Factors and Their Signaling Mechanisms Involved in the Regulation of Growth and the Function of Insulin-Secreting Cells

インスリン分泌細胞の増殖と機能の制御に関与する因子とその情報伝達

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Abbreviations

ANOVA, analysis of variance; β-CAS, β-casein gene promotor; bFGF, basic fibroblast growth factor; bGH, bovine growth hormone; BHA, butylated hydroxyanisol; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic Ca^{2+}; CHO cell, Chinese hamster ovary cell; CM, complete medium; CPT-cAMP, 8-(4-chlorophenylthio)cyclic AMP; ECM, extracellular matrix; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAS, interferon-γ-activated site; GH, growth hormone; GLP-1, glucagon-like peptide-1; HGF, hepatocyte growth factor; hGH, human growth hormone; HUVEC, human umbilical vein endothelial cells; IBMX, 3-isobutyl-1-methyl-xanthine; IDDM, insulin-dependent diabetes mellitus; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; iNOS, inducible isoform of nitric oxide synthase; IRF-1, interferon regulatory factor-1; IRS, insulin receptor substrate; JAK, Janus kinase; K^+_{ATP}-channel, ATP-sensitive K^+-channel; KRBH, Krebs-Ringer bicarbonate-HEPES buffer; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 tetrazolium bromide; NGF, nerve growth factor; NIDDM, non-insulin-dependent diabetes mellitus; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PRL, prolactin; RIA, radioimmunoassay; Rp-cAMPS, Rp-cyclic adenosine-3',5'-monophosphorothioate; SDS, sodium dodecyl sulfate; SFM, serum-free medium; SIE, *sis*-inducible element in the c-*fos* promotor gene; STAT, signal transducers and activators of transcription; T_3, triiodothyronine; TNF, tumor necrosis factor; VDCC, voltage-dependent Ca^{2+} channel

PURPOSE/BACKGROUND

Diabetes mellitus is a clinical syndrome in which absolute or relative insulin deficiency causes the derangement of glucose, protein, and lipid metabolism, ultimately leading to a variety of systemic complications. Hyperglycemia develops when the capacity of insulin secretion fails to meet a demand of the body, especially in the presence of insulin resistance as often observed in patients with non-insulin-dependent diabetes mellitus (NIDDM) [1]. Insulin-dependent diabetes mellitus (IDDM) is characterized by severe insulin deficiency due to the autoimmune destruction of pancreatic β-cells [2]. Although insulin secretory capacity is rather preserved in patients with NIDDM, they usually show functional defects in insulin secretion, such as loss of pulsatile fluctuations of plasma insulin levels [3] and impairment of acute-phase glucose-induced insulin release [4]. Moreover, it has been reported that β-cell mass is decreased in NIDDM compared with nondiabetics [5,6]. Thus in order to maintain the normal blood glucose regulation, sufficient insulin secretion as well as adequate pancreatic β-cell mass with preserved insulin biosynthesis are thought to be essential. It is therefore important to elucidate the precise mechanisms involved in the regulation of proliferation and the differentiated function, as well as those underlying the destruction, of pancreatic β-cells.

It has been known that pancreatic β-cells possess a limited potential for cell growth [7]. Nevertheless, there are some physiological or pathological situations in which replication of β-cells is accelerated, such as pregnancy [8,9], obesity [5], glucose infusion [10-12], and partial pancreatectomy [13,14]. A number of factors have been reported to stimulate β-cell growth (Table 1) [7,15-17]. These include glucose, amino acids, cAMP, and a variety of peptides (hormones, cytokines, and growth factors), although their intracellular signaling mechanisms are largely unknown. It can be presumed that investigation on the mode of action of these potent factors should provide insight into the major physiological mechanisms regulating growth and the differentiated function of β-cells. In this respect, growth hormone (GH) and its related peptides prolactin (PRL) and placental lactogen are of interest because of their potency in affecting growth and the function of β-cells. Reported effects of these hormones in various types of insulin-secreting cells are summarized in Table 2. Both hormones stimulate growth of β-cells [18-25], increase insulin biosynthesis [19,25,27-

29], promote cellular oxidative metabolism [19,20] which is believed to be essential for glucose-induced insulin secretion [32,33]. Moreover, these hormones are thought to promote the functional maturation (i.e. increase in sensitivity to glucose for the stimulation of insulin secretion) of β-cells [27,30,31]. In view of these diverse effects of GH-related peptides in β-cells, to study their mode of action might reveal crucial mechanisms for the regulation of growth and the function of these cells [7,15,17,34].

Table 1. Reported mitogenic factors for insulin-secreting cells.

Nutrients	Glucose, Amino acids, Fatty acids
Insulin-related factors	Insulin, IGF-I, IGF-II
The GH family	GH, PRL, placental lactogen
Growth factors	PDGF, EGF, bFGF, HGF
Gastrointestinal peptides	Gastrin, Cholecystokinin
Pharmacological agents	cAMP analogues*, Forskolin, Phorbol ester, Sulfonylureas*, Lithium, Nicotinamide

*contradictory

Table 2. Actions of GH and PRL reported in various types of insulin-secreting cells.

	GH	PRL
Proliferation/DNA synthesis	islets (adult [18,19], neonatal [20]) neonatal β-cells [23, 24] RIN-5AH cells [25]	islets (adult [18,21,22], neonatal [20, 21])
Insulin biosynthesis	islets (adult [19], fetal [26]) RIN-5AH cells [25,28]	islets (neonatal [27])
IGF-I production	islets (adult [19], fetal [26]) human fetal pancreas [29]	(-)
Glucose oxidation	islets (adult [19])	islets (neonatal [20])
Functional maturation	human fetal islet-like clusters [30]	islets (neonatal [27, 31])

References are indicated in parentheses.

Receptors for both GH and PRL belong to the cytokine/hematopoietic growth factor receptor superfamily which comprises receptors for a number of interleukins (ILs 2-7, 9-13), erythropoietin, colony stimulating factors and other trophic substances [35,36]. Recent investigations have established the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway as the major signaling paradigm of these factors [37]. The cytokines activate the JAK family of tyrosine kinases associated with the plasma membrane receptors through tyrosine phosphorylation, followed by the activation of STATs, which are tyrosine phosphorylated by JAK kinases and bind to the DNA to activate gene transcription (Fig. 1). In fact, many of the factors belong to the cytokine receptor superfamily [37,38], including GH [39] and PRL [40,41], induce tyrosine phosphorylation, and subsequently activation, of JAK2. Three different STAT proteins, namely STAT1, STAT3, and STAT5, have been reported to be activated by GH [42], whereas PRL activates only STAT5 which has been known as the mammary gland factor [43]. Some other signaling mechanisms have also been reported for the effects of GH [44] and PRL [45]. These include the rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) [45,46], the production of diacylglycerol followed by the activation of protein kinase C (PKC), tyrosine phosphorylation of various proteins such as insulin receptor substrate (IRS)-1 [47-49], IRS-2 [50], phosphatidylinositol 3'-kinase [48], the stimulation of G-protein-coupled mechanisms [44], and activation of the mitogen-activated protein (MAP) kinase pathway [51,52]. It should be noted that a proline-rich juxta-membrane cytosolic domain of the GH receptor has been implicated in the activation of both JAK2 and the MAP kinase pathway [53,54], and that the deletion of this portion leads to the selective loss of GH stimulation of mitogenesis [55]. Moreover, expression of a kinase-deficient form of JAK2 has been shown to abrogate erythropoietin-induced mitogenesis [56]. Thus it seems reasonable to postulate that either of these two distinct signaling pathways plays a major role in the regulation of β-cell growth.

