reported that forskolin stimulates glucagon secretion at 0 and 5 mmol/L glucose, but not at 10 and 20 mmol/L glucose, after 30 minutes of incubation. The effect of glucose on the intracellular machinery of the A cell and the mechanism of interaction of the glucose effect with the protein kinase A pathway are not understood. However, it is known that in the B cell, glucose must be metabolized to stimulate insulin secretion.[19-21]

Inhibition of glycolysis by the glucokinase inhibitor mannoheptulose[19-21] did not allow protein kinase A pathway activation to stimulate glucagon synthesis or secretion. Glucokinase is present in 95% of B cells and also in a significant fraction (35% to 75%) of A cells.[4] In addition, a decrease in insulin secretion through destruction of B cells with streptozotocin treatment or treatment with mannoheptulose did not permit forskolin plus IBMX stimulation of glucagon at a high concentration of glucose. Therefore, not only is glucose metabolism not required for the inhibitory effect of glucose on forskolin plus IBMX–induced glucagon production, but insulin does not appear to inhibit the response of A cells to forskolin plus IBMX. Insulin could

still be exerting a paracrine effect on A cells within the islet; however, this seems unlikely, since paracrine insulin secretion should also be decreased in studies such as those with streptozotocin, in which insulin content of the islets and insulin secretion were significantly decreased.

Citrate is a Krebs cycle intermediate, and it has been reported that Krebs cycle intermediates can modulate insulin secretion.[22-25] In the present study, citrate treatment allowed forskolin plus IBMX to stimulate glucagon biosynthesis and secretion at the high glucose concentration. In addition to being a Krebs cycle intermediate, citrate is also known to bind calcium. Since EGTA, a calcium chelator, did not permit an effect of forskolin plus IBMX on glucagon production, citrate is probably acting as a Krebs cycle intermediate. It is possible that citrate inhibits an inhibitory signal initiated by glucose; citrate does inhibit glycolysis by inhibiting the regulatory enzyme phosphofructokinase.[23] However, this seems unlikely, since mannoheptulose did not affect glucagon synthesis or secretion. Alternatively, citrate may be an essential cofactor for protein kinase A–induced glucagon synthesis and secretion at high glucose concentrations. It is possible that after the initial 48 hours of incubation in culture, islets were depleted of intracellular citrate stores. Thus, we speculate that replenishment of citrate may have permitted the effects of protein kinase A stimulation on glucagon synthesis and secretion. The mechanism by which citrate affects the A cell clearly requires further investigation.

In conclusion, it was found that activation of the protein kinase A pathway stimulates glucagon synthesis and secretion at low concentrations of glucose, whereas high glucose levels inhibit the effect of forskolin plus IBMX on glucagon synthesis and secretion. Although these effects are probably not mediated by insulin, it appears that the Krebs cycle intermediate, citrate, is required to inhibit the action of glucose on the response of glucagon to forskolin plus IBMX. Such a mechanism may be important for the fine-tuning of glucagon secretion in the face of elevated concentrations of protein kinase A stimulators such as epinephrine, particularly when glucose concentrations are high.

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