Pregnancy is a unique challenge to the β-cell. Pregnancy induces peripheral insulin resistance, which is normally compensated by increased β-cell proliferation, expansion of islet volume, and increased insulin synthesis and secretion. In addition to peripheral insulin resistance driving β-cell compensation, pregnancy-associated hormones can trigger changes in β-cell physiology and function. For example, increases in serum placental lactogen levels during pregnancy correlate with enhanced β-cell proliferation, insulin synthesis, and glucose-induced insulin release (24, 26). However, there are several other hormones that increase early during pregnancy that may participate in the regulation of islet function. Prolactin levels increase up to 10-fold in early pregnancy, progesterone increases fivefold, and glucocorticoids continue to rise throughout gestation, peaking during late pregnancy (2). In vitro, progesterone counteracts the effects of lactogenic hormones on insulin secretion and islet β-cell division (25). However, in vivo studies indicate that progesterone stimulates pancreatic cell proliferation but has no effect on insulin secretion (17, 18).

Data accumulated thus far suggest that prolactin and placental lactogen upregulate islet function by 1) increased insulin synthesis; 2) increased glucose utilization, oxidation, and metabolism; and 3) increased glucose-induced insulin release with a reduced glucose stimulation threshold (24, 26, 29, 30). The mechanisms underlying these observations have received little attention. Recently, a role for cAMP production via activation of PKA on amplifying glucose-induced insulin secretion has been suggested (10), which could play a role in pregnancy-induced insulin secretion. Many studies suggest that glucocorticoids inhibit insulin synthesis and glucose-stimulated insulin secretion by inhibiting the insulin-exocytotic process (13, 22). The increase in maternal glucocorticoids during late pregnancy has been suggested to contribute to reduced glucose-stimulated insulin secretion (11) and may counteract the effect of prolactin; however, the mechanisms underlying this effect have not been studied. These studies suggest that prolactin and glucocorticoids may regulate islet function at different sites, but the mechanisms are not clear.

The goal of present study was to determine the mechanism(s) whereby prolactin, progesterone, and dexamethasone regulate β-cell function and how they work together to either augment or suppress insulin secretion. With use of the glucose-responsive β-cell line MIN6, our results indicate that prolactin, and to a lesser extent progesterone, upregulate basal insulin release and glucose-stimulated insulin secretion primarily by increasing glucokinase activity. However, dexamethasone dominantly downregulates only glucose-stimulated insulin secretion from MIN6 cells, not only by reducing glucokinase activity but also by reducing exocytosis and by reducing cAMP levels via increased phosphodiesterase activity. This activity may in part help explain the basal hyperinsulinemia of pregnancy and possibly a reduced capacity for insulin secretion during late gestation.

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MATERIALS AND METHODS

Materials. Rat Prolactin (NIDDK rPRL, 30 IU/mg) was obtained from the National Hormone and Pituitary Program of NIDDK (Baltimore, MD). Progesterone (PRG), dexamethasone (DEX), phenylmethylsulfonyl fluoride (PMSF), and all other reagent-grade chemicals were from Sigma (St. Louis, MO). DMEM and fetal bovine serum were from Life Technologies (Rockville, Maryland). Insulin immunoassay kits were purchased from ALPCO (Windham, NH). Rat Prolactin (NIDDK rPRL, 30 IU/mg) was obtained from the National Hormone and Pituitary Program of NIDDK (Baltimore, MD). Progesterone (PRG), dexamethasone (DEX), phenylmethylsulfonyl fluoride (PMSF), and all other reagent-grade chemicals were from Sigma (St. Louis, MO). DMEM and fetal bovine serum were from Life Technologies (Rockville, Maryland). Insulin immunoassay kits were purchased from ALPCO (Windham, NH). Insulin immunoassay kits were purchased from ALPCO (Windham, NH). Insulin immunoassay kits were purchased from ALPCO (Windham, NH). Insulin immunoassay kits were purchased from ALPCO (Windham, NH).

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Cell culture. MIN6 β-cells were maintained and grown in DMEM containing 10% (vol/vol) heat-inactivated fetal bovine serum with 2 mM glutamine, 25 mM glucose, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C with 5% CO2. For determination of the effect of hormones, cells were cultured in the same medium but containing 5.5 mM glucose and 0.1% BSA, followed by incubation for 1 h in this buffer at 37°C. The reaction mixtures were applied to AG 1-X2 resin slurry, and the medium was equilibrated for 15 min at 30°C. The reaction mixtures were applied to AG 1-X2 resin slurry, thoroughly vortexed, and then allowed to equilibrate for 15 min at 3°C. The reaction mixtures were applied to AG 1-X2 resin slurry, thoroughly vortexed, and then allowed to equilibrate for 15 min at 3°C.