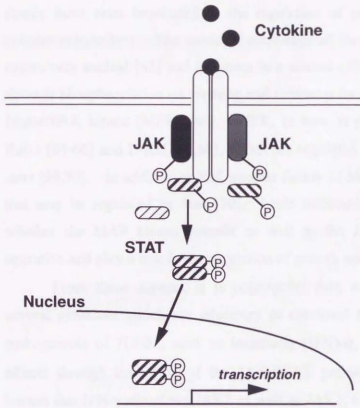


Fig. 1. The JAK-STAT pathway. A simplified scheme representing a typical mode of activation of the JAK-STAT pathway is shown. Cytokines binding to their transmembrane receptors activate JAK kinases associated with the receptor through tyrosine phosphorylation. STAT proteins, which are tyrosine phosphorylated by JAK kinases, form homo- or heterodimers, translocate to the nucleus, and bind to DNA to promote transcription of their target genes.

MAP kinase (also called extracellular signal-regulated kinase, ERK) comprises a family of serine/threonine kinases activated by a number of growth factors, hormones, and neurotransmitters in a cell type-specific manner [57-61]. The members of this family have been implicated in the regulation of proliferation, differentiation, and cellular metabolism. The mode of activation of the MAP kinase cascade has been extensively studied [62] and is shown in a scheme (Fig. 2). MAP kinase is activated through phosphorylation on tyrosine and threonine by the dual specificity kinase MAP kinase/ERK kinase (MEK) [63]. MEK, in turn, is phosphorylated and activated by Raf-1 [64-66] and B-Raf [67,68], which are regulated by Ras in its GTP-bound active state [69,70]. In addition to Raf, another family of MEK kinases has been discovered that may be regulated by Ras [70]. Little information is, however, available as to whether the MAP kinase cascade as well as the JAK-STAT pathway are indeed operative and play a role in the regulation of growth and the function of β-cells.

From these aspects, it is noteworthy that, aside from GH-related peptides, several cytokines which are inhibitory or cytotoxic to β-cells and implicated in the pathogenesis of IDDM, such as interferon (IFN)-α, IFN-γ, IL-6, could elicit their effects through activation of the JAK-STAT pathway [36,38,71]. Although it is known that IFN-γ stimulates JAK2 as well as JAK1, effects of this cytokine on β-cells [72,73] are quite different from those of GH or PRL that also activate JAK2. Treatment of insulin-secreting cells with IFN-γ, especially in combination with other cytokines such as IL-1β or tumor necrosis factor (TNF)-α, resulted in inhibition of insulin secretion and ultimately in apoptotic cell death [73,74]. Despite these clear effects, signal transduction of IFN-γ in β-cells has been less studied. IFN-γ is known to activate STAT1, which, being tyrosine phosphorylated, binds to the IFN-γ-activated site (GAS) in many IFN-γ-responsive genes, including interferon regulatory factor-1 (IRF-1) [75]. IRF-1 has been implicated in the induction of apoptosis by IFN-γ [76] and of the cytokine-inducible nitric oxide (NO) synthase (iNOS) [77,78] that catalyzes the conversion of L-arginine to L-citrulline thereby generating NO [79,80]. Thus NO may cause inhibition of mitochondrial metabolism [81], protein modification [82], and DNA cleavage [83], all of which could lead to the

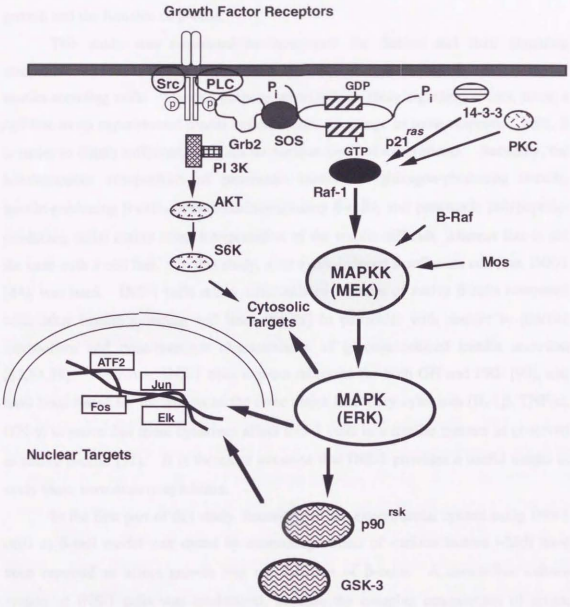


Fig. 2. The MAP kinase cascade. A typical mode of activation of the MAP kinase cascade through the growth factor receptor is shown.

impairment in insulin secretion and β-cell death. Taken together, it seems possible that the JAK-STAT pathway plays a central role both in stimulation and in inhibition of growth and the function of β-cells.

This study was conducted to investigate the factors and their signaling mechanisms involved in the regulation of growth and the differentiated function of insulin-secreting cells. For this purpose, especially to study signaling events, using a cell line as an experimental model system offers advantage in some respects. First, it is easier to obtain sufficient materials for various kinds of experiments. Secondly, the heterogeneous composition of pancreatic islets (i.e. glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing cells) makes often interpretation of the results difficult, whereas this is not the case with a cell line. In this study, a rat x-ray-induced insulinoma cell line, INS-1 [84], was used. INS-1 cells retain differentiated features of native β-cells compared with other insulin-secreting cell lines [85-88] in particular with respect to glucose metabolism and dose-response characteristics of glucose-induced insulin secretion [32,84,89]. Moreover, INS-1 cells express receptors for both GH and PRL [90], and have been tested for the effects of the three major inhibitory cytokines (IL-1β, TNF-α, IFN-γ) to prove that these cytokines affect INS-1 cells in a similar manner as observed in native β-cells [91]. It is therefore assumed that INS-1 provides a useful model to study these hormones or cytokines.

In the first part of this study, feasibility of the experimental system using INS-1 cells as β-cell model was tested by examining effects of various factors which have been reported to affect growth and the function of β-cells. A serum-free culture system of INS-1 cells was established, because the complex composition of serum renders difficult definition of individual factors. Using this system, effects of GH and PRL as β-cell mitogens, as well as those of the inhibitory cytokines IFN-γ and TNF-α were examined. In addition, factors maintaining insulin secretory function of INS-1 cells were investigated. In the second part, signal transduction of these potent factors were studied, with special reference to the JAK-STAT pathway. Finally, activation of the MAP kinase cascade in INS-1 cells was examined in conjunction with cell growth and insulin secretion.

