Endocrine, *vol. 12, no. 3, 273–278, June 2000* 0969–711X/00/12:273–278/$11.50

Regulation of In Vitro Maturation of Stimulus-Secretion Coupling in Fetal Rat Islet β-cells

Åke Sjöholm, Elvi Sandberg, Claes-Göran Östenson, and Suad Efendic

Department of Molecular Medicine (L6:01B), The Endocrine and Diabetes Unit, The Rolf Luft Center for Diabetes Research, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden.

We have studied the maturation of a glucose-responsive insulin release from fetal rat islets, and specifically investigated the impact of nutrients, α-adrenoceptors, imidazoline receptors, and cyclic adenosine monophosphate (cAMP). Islets were isolated from 21-d-old fetal rats and maintained for 7 d in tissue culture at 3.3 or 11.1 m*M* glucose and various supplements. Culture in the presence of the nonglucidic nutrient α-ketoisocaproic acid (KIC), markedly enhanced both basal and stimulated insulin release from islets cultured at either low or high glucose. Additionally, KIC significantly elevated the insulin content of islets maintained in low glucose, whereas it slightly lowered it in islets cultured at high glucose. Culture with phentolamine, an antagonist of α-adrenergic and imidazoline receptors, markedly amplified both basal and glucose-stimulated insulin secretion when added with islets cultured in either low or high glucose. By contrast, the pure α_2-adrenoceptor antagonist benextramine had no such effects. Addition to culture media of a membrane-permeant agonist (Sp-cAMP[S]) or antagonist (Rp-cAMP[S]) of cAMP-dependent protein kinases types I and II failed to influence basal or glucose-responsive insulin secretory rates at either glucose concentration during culture as well as islet insulin content. In conclusion, islet β-cell differentiation and functional maturation of the stimulus-secretion coupling can be accelerated in vitro in fetal rat pancreatic tissue by nutrient stimulation, and by interference with imidazoline receptors, whereas cAMP seems virtually ineffective in this respect. These effectors may be of regulatory significance in the in vivo development of glucose-sensitive β-cells.

Key Words: Islet; insulin; diabetes; cyclic AMP; adrenoreceptors; imidazolines.

Received September 21, 1999; Revised January 19, 2000; Accepted January 19, 2000.

Author to whom all correspondence and reprint requests should be addressed: Dr. Åke Sjöholm, Department of Molecular Medicine (L6:01B), The Endocrine and Diabetes Unit, The Rolf Luft Center for Diabetes Research, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden. E-mail:ake@enk.ks.se

Introduction

Insulin, secreted exclusively by the pancreatic islet β-cell, is the key hormone in maintenance of normoglycemia; consequently, an impaired insulin secretion ultimately will result in diabetes mellitus *(1)*. Glucose is a cardinal stimulator of pancreatic β-cell insulin secretion in postnatal life. By contrast, during fetal life β-cells do not respond to hyperglycemia but release insulin when stimulated with other fuels, e.g., amino acids *(2,3)*. The β-cell stimulus-secretion coupling is thus subject to maturation during fetal life *(2–5)*. Presently, little is known about the factors that regulate this functional development of the β-cells during fetal and early postnatal life. In this context, it is of interest that there are studies suggesting that impaired nutrition during fetal life constitutes an important risk factor for the development of noninsulin-dependent diabetes mellitus *(6)*. We have therefore studied the maturation of a glucose-responsive insulin release from fetal rat islets, and specifically investigated the impact of metabolic substrates, α-adrenoceptors, imidazoline receptors, incretins, and cAMP.