Preparation of protein extracts. After hormone treatment and stimulation, cells were washed twice with ice-cold PBS and lysed in cold lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 20 HEPES, pH 7.4, with 3 mM glucose and 0.1% BSA), followed by incubation for 1 h in this buffer at 37°C. The cells were then stimulated acutely with KRB supplemented with either basal glucose at 3 or 16 mM glucose or 10 mM α-ketoisocaproate (α-KIC) at 37°C, and the medium was collected at the end of 1 h of stimulation. The medium was collected and stored at −20°C for subsequent insulin assay. Insulin concentration was measured by ELISA using the ALPCO kits.

Immunoblotting. Equal amounts of protein (75 μg) were subjected to 7 or 10% SDS-PAGE and transferred to PVDF membrane. After blocking with 5% fat-free milk, the membrane was incubated with GLUT2 antibody [1:1,000 in Tris-buffered saline plus Tween (TBS-T) with 5% fat-free milk] or glucokinase antibody (1:500 in TBS-T with 5% milk) overnight at 4°C. The membranes were washed extensively with TBS-T and incubated for 1 h with HRP-conjugated goat anti-rabbit IgG (1:10,000) and then washed with TBS-T. The bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Arlington heights, IL) according to the manufacturer’s instructions.

Glucokinase activity assay. Glucokinase activity was measured as previously described (15, 23). Briefly, the cells were washed once with ice-cold PBS and lysed with lysis buffer (in mM: 10 Tris, pH 7.0, 100 KCl, 15 EDTA, 600 sucrose, and 15 β-mercaptoethanol and 10 μg/ml leupeptin, 1 mM benzamidine and 1 mM PMSF). After sonication for 10 s, the lysates were centrifuged at 10,000 g for 15 min at 4°C. The supernatants were collected, and protein concentration was determined by incubating with desired hormones in the presence or absence of the phosphodiesterase (PDE) inhibitor IBMX (500 μM). The cells were washed and incubated with KRB buffer for 1 h and then incubated with KRB buffer with 16 mM glucose for 1 h with or without IBMX. Excess culture medium was aspirated, and the cells were lysed with reagent materials supplied with the ELISA kit. The intracellular cAMP concentration was measured by ELISA (Amer...
and PRL together augment insulin levels in response to low glucose challenge, even in the presence of DEX. At higher (16 mM) glucose concentrations, a significant increase in insulin release in response to PRL and PRG was seen at all time points from 6 h on (Fig. 2B, P < 0.01), whereas DEX inhibited insulin secretion significantly at all time points (P < 0.01). In contrast to 3 mM glucose, when the three hormones were added together, insulin release at 16 mM glucose was inhibited beginning at the 12-h time point (Fig. 2B), suggesting that DEX has little effect at 3 mM glucose but exerts a dominant negative effect on insulin secretion over the other hormones when the cells are challenged at 16 mM glucose.

PRL and PRG increase glucokinase activity, but DEX dominantly inhibits glucokinase activity in MIN6 cells. It is well known that glucose oxidation in mitochondria increases the ATP/ADP ratio, thereby inducing a closure of the ATP-dependent K⁺ channel, leading to insulin secretion (10). Because glucokinase is one of the key regulatory enzymes in control of glucose metabolism in β-cells (6, 10), we measured glucokinase protein and activity levels in hormone-treated cells. There were no differences in glucokinase protein levels before or after treatment with any hormone (data not shown). However, as shown in Fig. 3, PRL treatment significantly increased glucokinase activity at all time points and maximally at 24 h (P < 0.05). PRG also increased glucokinase activity (P < 0.05) but to a lesser extent than in PRL-treated cells, reaching maximal levels at 12 h. Similar to insulin secretion, DEX treatment inhibited glucokinase activity in MIN6 cells with prolonged incubation, beginning at 6 h (P < 0.05). When the cells were treated with all three hormones together, glucokinase activity went down in a manner similar to DEX alone (P < 0.05), suggesting a dominant negative effect of DEX on glucokinase activity. We also measured GLUT2 protein levels in hormone-treated MIN6 cells. GLUT2 protein level was slightly increased after 6-h DEX treatment (P < 0.05) and then returned to normal levels by 48 h (data not shown). PRL and PRG had no effect on GLUT2 protein level even after 48 h of incubation.