MATERIALS AND METHODS

Materials

RPMI 1640 and 2-mercaptoethanol were purchased from Gibco (Life Technologies, Basel, Switzerland) and all the other supplements of the culture media were from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human GH (hGH) was kindly provided by Dr M. Dreano (Ares-Serono, Geneva, Switzerland) and recombinant bovine GH (bGH) by American Cyanamid Co. (Princeton, NJ, USA). Glucagon-like peptide-1 (GLP-1) was generously given by Dr G. K. Hendrick (Harvard Medical School, Boston, MA, USA) and Insulin-like growth factor (IGF)-I by Dr J. Zapf (University of Zürich, Zürich, Switzerland). Nerve growth factor-2.5S (NGF) from mouse submaxillary glands, recombinant human basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), phorbol 12-myristate 13-acetate (PMA), 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), forskolin, verapamil, sodium orthovanadate, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), bovine brain myelin basic protein, leupeptin, and Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5 tetrazolium bromide (MTT), hyamine hydroxide, N^G-monomethyl-L-arginine (NMMA), butylated hydroxyanisole (BHA), and quercetin were from Sigma. Pituitary adenylate cyclase-activating polypeptide (PACAP)38 of ovine origin was from Peninsula Laboratories, Inc. Recombinant murine IFN-γ was purchased from Genzyme Diagnostics (Cambridge, MA, USA), and recombinant murine TNF-α was from R&D Systems (Funakoshi, Tokyo, Japan). Rp-cyclic adenosine-3',5'-monophosphorothioate (Rp-cAMPS) was obtained from BioLog Life Science Institute (Bremen, Germany), calciseptine from Latoxan (Rosans, France) and lavendustin A from Calbiochem (La Jolla, CA, USA). [*Methyl*-^{3}H]thymidine (247.9 GBq/mmol, 37.0 MBq/ml), [3,4-^{14}C]glucose (1.11-2.22 GBq/mmol), and [1-^{14}C]pyruvic acid sodium salt (0.52 GBq/mmol) were from New England Nuclear Research Products (Boston, MA, USA). [α-^{32}P]dCTP, [γ-^{32}P]ATP, protein A-Sepharose, and the RIA kit for cAMP determination were purchased from Amersham Life Science (Little Chalfont, UK). A solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma (Matrigel®) was obtained from Becton Dickinson Labware (Bedford, MA, USA), and microtiter plates coated with bovine corneal endothelium-derived extracellular matrix (ECM) from Eldan Tech (Jerusalem,

Israel). Fura-2/acetoxy-methylester (AM) and bis-(1,3-diethylthiobarbiturate)trimethine-oxonol (bisoxonol) were from Molecular Probes (Eugene, OR, USA). Monoclonal anti-phosphotyrosine antibody (4G10) and rabbit polyclonal antiserum against human JAK1 and murine JAK2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Monoclonal antibodies against STAT proteins were from Transduction Laboratories (Lexington, KY, USA), and horseradish peroxidase-conjugated anti-mouse IgG antibodies from Sigma. Rabbit polyclonal antisera to the 12-amino acid carboxyterminal of 44-kDa MAP kinase and the 17-amino acid amino-terminal of MEK-1, synthesized by Neosystem (Strasbourg, France), were generated as described [92]. Bacteria expressing rat GST-44-kDa MAP kinase fusion protein was kindly provided by Dr. Joseph Avruch (Boston, MA, USA). Specific cDNA probe of the mouse macrophage iNOS was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

Cell cultures

INS-1 cells (passages 73-89) were cultured in the complete medium (CM) composed of RPMI 1640 supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol as previously reported [84]. Cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and detached by exposing to the same buffer containing 0.025% (w/v) trypsin and 2 mM EDTA. Then, cells were diluted in serum-free RPMI medium (4°C), centrifuged (1,000 x *g*, 10 min), and resuspended in the same medium for plating. The pheochromocytoma cell line PC12 and the hepatoma cell line (HepG2) were cultured according to standard techniques. Human umbilical vein endothelial cells (HUVEC) were prepared as described elsewhere [93] and were used during passages 1-3. These three cell types were cultured for 3 days in SFM, as for INS-1 cells. Subsequently, the media were collected, centrifuged (150*g*), and stored at -20°C until used as nondiluted conditioned media.

Assessment of cell growth, viability, and insulin content

The defined serum-free medium (SFM) was prepared according to Clark and Chick [94]: RPMI 1640 supplemented with 0.1% human serum albumin (fraction V, Sigma), 10 μg/ml human transferrin, 0.1 nM triiodothyronine (T_3), 1 nM PRL, 0.65 nM IGF-I, 50 μM ethanolamine, 50 μM phosphoethanolamine and 2 mM L-glutamine. In addition and at variance with the previously described medium [94], the medium also contained 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 mM HEPES, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

To determine cell growth rate in the medium, cells were seeded at densities of 100,000 or 500,000 cells/well in 24-well multiplates coated with poly-L-ornithine (Sigma), and cultured in SFM or CM. On the day of experiment, cell number was counted using a hemocytometer after detachment with PBS containing 0.025% (w/v) trypsin. Cellular viability was assessed by the MTT colorimetric assay [95]. After washing twice with PBS, cells were incubated with 0.5 mg/ml MTT for 30 min in a modified Krebs-Ringer bicarbonate-HEPES buffer (KRBH) composed of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 2 mM $NaHCO_3$, 0.1% BSA and 11.2 mM glucose. Thereafter, cells were solubilized with 'extraction solution' containing 20 % sodium dodecyl sulfate (SDS) and 50 % N,N-dimethylformamide. Optical density was measured at 550 nm with an automated spectrometer (Dynatech, Embrach, Embraport, Switzerland). Cellular insulin content was determined by radioimmunoassay (RIA) after acid-ethanol extraction using rat insulin as standard [84,85]. Cellular DNA content was measured fluorimetrically [96] after overnight treatment with 0.2 M NaOH and freeze-thawing, with the fluorescent reagent Hoechst 33258 and calf thymus DNA (Type I, Sigma) as standard.

Preparation of rat islet cells

Pancreatic islets were isolated from overnight-fasted male Wistar rats (200-240 g). Dissociated cells were obtained by trypsin-EDTA digestion as described previously [97], resuspended in SFM or CM, and seeded at a density of 100,000 cells/well in 48-well multiwell plates coated with poly-L-ornithine. Cells were cultured in each of the media for 3 days before the static incubation. The conditioned

medium from islet cell monolayers or free-floating islets cultured for 3 days in SFM was collected for testing in the culture of INS-1 cells, without dilution.

[^{3}H]Thymidine incorporation assay

DNA synthesis in INS-1 cells was assessed by incorporation of [*methyl*-^{3}H]thymidine into the cells. Cells (20,000 cells/well) were seeded and cultured in SFM for 3 days. The medium was then changed to one containing test substances, in which cells were cultured for a further 3 days. During the final 4 h of the 24-h or 24 h of the 72-h incubation period , 0.5 μCi/well of [*methyl*-^{3}H]thymidine was added to the medium. In experiments in which effects of various inhibitors (verapamil, lavendustin A, diazoxide and Rp-cAMPS) were examined on GH-induced mitogenesis, cells were incubated for 6 h with stimuli in the absence or presence of these agents, followed by extensive washing, and continuous culture for 14 h in the same medium without any agents before a 4-h pulse of [^{3}H]thymidine. The labeled cells were harvested onto paper filters with a multichannel harvester (PHD, Cambridge Technology, Cambridge, MA, USA). After the filters were dissolved in liquid scintillant (Hydroluma, Lumac•LSC, Groningen, The Netherlands), radioactivity on the filters was measured with a liquid scintillation counter.