Results

Figure 1A reveals that in islets cultured in 3.3 m*M* glucose, acutely raising the ambient glucose concentration from 3.3 to 16.7 m*M* evoked a modest stimulation of short-term insulin release. However, as is evident from Fig. 1B, the stimulation by glucose was more pronounced in islets maintained in 11.1 m*M* glucose. Moreover, the insulin content was much higher in islets cultured in 11.1 than in 3.3 m*M* glucose (Fig. 1C). Culture in the presence of the nonglucidic nutrient α-ketoisocaproic acid (KIC) (20 m*M*), the deamination product of L-leucine, markedly enhanced both basal (3.3 m*M* glucose) and stimulated (16.7 m*M* glucose) insulin release from islets cultured at 3.3 or 11.1 m*M* glucose (Figs. 1A,B). Additionally, KIC significantly elevated the insulin content of islets maintained in 3.3 m*M* glucose, whereas it slightly lowered it in islets cultured at 11.1 m*M* glucose (Fig. 1C). The α-adrenoceptor antagonist phentolamine (10 μ*M*) markedly amplified both basal and glucose-stimulated insulin secretion when added with

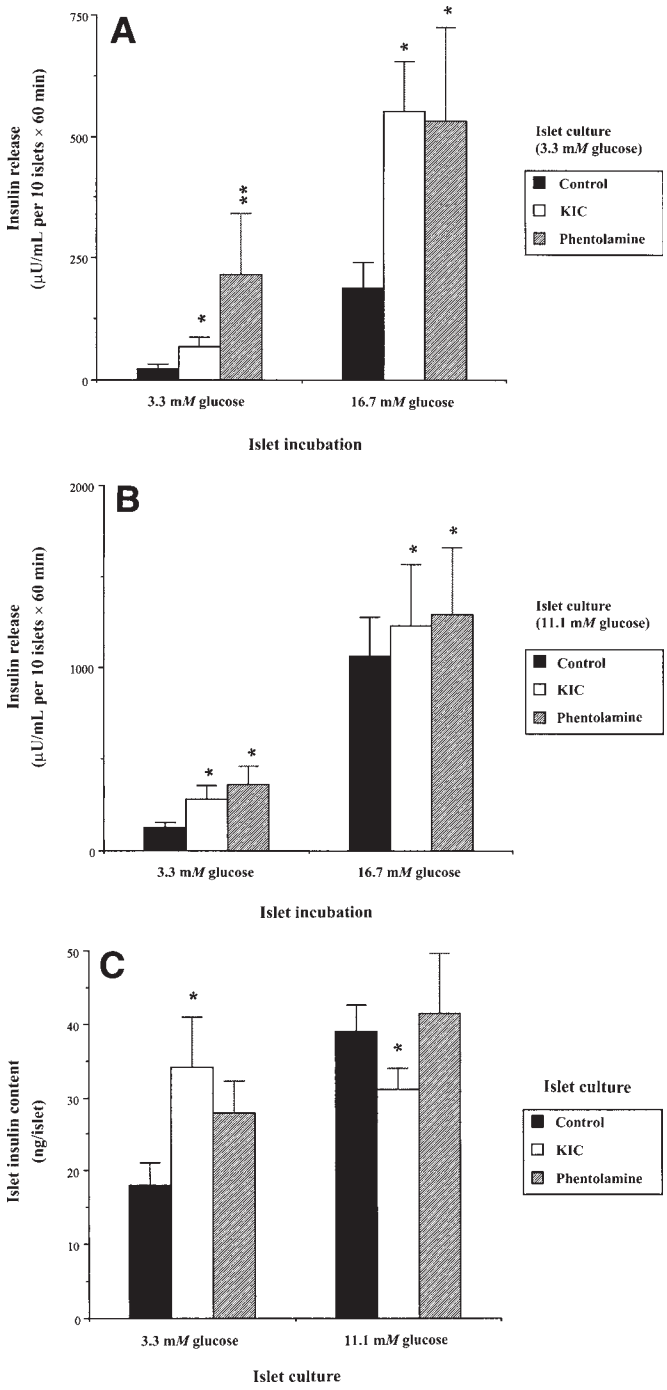


Fig. 1. **(A–C)** Effects of glucose, KIC, and phentolamine on fetal β-cell differentiation. Fetal rat pancreatic islets were cultured for 7 d in RPMI-1640 medium with 10% fetal calf serum (FCS) in 3.3 (A) or 11.1 m*M* (B) glucose and in the presence or absence of the indicated compounds. Concentrations used were 20 m*M* KIC and 10 μ*M* phentolamine. Insulin release (A,B) was studied in batches of 10 islets exposed to two consecutive incubations in 3.3 and 16.7 m*M* glucose, respectively. The insulin content of islet homogenates (C) was measured by radioimmunoassay (RIA). Bars represent means ± SEM for 5–18 observations. * and ** denote $p < 0.05$ and $p < 0.01$ for chance differences vs control islets using two-way analysis of variance (ANOVA) in conjunction with Bonferroni's transformation. Note the difference in the *y*-axis in (A–C).