To determine the contribution of glucokinase in regulation of insulin secretion in hormone-treated cells, we incubated hormone-treated cells with the glucokinase inhibitor 5-thio-D-glucose under low and high glucose concentrations (27). Consistent with previous studies, 5-thio-D-glucose had much less inhibitory effect on insulin release at 3 mM compared with 16 mM glucose (Fig. 4). With the exception of PRL-treated cells,
5-thio-d-glucose significantly reduced 3 mM glucose-induced insulin secretion to levels seen in control, nonhormone-treated cells \( (P < 0.05) \). Interestingly, at 16 mM glucose, 5-thio-d-glucose did not completely inhibit insulin secretion in PRL and Mix-treated cells compared with control cells under the same glucose concentration.

**α-KIC-induced insulin secretion in hormone-treated cells.**

α-KIC is a leucine derivative that increases β-cell stimulus secretion coupling by generating ATP distally via oxidative phosphorylation and by direct inhibition of potassium-dependent ATP \( (K_{ATP}) \) channels to increase insulin secretion (1). We used α-KIC to test whether PRL, PRG, or DEX had any additional effects on this distal mechanism for increasing insulin secretion. As shown in Fig. 5, there was no significant difference in α-KIC-induced insulin secretion in either PRL- or PRG-treated cells compared with nonhormone-treated cells. However, in DEX- and Mix hormone-treated MIN6 cells, after a 12-h incubation, α-KIC-stimulated insulin secretion was lower compared with α-KIC-treated control cells, reaching a maximum at 48 h.

**DEX counteracts PRL effects through increased PDE activity.** cAMP is known to play an important role in the regulation of insulin secretion in response to glucose via activation of PKA (9, 10). At low glucose concentrations, the augmentation of cAMP content has very little if any effect on insulin secretion. However, in response to high glucose, cAMP can amplify or potentiate glucose-stimulated insulin secretion (10). We sought to determine the role of cAMP in glucose-stimulated insulin secretion in hormone-treated cells. After 24 h of treatment with different hormones and 1 h of stimulation with 16 mM glucose, intracellular cAMP levels were significantly increased in PRL-treated cells (Fig. 6A, \( P < 0.05 \)). There was no change in cAMP concentration in PRG-treated cells. However, in DEX-treated cells, and to a lesser extent in Mix-treated cells, cAMP levels were lower by nearly 50% compared with control cells \( (P < 0.05) \).

To examine the possible underlying mechanisms for cAMP regulation in response to hormone treatment, we used the PDE inhibitor IBMX \((500 \mu M)\), which inhibits cAMP breakdown, thereby allowing the measurement of cAMP production in the absence of catabolism. MIN6 cells were treated with hormones as above and stimulated for 1 h with 16 mM glucose and IBMX (Fig. 6B). In all cells, IBMX increased the cAMP concentration 10- to 50-fold. In PRL-treated cells, IBMX had no significant effect on intracellular cAMP content compared with control cells. The lack of change using the PDE inhibitor suggests that the increase in cAMP in PRL-treated cells could be due to inhibition of PDE as well as increased cAMP production. In Fig. 7, PDE activity was determined in PRL-treated cells and was unchanged. In PRG-treated cells, cAMP levels were not significantly changed with 16 mM glucose with or without IBMX compared with control cells. Because PDE activity in PRG-treated cells shown was increased (Fig. 7), this suggests that PRG may have stimulated an increase in cAMP production offset by greater PDE activity. DEX or the combination of hormones reduced cAMP levels but was increased significantly \( (P < 0.05) \) in the presence of PDE inhibitor IBMX.
are means /H11006 maintained in culture. Tagogues, including cAMP, but may differ from neonatal islets maintain a robust response to glucose in culture (12). In secretion in incubated neonatal rat islets. The reasons for these progesterone treatment had an inhibitory effect on insulin (3, 16). However, Sorenson et al. (25) found that 3 days of testosterone treatment enhances insulin secretion during pregnancy (7, 20). In the present study, we show that prolactin has a marked effect on increasing both basal and glucose-induced insulin secretion from MIN6 cells, whereas progesterone alone had a stimulatory effect but to a lesser extent. These results are consistent with earlier studies performed on isolated islets (24, 26) and support the concept that placental lactogen(s) upregulate β-cell insulin secretion during pregnancy. Both in vivo and organ culture experiments suggest that short-term (<12 h) progesterone treatment enhances insulin secretion during pregnancy (3, 16). However, Sorenson et al. (25) found that 3 days of progesterone treatment had an inhibitory effect on insulin secretion in incubated neonatal rat islets. The reasons for these differences are not clear but could be due to differences between cultured fetal islets and the mouse-derived MIN6 cells. MIN6 cells are mature, well-differentiated cells that maintain a robust response to glucose in culture (12). In addition, MIN6 cells, although they are not true islets, maintain their response to a number of physiologically relevant secretagogues, including cAMP, but may differ from neonatal islets maintained in culture.