Static incubation for determination of insulin secretion and MTT reduction

INS-1 cells (approximately 50,000 cells/well) were seeded in 96-well microtiter plates coated with poly-L-ornithine, except for experiments with Matrigel or cornea-derived ECM, to facilitate cell attachment to the substratum and cultured in CM or SFM in the absence or presence of test agents as indicated for 3 days. After washing twice with glucose-free KRBH, cells were preincubated in the same buffer for 30 min at 37°C. Thereafter, the medium was changed and cells were incubated for 30 min in KRBH containing various stimuli for determination of insulin secretion by RIA using rat insulin as standard. After the incubation, the MTT colorimetric assay [32,89] was performed by incubating the cells for a further 30 min in the presence of the stimuli as described above. Static incubation of primary islet cells was performed in 48-well multiplates in the same manner as for INS-1 cells, except that the MTT assay was not performed. Cellular content of insulin and DNA content was measured as described above.

Perifusion for insulin secretion

Cells were kept in spinner culture for 3 h in RPMI 1640 supplemented with 25 mM HEPES and 1% FCS. Cells were then distributed into small perifusion chambers and perifused in KRBH at a rate of 1 ml/min [84].

Measurement of glucose and pyruvate oxidation

Approximately 500,000 cells were seeded in 35-mm Petri dishes coated with poly-L-ornithine and cultured for 3 days in SFM or CM. After washing twice with glucose-free KRBH, the cells were preincubated in the same buffer for 30 min at 37°C. After removal of the medium, 1 ml of fresh KRBH containing ^{14}C-labeled glucose or pyruvate as specified was added. The dish was then transferred into a sealed glass chamber fitted with a holder, which was kept at 37°C for 2 h. The incubation was stopped by injection of 0.5 ml of 0.1 M HCl through a membrane in the screw-top of the chamber. Immediately thereafter, 0.5 ml of hyamine hydroxide was injected through another membrane onto a filter paper placed on the bottom of the chamber. The filter paper was retrieved after 1 h at room temperature and its radioactivity was measured as for [^{3}H]thymidine incorporation.

Measurement of cytosolic Ca^{2+}

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was assessed in fura-2/AM-loaded cells using a microfluorimeter system or with a video imaging system [98]. In the former, INS-1 cells were plated at 50,000 cells/glass coverslip (21 x 26 mm) precoated with poly-L-ornithine. After 3 days, cells were loaded with 1 μM fura-2/AM added to CM for 30 min preceding the experiment. The coverslips were then mounted in a recording chamber held at 37°C. Cells were superfused with KRBH containing 2.8 mM glucose. Stimuli were delivered as square pulses by two large orifice glass pipettes connected to microsyringe pumps [97]. The microfluorimeter system consisted of a Nikon Diaphot microscope and a rotating filter-wheel fluorimeter (constructed in the workshop of the Geneva University Medical Center). In experiments with the video imaging, approximately 300,000 cells were seeded on glass coverslips and cultured as described above. Cells were loaded with 1 μM fura-2/AM for 30-45 min in glucose- and glutamine-free RPMI 1640 containing 10 mM HEPES in the absence (for cells in SFM) or presence (for cells in CM) of 10% FCS, and perifused at 1.0 ml/min in KRBH

containing 2.8 mM glucose. Approximately 10 cells were examined in one field (40x objective). The cells were excited at 340 and 380 nm and emitted light recorded at 500 nm. $[Ca^{2+}]_i$ was calculated from the ratio 340/380 using equations and constants as previously defined [97,98].

Measurement of membrane potential

Membrane potential was evaluated either with the fluorescent probe bisoxonol [84,87,99] or with the patch-clamp technique. Measurements with bisoxonol were performed in cells attached on poly-L-ornithine-coated glass coverslips after culture of 2-5 days in CM, or in cell suspension (10^6 cells in 2 ml KRBH containing 2.8 mM glucose). Fluorescence signal was monitored in a glass cuvette in the presence of 100 nM bisoxonol at excitation and emission wavelengths of 535 nm and 570 nm, respectively. The patch-cramp experiments were performed in the perforated configuration [100] in a bathing solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2.5 mM glucose, and 10 mM HEPES (pH 7.4, at 25°C). Sylgard-coated patch pipettes had resistance between 2-4 MΩ after filling with the standard solution, which contained 120 mM K-glutamate, 30 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM EGTA, 30 mM mannitol, 10 mM HEPES (pH 7.2, 25°C) and 240 mg/ml amphotericin B (to permeabilize selectively the membrane to monovalent ions) or 145 mM K-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 2 mM ATPMg and 10 mM HEPES (pH 7.2, 25°C). Experiments were carried out at room temperature under continuous perfusion. Stimulation was achieved by switching to a bathing solution containing 22 mM glucose final concentration, while tolbutamide (0.2 mM) was applied by local pressure from a wide-tipped micropipette (5-10 μm). The membrane potential was recorded at a sampling rate of 1 kHz by a computer based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany) controlled by the Pulse software (HEKA), and later analyzed by the Igor software (Wavemetrics, Lake Oswego, OR, USA). All voltages were corrected for a liquid junction potential of 8 mV between external and internal solutions. Registration was started when the access resistance was stable and below 50 MΩ. Before recording, capacitative currents were canceled in the voltage-clamp mode, using the automatic capacitance compensation of the EPC-9.

Determination of cAMP levels

Cell suspensions were prepared in KRBH (10^6 cells/ml), and incubated for 5 min with stimuli. cAMP levels were measured by RIA as described previously [101].

Immunoprecipitation and Western blotting

Cells (5-10 x 10^6 cells/dish) were cultured in CM for 5-7 days and incubated overnight in a serum-free RPMI 1640 medium containing 1% BSA, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. After washing twice with PBS, cells were preincubated in glucose-free KRBH for 60 min and stimulated with agonists in the same buffer. Incubation was stopped by washing twice with ice-cold PBS and by adding a "lysis buffer" containing 20 mM Tris-HCl, pH 7.4, 30 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1% Triton-X100, 1% deoxycholate, 1 mM PMSF and 2 mM sodium orthovanadate. Cell lysates were sonicated briefly and centrifuged at 12,000 x *g* for 60 min. For immunoprecipitation, supernatants were incubated with anti-JAK2 serum (UBI) or with monoclonal antibodies against STAT proteins (Transduction Laboratories) by rotating end over end overnight at 4°C. The immune complex was then precipitated by incubation for 60 min at 4°C with protein A-Sepharose beads (Pharmacia Biotech Europe, Brussels, Belgium), and eluted by boiling in SDS-sample buffer for 5 min. All the samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking with Tris-buffered saline containing 1% BSA for 120 min, the nitrocellulose membrane was incubated at 4°C overnight in the same buffer with the monoclonal anti-phosphotyrosine antibody 4G10. Blots were washed, exposed to horseradish peroxidase-conjugated goat anti-mouse IgG for 90 min, and detected by the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham).

Northern Blotting

After incubation with test agents, total RNA was extracted from INS-1 cells by the acid guanidium thiocyanate-phenol-chloroform method [102]. *jun*B, *nur*77, and *zif*268 mRNAs were detected by Northern blot hybridization with antisense ^{32}P-labeled riboprobes. The linearized plasmids used were pGEM-2-*jun*B containing a 1180-base

pair *Bam*Hi-*Eco*RI fragment (positions 423-1576), pGEM-2-*nur*77 containing a *nur*77 cDNA probe kindly provided by Dr L. Lau (University of Illinois, Chicago, USA), and pBS-KS-*zif*268 containing a 690-base pair *Nde*I-*Bgl*II fragment (positions 1273-1963). For the determination of iNOS mRNA expression, a specific probe of the mouse macrophage iNOS (Cayman Chem., USA) was used. The DNA probe of the ribosomal RNA 18 S, kindly provided by Dr I. Oberbäumer (Max-Planck-Institut, Martinsried, Germany), was used as invariant control.