islets cultured in either 3.3 or 11.1 m*M* glucose (Figs. 1A,B). However, phentolamine did not cause a discernible change in the islet content of insulin (Fig. 1C). By contrast, the pure α_2-adrenoceptor antagonist benextramine (10 μ*M* for 7 d) had no such effects (Table 1).

In the next series of experiements (Figs. 2A–C), the cAMP system was studied. Addition to culture media of the membrane-permeant agonist of cAMP-dependent protein kinases types I and II, Sp-cAMP[S] (50 μ*M*), failed to influence basal or glucose-responsive insulin secretory rates at either glucose concentration during culture (Figs. 2A,B); also, the cAMP analog did not affect the islet insulin content (Fig. 2C). Likewise, the membrane-permeant antagonist of cAMP-dependent protein kinases, Rp-cAMP[S] (50 μ*M*), did not affect any of these parameters (Figs. 2A–C). Note the difference in the *y*-axis in Figs. 1A–C and 2A–C.

Figure 3 presents the time-course of the impact of glycemic stimulation on a glucose-sensitive insulin secretion in fetal pancreatic explants. It is evident that culture in a substimulatory (3.3 m*M*) glucose concentration retarded the development of a mature glucose-sensitive insulin secretion, whereas culture in a high (16.7 m*M*) glucose concentration accelerated this process (Figs. 3A,B). Likewise, the insulin content in the fetal pancreatic tissue was significantly elevated by culture in a stimulatory glucose concentration (Fig. 3C). Additionally, high glucose increased the fractional insulin release, i.e., the proportion of hormone released of total pancreatic insulin content (Fig. 3D).

Discussion

The present study shows that β-cell functional maturation can be modulated in vitro by nutrients and by phentolamine, whereas cAMP seems virtually ineffective in this respect. The dependency of maturation of the stimulus-secretion coupling on a stimulatory glucose level has been confirmed in many previous reports studying rodent islets *(2,3)*. Aside from being the cardinal short-term stimulator of insulin biosynthesis and release *(7–9)*, the sugar is also an important mitogenic stimulus for the β-cell *(4,5,10,11)*. It is thus possible that the increased insulin content of islets cultured in a high-glucose concentration reflects not only increased insulin biosynthesis by individual β-cells, but also an increased number of β-cells recruited either through mitotic division or by differentiation of endocrine precursor cells, as has been shown for another β-cell differentiating agent, nicotinamide *(12)*. Because we have made no attempt to discriminate among effects on preexisting β-cells or new β-cells formed through recruitment of undifferentiated precursor cells or through replication of β-cells, we cannot differentiate among effects on individual β-cells or the neoformation process per se. Infants of diabetic mothers show "mature" insulin secretory responses to glucose in contrast to normal infants, who generally have blunted insulin responses to glu-

Table 1
Effects of Benextramine on Functional Differentiation of Fetal β-Cells[a]

Culture (7 d)		Incubation (60 min)	Insulin release	Insulin
Glucose (m*M*)	Benextramine	(m*M* glucose)	(μU[20 islets·60 min])	(mU/islet)
3	–	3	351 ± 155	1.99 ± 0.42
3	–	16.7	445 ± 131	
3	+	3	638 ± 404	2.54 ± 0.64
3	+	16.7	746 ± 248	
11	–	3	568 ± 266	7.34 ± 1.59*c*
11	–	16.7	2229 ± 465*b*	
11	+	3	832 ± 404	4.93 ± 1.29
11	+	16.7	2081 ± 316	

[a]Fetal rat pancreatic islets were cultured for 7 d in RPMI-1640 medium with 1% FCS in 3.3 or 11.1 m*M* glucose and in the presence (+) or absence (–) of benextramine (10 μ*M*). Insulin release was studied in batches of 10 islets exposed to two consecutive incubations in 3.3 and 16.7 m*M* glucose, respectively. Values represent means ± SEM for six observations.