An important finding of the present study is that dexamethasone, alone or in combination with prolactin and progesterone, inhibited insulin secretion in response to 16 mM glucose-stimulating concentrations. However, in the basal state (3 mM glucose), the insulin levels in response to dexamethasone treatment were unchanged, and three hormones together maintained higher insulin release. These data suggest that the adaptive response to dexamethasone primarily affects only glucose-stimulated insulin secretion and counteracts the effects of prolactin and progesterone. Our results show that a major mechanism whereby dexamethasone suppresses insulin secretion involves a decrease in glucokinase activity. Glucokinase is the major rate-limiting enzyme of glucose metabolism in the β-cell and serves as a glucose sensor in regulating insulin secretion. Previous studies showed that glucose utilization and oxidation are increased in islets during pregnancy (31). We found that prolactin and progesterone increased glucokinase activity without a change in the level of expression of the protein, whereas dexamethasone suppressed glucokinase activity. It is very possible that glucokinase activity can be induced by changes in the conformational state of the enzyme, which would not be picked up by measuring the protein level by Western blotting. To determine the contribution of glucokinase to the responsiveness to glucose, the glucokinase inhibitor 5-thio-d-glucose was used in basal and glucose-stimulated cells. Basal and 16 mM glucose-stimulated insulin release in prolactin-treated cells was still higher than in control cells at the same glucose concentration using 5-thio-d-glucose, whereas in progesterone- and dexamethasone-treated cells the glucokinase inhibitor reversed the insulin release to control levels. These results suggest that progesterone and dexamethasone may operate through glucokinase to control insulin release but that prolactin may have more than one mechanism in addition to glucokinase activation to enhance insulin secretion. For example, prolactin treatment enhances the production of cAMP levels in response to glucose, and this may be an important factor underlying the potentiation of glucose-stimulated insulin secretion.

α-KIC was used to determine whether these hormones act on a distal pathway to stimulate insulin secretion by enhancing mitochondrial ATP production and possibly by triggering a release of cytosolic calcium to close KATP channels of the plasma membrane (8). The ability of α-KIC to stimulate this pathway for insulin release was unchanged in prolactin- and progesterone-treated cells; however, dexamethasone alone or in combination with prolactin and progesterone dominantly suppressed α-KIC-mediated insulin release. These data suggest that, in addition to reducing glucokinase activity, dexamethasone alone may also interfere with β-cell stimulus secretion coupling either by reduced generation of mitochondrial ATP or perhaps by modulation of the KATP channels. Reduced insulin synthesis cannot account for the inhibition of α-KIC-induced insulin secretion, because dexamethasone increases the proinsulin mRNA and insulin content in islets (13, 19, 30).

It is well established that glucose stimulates insulin release through a KATP channel-dependent triggering pathway and by a K+ channel-independent amplifying pathway (4, 10). The triggering pathway is well characterized and includes entry of glucose, glucose oxidation, and increased ATP/ADP ratio, closure of KATP channels, opening of Ca2+ channels, increases in cytoplasmic free Ca2+, and activation of the exocytotic process. The amplifying pathway includes Ca2+-dependent and Ca2+-independent augmentation pathways. It has been suggested that glucose’s effect on augmenting insulin secretion is activated by increases in cellular cAMP that potentiate insulin secretion through an increase in PKA and small G protein pathway (10). The mechanism may involve an increase in increased rates of phosphorylation of ion channels, leading to accelerated exocytosis (10). Our results show that, similar to a previous observation in islets (29), 24 h of prolactin treatment increased cAMP content 86% in MIN6 cells at high glucose concentrations, whereas progesterone had no effect on cAMP levels compared with control cells incubated under similar glucose concentrations. Our results extend these observations to show that dexamethasone dominantly suppresses cAMP concentration alone and in combination with prolactin and progesterone. These data, together with a previous study show-