Immunopurification of 44-kDa MAP Kinase and MEK-1 from INS-1 Cell Extracts

The following procedure was used to prepare the cells for the measurements of MAP kinase activity, with all incubations being at 37°C in 92.5% atmospheric air, 7.5% CO_2. Confluent cell monolayers (4.5 cm^2 and 10 cm^2/condition for MAP kinase and MEK assay, respectively) were preincubated for 1 h in glucose-free KRBH. After incubation with test agents, experiments were stopped by quickly aspirating the medium (which was saved for measurement of secreted insulin) and solubilizing cells for 15 min on ice with solubilization buffer containing 1% (v/v) Triton X-100, 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 100 U/ml aprotinin, 20 μM leupeptin, and 0.2 mg/ml PMSF. Cell extracts were clarified by centrifugation for 15 min at 18,000 x *g* and then incubated for 2 h with antibody to 44 kDa MAP kinase or MEK-1 preadsorbed to protein A-Sepharose beads. Following the immunoprecipitation period, the beads were washed three times with solubilization buffer. All manipulations were performed at 4°C.

MAP kinase assay

Protein A-Sepharose beads with immunoprecipitated 44 kDa MAP kinase were washed two times with HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100) with 0.2 mM sodium orthovanadate, dehydrated with a syringe, and resuspended in 50 μl of HNTG buffer supplemented with 0.2 mM sodium orthovanadate, 100 U/ml aprotinin, 20 μM leupeptin, and 0.2 mg/ml PMSF. The kinase reaction was started by the addition of the following, given at final concentrations: 150 μg/ml myelin basic protein (as a MAP kinase substrate), 10 mM magnesium acetate, 1 mM dithiothreitol, and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol).

The phosphorylation reaction was allowed to proceed for 15 min at room temperature (linear assay condition) and was stopped by spotting Whatman P-81 filter papers with an aliquot of the reaction mixture and dropping them into 0.1% (v/v) orthophosphoric acid. The papers were washed overnight in this solution with several changes, washed once in ethanol, and dried. Radioactivity associated with the papers was determined by Cerenkov-counting. The reaction blank (a sample in which cell lysate had been omitted during immunoprecipitation but otherwise treated like the rest of the samples) was subtracted from all values.

MEK Assay

The kinase activity of MEK-1 was measured in a reconstitution assay by the ability of immunopurified MEK-1 to activate bacterially expressed, rat 44-kDa MAP kinase, the activity of which was measured using myelin basic protein as a substrate. Protein A-Sepharose beads with immunoprecipitated MEK-1 were washed twice with 50 mM HEPES, pH 7.4, dehydrated with a syringe, and resuspended in 50 µl of HEPES buffer, pH 7.4, containing recombinant 44-kDa MAP kinase, 0.2 mM sodium orthovanadate, 100 U/ml aprotinin, 20 µM leupeptin, and 0.2 mg/ml PMSF. The *in vitro* phosphorylation cascade was started by the addition of (final concentrations) 50 µM [γ-^{32}P]ATP (33 Ci/mmol), 150 µg/ml myelin basic protein, 15 mM $MgCl_2$, and 1 mM EGTA. The phosphorylation reaction was allowed to proceed for 10 min at room temperature and was stopped by spotting Whatman P-81 filter papers with an aliquot of the reaction mixture and dropping them into 0.1% (v/v) orthophosphoric acid. The papers were washed overnight in this solution with several changes, washed once in ethanol, and dried. Radioactivity associated with the papers was determined by Cerenkov counting. The reaction blank (a sample in which cell lysate had been omitted during immunoprecipitation but otherwise treated like the rest of the samples) was subtracted from all values. Control experiments showed that omission of myelin basic protein or recombinant MAP kinase reduced radioactivity in the filter papers by 90%.

Electrophoretic mobility shift assay (EMSA)

INS-1 cells were cultured and stimulated as described for *Immunoprecipitation and Western blotting*. Nuclear extract was prepared from the cells as previously

described [103]. Three different synthetic double-strand oligonucleotides end-labeled with [γ-^{32}P]ATP by T_4 polynucleotide kinase were used as probes. These represent GAS in the promoter of the IRF-1 gene, 5'-GATCCGATTTCCCCGAAAT-3' [104]; GAS in the iNOS gene promoter, 5'-CTTTTCCCCTAACAC-3' [105], and the consensus NF-κB binding sequence (κB site), 5'-GATCCCAACGGCAGGGGAATTCCCCTCTCC-3' [106]. EMSA was performed according to a method described elsewhere [107]. The probe (10^4 cpm) was incubated with 10 μg of the nuclear protein along with 1 μg of poly(dI-dC) in each reaction for 30 min at room temperature. DNA-protein complexes were resolved on 4% non-denaturing acrylamide gels, dried, and visualized by autoradiography.

Statistical analysis

Results are presented as mean ± SEM (at least three experiments were conducted) for the number of preparations as indicated. Statistical significance was determined by paired or unpaired Student's t test where appropriate, and by Mann-Whitney *U* test. In case of multiple comparisons, data were evaluated by one-way analysis of variance (ANOVA) with Sheffe's multiple range test. Differences between experimental and control groups were considered "significant" at $p < 0.05$.

RESULTS

I. Factors Affecting Growth and the Function of INS-1 Cells

I-1. Factors affecting growth of INS-1 cells

I-1-a. Growth of INS-1 cells cultured in CM and SFM

Growth and viability criteria of INS-1 cells in CM and SFM were first examined and the results are shown in Fig. 3. Cell numbers in both culture conditions increased in a similar manner up to 10 days, although there was a tendency at the earlier time points for a slower growth of the cells in SFM. After 7 days, neither cell number nor viability was significantly different between the two culture conditions. There was a good parallelism between the increases in cell number and the degree of MTT reduction (Fig. 3). By using this SFM, INS-1 cells were able to be propagated for at least two months.

It should be noted that INS-1 cells grown in SFM showed morphological changes similar to those reported in other cell lines [94,108,109]. In the absence of any attachment factor, the cells remained round, tended to grow in clusters, and eventually detached from the culture dish to become free-floating "islet-like" aggregates (Fig. 4A). However, when plated on poly-L-ornithine, they were firmly attached to the bottom and grew as monolayers (Fig. 4B), although still being less flattened out than the cells cultured in CM (Fig. 4C). In the following experiments, the cells were always grown on poly-L-ornithine-coated substratum unless otherwise stated.