[b]$p < 0.01$ for a chance difference vs islets cultured in 11 m*M* glucose without benextramine and incubated in 3 m*M* glucose, using two-way ANOVA in conjunction with Bonferroni's transformation.

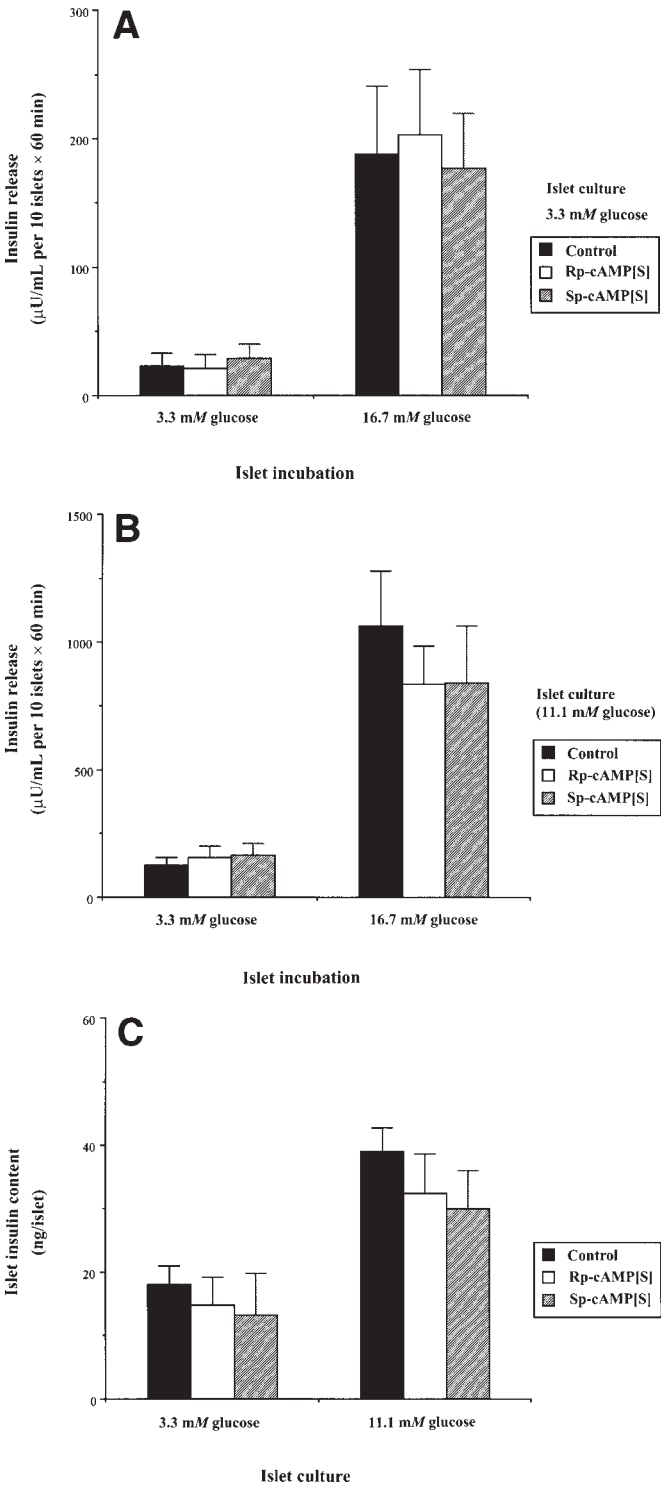
[c]$p < 0.05$ for a chance difference vs islets cultured in 3 m*M* glucose without benextramine.

cose *(6)*. Although this may well influence *in utero* development and immediate postnatal events such as macrosomia and hypoglycemia, there is no clear evidence to suggest that low nutrient concentrations may permanently impair insulin secretion.