**DISCUSSION**

It is well known that glucocorticoids can impair the function of the endocrine pancreas; however, the cellular mechanism(s) whereby prolactin, progesterone, and dexamethasone work together to either stimulate or suppress insulin secretion in the islet has received relatively little attention. It has been reported that, during pregnancy, prolactin and progesterone increase β-cell mass and insulin gene expression, and, synergistically with glucose, stimulate insulin production (7, 20). In the present study, we show that prolactin has a marked effect on increasing both basal and glucose-induced insulin secretion from MIN6 cells, whereas progesterone alone had a stimulatory effect but to a lesser extent. These results are consistent with earlier studies performed on isolated islets (24, 26) and support the concept that placental lactogen(s) upregulate β-cell insulin secretion during pregnancy. Both in vivo and organ culture experiments suggest that short-term (<12 h) progesterone treatment enhances insulin secretion during pregnancy (3, 16). However, Sorenson et al. (25) found that 3 days of progesterone treatment had an inhibitory effect on insulin secretion in incubated neonatal rat islets. The reasons for these differences are not clear but could be due to differences between cultured fetal islets and the mouse-derived MIN6 cells. MIN6 cells are mature, well-differentiated cells that maintain a robust response to glucose in culture (12). In addition, MIN6 cells, although they are not true islets, maintain their response to a number of physiologically relevant secretagogues, including cAMP, but may differ from neonatal islets maintained in culture.

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**Fig. 7.** Effect of hormone treatment on phosphodiesterase (PDE) activity in MIN6 cells. Cells were incubated for 24 h with PRL (500 ng/ml), PRG (100 nM), DEX (100 nM), or all 3 hormones (Mix) together. Cells were lysed, and PDE activity was measured as described in MATERIALS AND METHODS. Values are means ± SE from 4 separate experiments. *P < 0.05 vs. control.
ing a strong linear correlation between cAMP and the rate of glucose oxidation in islets (29), suggest that reduced metabolism of glucose via suppressed glucokinase activity may lower levels of cAMP and could be a major contributor to dexamethasone’s suppressive effects on prolactin-induced insulin secretion. Moreover, it has been reported that the inhibitory effects of dexamethasone on insulin secretion can be reversed by incubation with cAMP analogs (13, 21), suggesting that dexamethasone may inhibit both the triggering and the amplifying pathways for glucose-mediated insulin release in the islet. The stimulatory effects of progestrone on insulin release may, however, be different from those of prolactin; that is, we found no increase in cAMP levels compared with control cells.

The steady-state levels of cAMP are regulated by the relative activity of adenylate cyclase and phosphodiesterase activity (9, 14). Our results show that, in the presence of the phosphodiesterase inhibitor IBMX, the increase in cAMP concentration in prolactin-treated cells was reduced to control levels. These data imply that prolactin enhances the net production of cAMP primarily by increasing adenylate cyclase activity. This was confirmed by measuring phosphodiesterase activity directly in MIN6 cells, which was unchanged in response to prolactin treatment. Progesterone on the other hand, increased cAMP levels in the presence of IBMX, and phosphodiesterase activity was increased in progestrone-treated cells. These data imply that progesterone increases cAMP synthesis and catalysis equally, and thus cAMP levels remained unchanged.

In our study, we found that 24-h incubation with dexamethasone reduced cAMP levels nearly 50% in MIN6 cells. After inhibition of phosphodiesterase activity with IBMX, cAMP levels increased beyond control cells. This suggests that an increment in phosphodiesterase activity in glucocorticoid-treated cells may be proportionally higher than biosynthesis via adenylate cyclase to lower cAMP levels. Direct measurement of phosphodiesterase activity confirmed this observation. This mechanism also applies to the inhibitory effects of dexamethasone on prolactin and progesterone. Incubation with dexamethasone in combination with prolactin and progesterone reduced cAMP level nearly 40% in MIN6 cells, associated with increased phosphodiesterase activity. Thus, under these in vitro conditions, dexamethasone dominantly reduces cAMP levels in part due to increased phosphodiesterase activity to impair insulin secretion.

In summary, our study confirms the hypothesis that elevated prolactin and progesterone coordinately upregulate insulin secretion, whereas glucocorticoids counter these effects only at higher glucose concentrations in MIN6 cells. An increase in glucokinase activity is one of the main mechanisms for up-regulating insulin secretion by prolactin and progesterone under basal conditions. Dexamethasone counteracts the upregulating effects of prolactin and progesterone at high glucose concentration by dominantly inhibiting glucose-stimulated glucokinase activity and by affecting the distal exocytotic process by increasing phosphodiesterase activity to reduce cAMP levels. These changes were observed primarily at high rather than basal glucose concentrations, which suggests that these adaptations in the β-cell could play an important role in maintaining the basal hyperinsulinemia of pregnancy while limiting the capacity of prolactin and progesterone to promote glucose-stimulated insulin secretion during late gestation. It is possible that the countering effects of glucocorticoids may be an important factor(s) underlying the inadequate insulin-secretory response that characterizes women with impaired glucose tolerance during pregnancy.

ACKNOWLEDGMENTS

We thank Dr. Boris Draznin for support of this project and critical reading of the manuscript.

REFERENCES