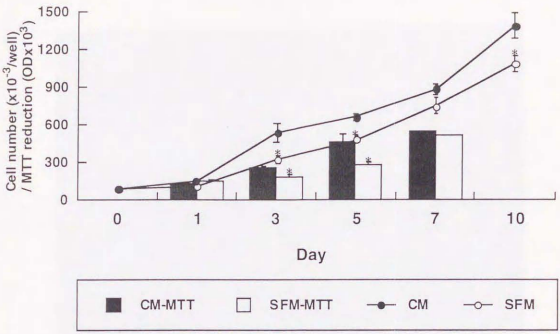
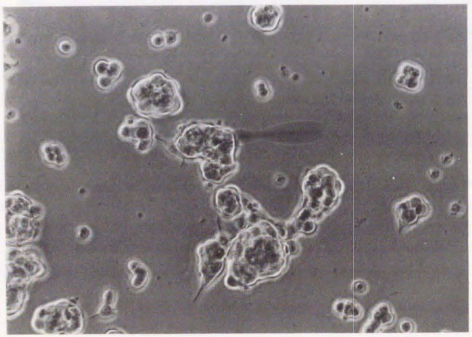
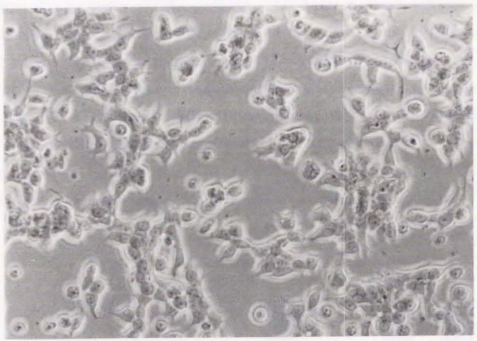


Fig. 3. Growth and viability of INS-1 cells cultured in either SFM or CM. Cells were cultured in either CM or SFM for up to 10 days. Cell number was counted in a hemocytometer, and viability was evaluated by the MTT colorimetric assay. Values are mean ± SE of six observations from two independent experiments. Statistical analysis by unpaired Student's t test, *$p<0.05$ vs. CM.

A



B



C

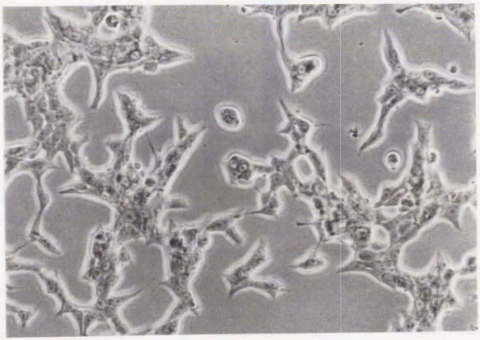


Fig. 4. Morphological changes of INS-1 cells cultured in either SFM. After 7 days of culture without any attachment factor in SFM, INS-1 cells tend to form clusters and become free-floating "islet-like" aggregates (A, *previous page*). INS-1 cells plated on poly-L-ornithine grow in monolayer in SFM (B), but remain less flattened compared with the cells cultured in CM (C).

I-1-b. DNA synthesis in INS-1 cells cultured in CM and SFM

Replication of INS-1 cells was assessed by the measurement of [^{3}H]thymidine incorporation as a parameter of DNA synthesis. As shown in Fig. 5, [^{3}H]thymidine incorporation in SFM was comparable to that in CM after 3 days. To evaluate mitogenic activities of each component in SFM, DNA synthesis was examined with omission of various combinations of the components. [^{3}H]thymidine incorporation in media lacking PRL was reduced by 50% whereas omission of IGF-I did not affect DNA synthesis (Fig. 5), although both of these factors have been reported to stimulate β-cell replication [18,110,111]. Omission of T_3 from the medium lacking both PRL and IGF-I resulted in a further reduction in [^{3}H]thymidine incorporation (Fig. 5).

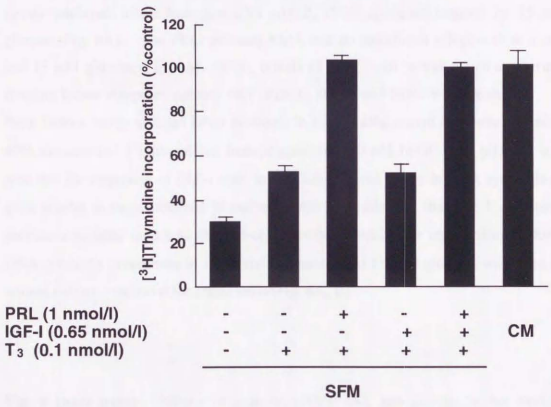
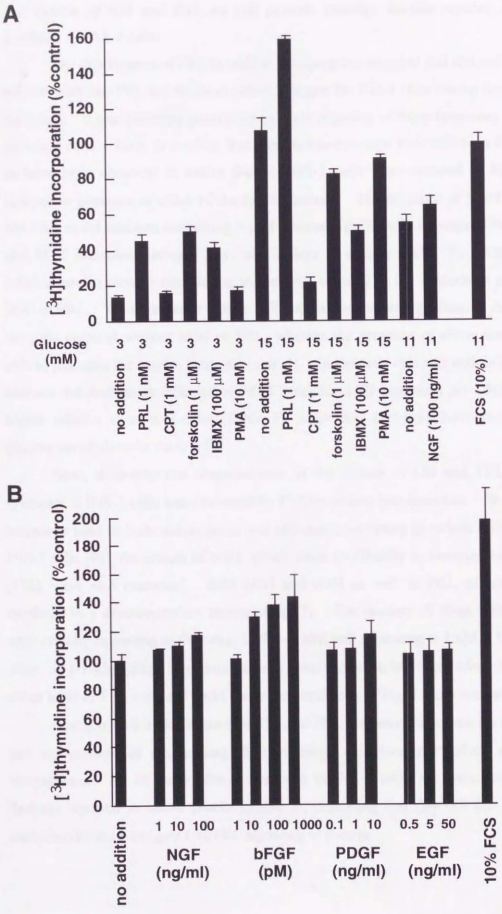


Fig. 5. DNA synthesis in INS-1 cells cultured in either SFM or CM. Cells (20,000/well) were cultured for 3 days in SFM, and incubated for a further 3 days in CM, SFM, or SFM lacking the indicated components (PRL, prolactin; T_3, triiodothyronine). Incorporation of [^{3}H]thymidine (0.5 μCi/well) was determined during the final 24 h of the incubation period. Values are mean ± SE from three independent experiments performed in triplicates.

I-1-c. Effects of various factors on DNA synthesis in INS-1 cells

Mitogenic activities of various factors were evaluated by the [^{3}H]thymidine incorporation into INS-1 cells. Their effects were examined in SFM without PRL, since this potent mitogen for INS-1 cells may mask the effect of each factor. [^{3}H]thymidine incorporation was approximately 6-times higher in the presence of 15 mM glucose than that in the presence of 3 mM glucose (Fig. 6A). A role of cAMP was examined using a membrane-permeant cAMP analogue CPT-cAMP, and two agents that raise intracellular cAMP levels, forskolin and IBMX. CPT-cAMP had no effect on DNA synthesis in low (3 mM) glucose medium, but inhibited high (15 mM) glucose-induced DNA synthesis (Fig. 6A). Forskolin and IBMX were found to increase [^{3}H]thymidine incorporation in the presence of 3 mM glucose, whereas these agents inhibited, albeit less than CPT-cAMP, DNA synthesis induced by 15 mM glucose (Fig. 6A). The PKC activator PMA had no significant effect both at 3 mM and 15 mM glucose (Fig. 6A). Next, effects of the growth factors which act through tyrosine kinase receptors, namely EGF, PDGF, bFGF, and NGF, were examined. All these factors hardly affected DNA synthesis in INS-1 cells, except that there was about 40% increase in [^{3}H]thymidine incorporation by 100 pM bFGF (Fig. 6B). It is of note that the responses of INS-1 cells to the factors noted above in DNA synthesis are quite similar to those obtained in native β-cells [7], indicating that INS-1 cells could provide a suitable model to study β-cell growth. Finally, for comparison is shown DNA synthesis in response to 10% fetal calf serum and 11 mM glucose, which are the normal culture conditions for INS-1 cells (Fig. 6A, B).