The metabolism of glucose involves its catabolism in the cytosolic Emden-Meyerhof pathway and, after funneling into mitochondria, further breakdown in the Krebs cycle *(13)*. It is not clear which metabolic signals are required for the differentiating actions of the sugar on the β-cell. However, because qualitatively similar results were recorded when islets were cultured with the nonglucidic nutrient KIC, i.e., the deamination product of L-leucine, which enters metabolism directly at the Krebs cycle level *(14)*, it appears that signals generated in the glycolytic pathway are of less importance for this process. It can be speculated that the high amount of adenosine triphosphate (ATP) and reduced pyridine nucleotides generated within the mitochondrion may be required in this context.

Sympathetic nerve fibers are abundant in the pancreatic islets and sympathetic stimulation inhibits insulin secretion *(15,16)*. In the present study, culture with phentolamine caused the development of an enhanced basal and glucose-regulated insulin secretion, thus suggesting that α-adrenergic stimulation represses such a development, consistent with the previously reported inhibitory role of this system in β-cell growth *(17)* and short-term insulin release *(15,16)*. However, phentolamine is a compound with an imidazoline structure. Therefore, it cannot be excluded that the effect of phentolamine may be mediated through the imidazoline receptor system. In fact, previous studies have shown that the short-term insulin-releasing property of phentolamine is not mediated by α-adrenoceptor blockade *(18,19)*. The pure α_2-adrenoceptor antagonist benextramine did not mimic the phentolamine. Previous studies have shown that benextramine does not differentiate between clonidine and noradrenaline in rat islets of Langerhans but that it does show preference for α-adrenoceptors in this tissue *(20)*. Thus, our findings tend to indicate that the differentiating effects of phentolamine may in part be accounted for by interference with imidazoline receptors, although additional and unknown actions of phentolamine may also be involved.

Rp-cAMP[S] is a chemically stable, membrane-permeant, inhibitory cAMP analog that competes with natural cAMP in interacting with the cAMP-dependent protein kinases (protein kinase A [PKA]) and is totally resistant to phosphodiesterases *(21)*. It prevents dissociation of the kinase holoenzyme into catalytic and regulatory subunits resulting in complete loss of phosphorylating activity *(21)*. Given that these kinases exhibit much higher affinity for Rp-cAMP[S] than for cAMP *(21)*, and that the intracellular concentration of Rp-cAMP[S] can be calculated to be at least 50-fold higher than that of cAMP, the use of Rp-cAMP[S] seems to be a valid instrument when probing the biological significance of the cAMP system. Conversely, Sp-cAMP[S] is the stimulatory diastereomer that is a potent PKA agonist *(21)* and stimulates β-cell mitogenesis and insulin secretion *(22)*. By contrast, in the presently investigated islets, neither the cAMP agonist nor the antagonist affected the development of glucose-sensitive insulin release, suggesting that this process seemingly occurs independently of cAMP. Early reports *(23)* have carefully characterized the adenylyl cyclase and phosphodiesterase systems operative in these islets.