Fig. 6 (next page). Effects of glucose, cAMP (A), and growth factors working through receptor tyrosine kinases (B) on DNA synthesis in INS-1 cells. Cells were cultured in CM for 3 days, and incubated for a further 3 days in SFM without PRL (except *PRL (1 nM)* at 3 or 15 mM glucose in A) containing 3, 11, or 15 mM glucose as indicated in A, or 11 mM glucose in B. Incorporation of [^{3}H]thymidine (0.5 μCi/well) was determined during the final 24 h of the incubation period. Values are mean ± SE from three independent experiments performed in triplicates.


A
[3H]thymidine incorporation (%control)
160
140
120
100
80
60
40
20
0
Glucose (mM)
3 3 3 3 3 3 15 15 15 15 15 15 11 11 11
no addition
PRL (1 nM)
CPT (1 mM)
forskolin (1 μM)
IBMX (100 μM)
PMA (10 nM)
no addition
PRL (1 nM)
CPT (1 mM)
forskolin (1 μM)
IBMX (100 μM)
PMA (10 nM)
no addition
NGF (100 ng/ml)
FCS (10%)
B
[3H]thymidine incorporation (%control)
200
180
160
140
120
100
80
60
40
20
0
no addition
1 10 100
NGF (ng/ml)
1 10 100 1000
bFGF (pM)
0.1 1 10
PDGF (ng/ml)
0.5 5 50
EGF (ng/ml)
10% FCS

I-2. Effects of GH and PRL on cell growth, viability, insulin content, and DNA synthesis of INS-1 cells

The experiments of [^{3}H]thymidine incorporation revealed that GH and its related peptide hormone PRL are the most potent mitogen for INS-1 cells among the factors so far tested. It was therefore interesting to study signaling of these hormones in insulin-secreting cells. First, to confirm that these hormones elicit their effects in INS-1 cells as have been observed in native β-cells, INS-1 cells were cultured in SFM in the absence or presence of either of the two hormones. The presence of 5 nM hGH or 5 nM PRL in the medium containing 3 mM glucose significantly increased DNA content and MTT reduction during 3 days and 7 days of culture (Table 3). After 3 days, cellular insulin content was similar in the cells cultured in the absence or presence of hGH or PRL. However, after 7 days, cellular insulin content significantly decreased in the cells cultured without hGH or PRL, whereas the presence of either hormone was able to maintain the insulin content (Table 3). In the cells cultured with hGH or PRL, nutrient metabolism as reflected by MTT reduction [32] expressed per DNA was also higher relative to control cells (Table 3), suggesting that both hormones stimulate glucose metabolism in these cells.

Next, dose-response characteristics of the effects of GH and PRL on DNA synthesis in INS-1 cells was evaluated by [^{3}H]thymidine incorporation. Since hGH is known to bind to both somatogenic and lactogenic receptors in rodent cells including INS-1 cells [90], the effects of bGH, which binds specifically to somatogenic receptors [112], were also examined. Both hGH and bGH as well as PRL stimulated DNA synthesis in a dose-dependent manner (Fig. 7). The potency of these hormones was very similar, increasing in the range 1 pM - 1 nM, and saturating at 1 nM. No additive effect on [^{3}H]thymidine incorporation was observed when bGH was administered with either hGH or PRL each at 10 nM under the conditions of Fig. 1 (data not shown).

These results indicate that both GH and PRL are potent mitogens for INS-1 cells and are capable of maintaining their viability, oxidative metabolism and insulin biosynthesis. All of these effects observed in INS-1 cells are consistent with the findings reported in native β-cells (Table 2), indicating that this cell line provides a useful model to investigate GH/PRL signaling in β-cells.

Table 3. Effects of hGH and PRL on replication, insulin content, viability of INS-1 cells cultured in SFM.

	Control	+hGH	+PRL
3 days			
DNA content (μg/well)	0.78 ± 0.03	1.15 ± 0.06**	1.32 ± 0.10*
Insulin content (ng/well)	81.4 ± 16.9	118.8 ± 6.8	156.9 ± 18.6*
Insulin content (ng/μgDNA)	102.7 ± 20.2	103.9 ± 5.6	114.5 ± 6.7
MTT reduction (10^3xOD$_{550}$/well)	55 ± 4	151 ± 12**	200 ± 18**
MTT reduction (10^3xOD$_{550}$/μgDNA)	71 ± 5	130 ± 8**	154 ± 4**
7 days			
DNA content (μg/well)	1.38 ± 0.14	2.12 ± 0.14**	2.11 ± 0.21*
Insulin content (ng/well)	57.3 ± 11.6	254.2 ± 9.6**	177.2 ± 10.2**
Insulin content (ng/μgDNA)	45.7 ± 11.9	120.1 ± 7.9**	89.8 ± 9.3*
MTT reduction (10^3xOD$_{550}$/well)	75 ± 7	231 ± 20**	286 ± 37**
MTT reduction (10^3xOD$_{550}$/μgDNA)	57 ± 9	112 ± 13*	131 ± 12**

INS-1 cells were cultured in SFM for 3 or 7 days in the absence or presence of 5 nM hGH or 5 nM PRL. Data are mean ± SE of three independent experiments. Statistical significance was evaluated by one-way ANOVA (*P < 0.05, **P < 0.01 vs. control).

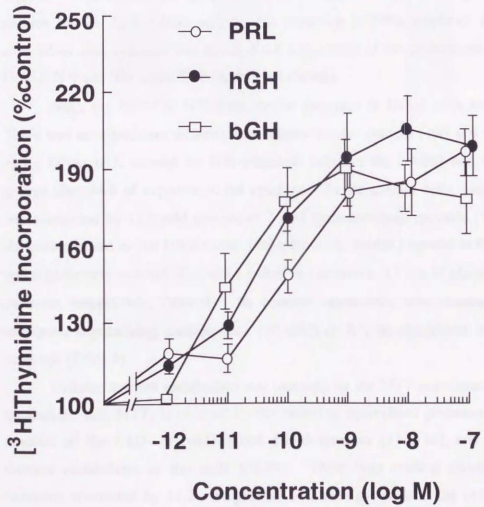


Fig. 7. Effects of hGH, bGH and PRL on DNA synthesis in INS-1 cells. Cells were incubated for 72 h in SFM containing various concentrations of the hormones. Values are expressed as % of control, taking the condition without hormone addition as control. Each bar represents mean ± SE of three independent experiments (each comprising 3-4 wells).

I-3. Effects of IFN-γ on growth and insulin secretion of INS-1 cells

Effects of IFN-γ on growth and viability of INS-1 cells were first evaluated by the measurement of DNA content as well as that of DNA synthesis with [^{3}H]thymidine. In this case, the culture media contained 10% FCS because the effect of the cytokine was known to be rather inhibitory for insulin-secreting cells. After a 24-h incubation with 100 U/ml IFN-γ, no reduction in DNA synthesis was observed (0.92 ± 0.17 vs. 1.03 ± 0.20 μg/well, n=6, control vs. IFN-γ-treated). Similarly, 100 U/ml IFN-γ did not affect DNA synthesis of INS-1 cells during the 72-h incubation (Fig. 8). Since it has been known that IFN-γ elicits cytotoxicity in combination with TNF-α in β-cells, effects of TNF-α were also examined. The combination of 100 U/ml IFN-γ and 50 ng/ml TNF-α markedly inhibited DNA synthesis, whereas TNF-α alone had no effect (Fig. 8). Thus most of the cells exposed to the combination of these cytokines did not survive for the 72-h culture period. No reduction in DNA synthesis was observed even when each cytokine was tested at ten times more of the concentration (i.e. 1,000 U/ml IFN-γ and 500 ng/ml TNF-α, data not shown).