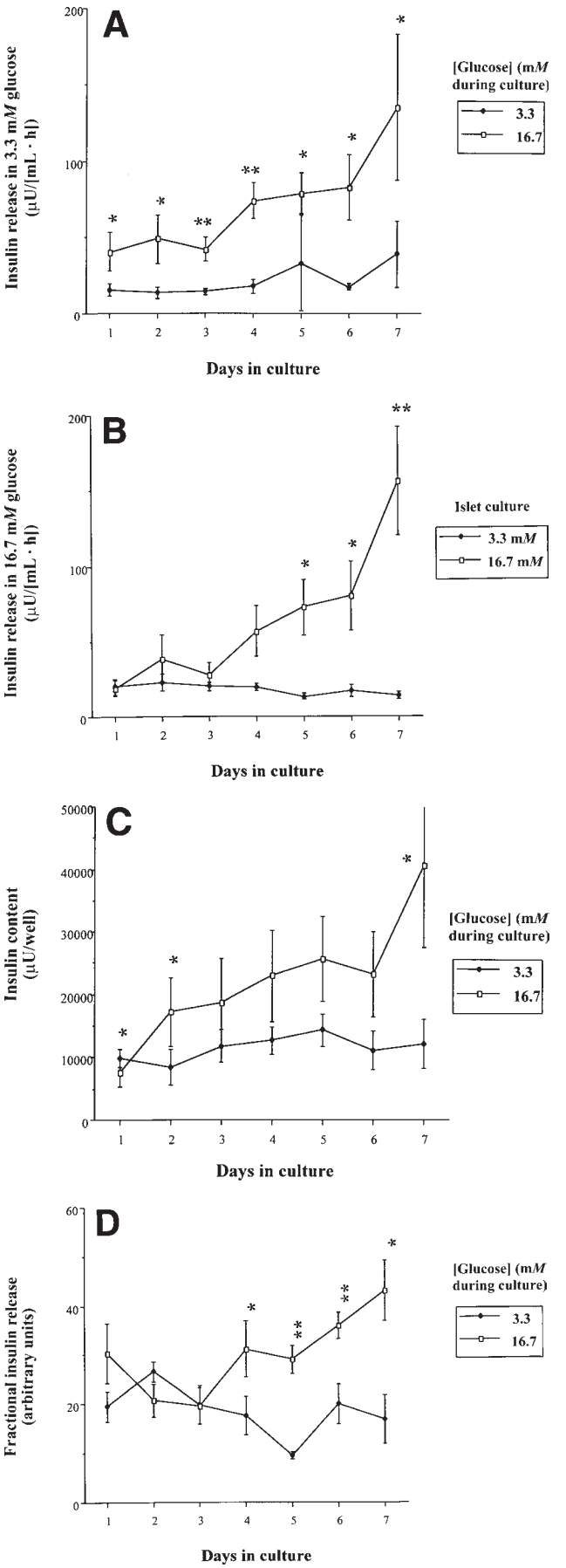


Fig. 2. **(A–C)** Effects of cAMP on fetal β-cell differentiation. Fetal rat pancreatic islets were cultured for 7 d in RPMI-1640 medium with 10% FCS in 3.3 (A) or 11.1 m*M* (B) glucose and in the presence or absence of the indicated compounds. Concentrations used were: 50 μ*M* Sp-cAMP[S] and 50 μ*M* Rp-cAMP[S]. Insulin release (A,B) was studied in batches of 10 islets exposed to two consecutive incubations in 3.3 and 16.7 m*M* glucose, respectively. The insulin content of islet homogenates (C) was measured by RIA. Bars represent means ± SEM for 6–18 observations. Note the difference in the *y*-axis in (A–C).

In conclusion, the present study shows that islet β-cell differentiation and functional maturation of the stimulus-secretion coupling can be accelerated in vitro in fetal rat pancreatic tissue by nutrient stimulation, and by phentolamine possibly acting through imidazoline receptors, whereas cAMP seems essentially ineffective in this respect. These effectors may be of regulatory significance in the in vivo development of glucose-sensitive β-cells.

Materials and Methods

Materials

Glucose, KIC, ATP, carbamylcholine, benextramine, and phentolamine were obtained from Sigma (St. Louis, MO). The membrane-permeant cAMP analogs Sp-cAMP[S] and Rp-cAMP[S] were delivered by the Biolog Life Science Institute (Bremen, Germany). Collagenase type CLS (EC 3.4.24.3) was obtained from Boehringer-Mannheim (Mannheim, Germany). Culture RPMI-1640 medium, FCS, L-glutamine, benzylpenicillin, and streptomycin were from Flow Laboratories (Irvine, UK). Antiporcine insulin serum was raised in guinea pigs in our laboratory *(24)*. Crystalline rat insulin was from Novo (Copenhagen, Denmark), and porcine ^{125}I-insulin was prepared in our laboratory. All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany).

Preparation and Culture of Fetal Islets

Pregnant Wistar rats, purchased from B & K Universal (Sollentuna, Sweden), were killed by decapitation on d 21 of gestation and their fetuses were rapidly removed. Fetal rat islets were prepared from pancreatic glands as previously described *(10,22,25)*. Briefly, the pancreata were finely chopped and digested for a short time with collagenase. The carefully washed digest was plated in ø 25-mm culture dishes allowing cell attachment (Nunc, Roskilde, Denmark) and cultured for 7 d at 37°C in a humidified atmosphere of 5% CO_2 in ambient air in RPMI-1640 medium containing 3.3 or 11.1 m*M* glucose, 10% FCS, 2 m*M* L-glutamine, 100 U/mL of benzylpenicillin, and 0.1 mg/mL of streptomycin. The desired test substances were added 24 h after seeding, and media were exchanged every 48 h. At the end of the culture period, groups of 10 islets were transferred to 1 mL of preincubation media (next section) in 24-multiwell plates or Ellerman tubes for subsequent secretion experiments.

In one series of experiments (Fig. 3), the pancreatic digest was aliquoted directly into 24-multiwell plates containing RPMI-1640 medium with 3.3 or 16.7 m*M* glucose, 10% FCS, 2 m*M* L-glutamine, 100 U/mL of benzylpenicillin, and 0.1 mg/mL of streptomycin. In this setting, media were exchanged every 24 h in connection with short-term insulin release experiments.

Insulin Secretion and Islet Insulin Content

Batches of 10 islets were selected and preincubated at 37°C for 45 min in a bicarbonate buffer *(26)* supplemented with 2 mg/mL bovine serum albumin (BSA), 3.3 m*M* glucose, and 10 m*M* HEPES (pH 7.4). The preincubation media were discarded and incubations continued for another 60 min in fresh buffer, and media were frozen for subsequent analysis of their insulin concentration *(24)*. Fresh media, now containing 16.7 m*M* glucose, were added to the same islets, and incubations continued for another 60 min, whereafter media were frozen for subsequent analysis of their insulin concentration *(24)*. Test substances were not present during these short-term experiments. The islet insulin content (extracted from sonicates overnight at 4°C in 70% ethanol plus 0.18 *M* HCl) was measured by RIA *(24)*.

In one series of experiments (Fig. 3), insulin release directly from the crude pancreatic digest was studied in 24-multiwell plates. Thus, culture media were discarded and 1 mL of 37°C preincubation media (a bicarbonate buffer [*26*] supplemented with 2 mg/mL of BSA, 3.3 m*M* glucose, and 10 m*M* HEPES [pH 7.4]) was carefully added to the wells for 45 min. These media were carefully removed and replaced with similar fresh media, and incubations were continued for 60 min at 37°C. Aliquots (300 μL) of these media were frozen for subsequent insulin analysis *(24)*. The remainder of the media was removed and replaced with fresh media, now containing 16.7 m*M* glucose, and incubations were extended for another 60 min. Aliquots (300 μL) of these media were frozen for subsequent insulin analysis *(24)*. The remainder of the media was removed and the tissue content of the wells scraped into Eppendorf tubes in 1 mL of phosphate-buffered saline. The tissue was pelleted by centrifugation (12,000*g*, 5 s) in an Eppendorf centrifuge and ultrasonically homogenized in 70% ethanol containing 0.18 *M* HCl, whereafter its insulin content was determined by RIA *(24)*. Immunoreactive insulin was measured using antibodies against porcine insulin, raised in guinea pigs at our laboratory, using ^{125}I-labeled insulin as tracer and rat insulin as standard. Dextran-coated charcoal in 0.2 *M* glycine buffer was used to separate bound and free insulin.

Statistical Analysis

Results presented are derived from four independent experiments performed on different days. Means ± SEM

Fig. 3. **(A–D)** Time course of glucose-promoted β-cell differentiation. Fetal rat pancreatic islets were cultured for 7 d in RPMI-1640 medium with 10% FCS in 3.3 or 16.7 m*M* glucose. Insulin release was studied daily by exposing islets to two consecutive incubations in 3.3 (A) and 16.7 mM (B) glucose, respectively. The insulin content of islet homogenates (C) was measured by RIA. In (D) the fractional release, i.e., the amount of insulin released during 60 min of stimulation in 16.7 m*M* glucose of total cellular insulin content, is shown (in arbitrary units). Values represent means ± SEM for six observations. * and ** denote $p < 0.05$ and $p < 0.01$ for chance differences vs islets cultured in 3.3 m*M* glucose using two-way ANOVA in conjunction with Bonferroni's transformation.

were calculated and groups of data compared using two-way ANOVA in conjunction with Bonferroni's transformation.

Acknowledgments

Financial support was received from Barndiabetesfonden, Funds of the Karolinska Institute, the Swedish Society of Medicine, the Nordic Insulin Fund, Åke Wiberg's Foundation, the Swedish Medical Research Council (grant K98-03X-12550-01A, 00034), the Swedish Diabetes Association, Harald Jeansson's and Harald and Greta Jeansson's Foundations, Fredrik and Inger Thuring's Foundation, Novo-Nordisk Sweden Pharma AB, Torsten and Ragnar Söderberg's Foundations, Magn. Bergvall's Fund, Tore Nilsson's Foundation for Medical Research, and Syskonen Svensson's Fund.

References

1. Efendic, S., Luft, R. and Wajngot, A. (1984). *Endocrine Rev.* **5,** 395–410.
2. Freinkel, N., Lewis, N. J., Johnson, R., Swenne, I., Bone, A., and Hellerström, C. (1984). *Diabetes* **33,** 1028–1038.
3. Hellerström, C. and Swenne, I. (1991). *Diabetes* **40(Suppl. 2),** 89–93.
4. Sjöholm, Å. (1996). *J. Intern. Med.* **239,** 211–220.
5. Sjöholm, Å. (1993). *Am. J. Physiol.* **264,** C501–Cß518.
6. Hales, C. N. (1994). *Diabetologia* **37(Suppl. 2),** S162–S168.
7. Sjöholm, Å. (1998). *Endocrine* **9,** 1–13.
8. Berggren, P. -O., Arkhammar, P., Islam, M. S., Juntti-Berggren, L., Khan, A., Kindmark, H., Köhler, M., Larsson, K., Larsson, O., Nilsson, T., Sjöholm, Å., Szecowska, J., and Zhang, Q. (1993). *Adv. Exp. Med. Biol.* **334,** 24–45.
9. Newgard, C. B. and McGarry, J. D. (1995). *Annu. Rev. Biochem.* **64,** 689–719.
10. Sjöholm, Å. and Hellerström, C. (1991). *Am. J. Physiol.* **260,** C1046–C1051.
11. Sjöholm, Å. (1992). *FEBS Lett.* **311,** 85–90.
12. Sjöholm, Å., Korsgren, O., and Anderson, A. (1994). *Endocrinology* **135,** 1559–1565.
13. Meglasson, M. D. and Matschinsky, F. M. (1986). *Diabetes/Metab. Rev.* **2,** 163–214.
14. Sjöholm, Å. (1996). *Am. J. Physiol.* **270,** C1105–C1110.
15. Miller, R. E. (1981). *Endocrine Rev.* **2,** 471–494.
16. Smith, P. H. and Porte, D. Jr. (1976). *Annu. Rev. Pharm. Toxicol.* **16,** 269–285.
17. Sjöholm Å. (1991). *Biochem. Biophys. Res. Commun.* **180,** 152–155.
18. Östenson, C.-G., Pigon, J., Doxey, J. C., and Efendic´, S. (1988). *J. Clin. Endocrinol. Metab.* **67,** 1054–1059.
19. Östenson, C.-G., Cattaneo, A. G., Doxey, J. C., and Efendic´, S. (1989). *Am. J. Physiol.* **257,** E439–E443.
20. Hurst, R. D., Chan, S. L., and Morgan, N. G. (1989). *J. Mol. Endocrinol.* **2,** 99–105.
21. Rothermel, J. D. and Botelho, L. H. (1988). *Biochem. J.* **251,** 757–762.
22. Sjöholm, Å. (1992). *Am. J. Physiol.* **263,** C114–C120.
23. Mintz, D. H., Levey, G. S., and Schenk, A. (1973). *Endocrinology* **92,** 614–617.
24. Herbert, V., Lau, K. -S., Gottlieb, C. H., and Bleicher, S. J. (1965). *J. Clin. Endocrinol. Metab.* **25,** 1375–1384.
25. Hellerström, C., Lewis, N. J., Borg, H., Johnson, R., and Freinkel, N. (1979). *Diabetes* **28,** 769–776.
26. Krebs, H. A. and Henseleit, K. (1932). *Hoppe-Seyler's Z. Physiol. Chem.* **210,** 33–66.