Next, the effect of IFN-γ on insulin secretion in INS-1 cells was examined. There was no significant difference in cellular insulin content (190 ± 6 vs. 212 ± 27 ng/μg DNA, n=3, control vs. IFN-γ-treated) between the control and IFN-γ-treated groups after 24-h of exposure to the cytokine. In the control cells, insulin secretion was stimulated by 11.2 mM glucose or 2 mM pyruvate about two-fold (Table 4). In the cells exposed to 100 U/ml mouse IFN-γ for 24 h, insulin response to these nutrients was significantly reduced (1.5- and 1.6-fold in response to 11.2 mM glucose and 2 mM pyruvate, respectively, Table 4). By contrast, when cells were stimulated with the membrane-depolarizing concentration (30 mM) of K^+, no significant inhibition was observed (Table 4).

Cellular nutrient metabolism was assessed by the MTT colorimetric assay. A tetrazolium salt, MTT, is reduced by the reducing equivalents generated through the reaction of the FAD- or NAD-linked dehydrogenases [113,114], and thus reflects nutrient metabolism in the cells [32,89]. There was marked decrease in MTT reduction stimulated by 11.2 mM glucose or 2 mM pyruvate in the cells exposed to IFN-γ for 24 h compared with the control, whereas the MTT reduction at the basal condition (2.8 mM glucose) was not affected by the exposure to IFN-γ (Table 4).

After a 30-min exposure to the cytokine, there was no difference in glucose-stimulated MTT reduction between the two groups (Table 4).

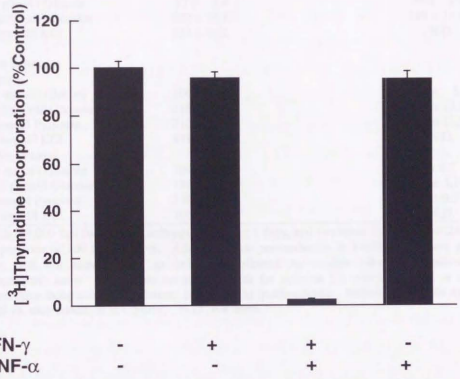


Fig. 8. Effects of IFN-γ and TNF-α on DNA synthesis in INS-1 cells. Cells were cultured in CM containing 1% FCS in the absence or presence of 100 U/ml IFN-γ and/or 50 ng/ml TNF-α for 3 days. During the final 24 h, 0.5 μCi/well of [*methyl*-^{3}H]thymidine was added and radioactivity incorporated into the cells was measured with a liquid scintillation counter. Values are mean ± SE with the mean value without cytokine as 100% from three independent experiments performed in quadruplicate.

Table 4. Effect of IFN-_ on insulin secretion and MTT reduction in INS-1 cells.

	Insulin secretion (%control)	MTT reduction (%control)
30-min exposure		
Control		
2.8 mmol/l Glucose	100 ± 2.4	100 ± 7.8
11.2 mmol/l Glucose	196 ± 7.9	202 ± 10.1
30 mmol/l KCl	311 ± 22.4	N.D.
IFN-_-treated		
2.8 mmol/l Glucose	97 ± 3.4	96 ± 8.8
11.2 mmol/l Glucose	202 ± 32.9	189 ± 12.1
30 mmol/l KCl	383 ± 30.2	N.D.
24-h exposure		
Control		
2.8 mmol/l Glucose	100 ± 5.6	100 ± 5.1
11.2 mmol/l Glucose	218 ± 17.9	228 ± 12.5
2 mmol/l Pyruvate	210 ± 12.9	255 ± 11.2
30 mmol/l KCl	441 ± 20.0	N.D.
IFN-_-treated		
2.8 mmol/l Glucose	100 ± 8.1	97 ± 0.7
11.2 mmol/l Glucose	145 ± 8.3*	110 ± 5.1*
2 mmol/l Pyruvate	159 ± 7.9*	128 ± 9.5*
30 mmol/l KCl	405 ± 30.4	N.D.

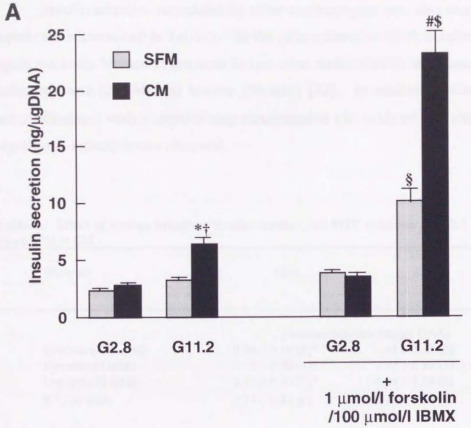
Cells (50,000 cells/well) were cultured in CM for 3 days, and incubated for 30 min or 24 h in the absence or presence of 100 U/ml IFN-γ. After a 30-min preincubation in KRBH, cells were stimulated for 30 min with the secretagogues as indicated, followed by another 30-min incubation for the MTT colorimetric assay. Values are mean ± SE with the value at 2.8 mmol/l glucose in *Control* as 100% from three independent experiments performed in quadruplicate. Statistical analysis by ANOVA: *$P <$ 0.05 vs. each condition in *Control*. N.D., not done.

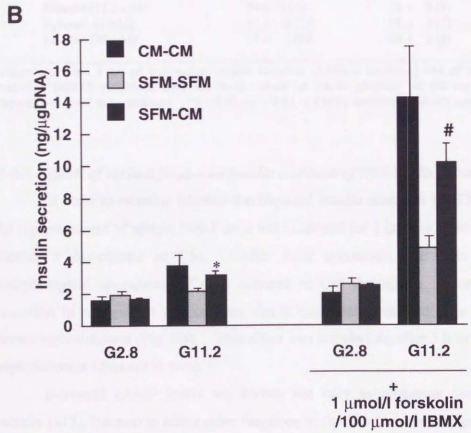
I-4. Factors maintaining insulin secretory function of INS-1 cells

I-4-a. Insulin secretory function of INS-1 cells cultured in SFM

Using the serum-free culture system of INS-1 cells, factors maintaining insulin secretory function of insulin-secreting cells were investigated. The function was examined after 3 days of culture in SFM and was compared with that in the control cells cultured in CM. After 3 days of culture, there was no significant difference either in total DNA content (1.63 ± 0.11, 1.79 ± 0.12 μg/well, n=21; SFM, CM, respectively) or in cellular insulin content (120 ± 22, 134 ± 26 ng/μgDNA, n=16; SFM, CM, respectively) on the day of experiment between the two culture conditions. Although insulin secretion at a substimulatory concentration (2.8 mM) of glucose was similar between both groups, stimulation by 11.2 mM glucose was markedly blunted in the cells cultured in SFM as compared with those in CM (Fig. 9A) The incremental secretion at 11.2 over 2.8 mM glucose in the former was 29% of that in the latter. Addition of a combination of an adenylate cyclase stimulator, forskolin (1 μM), and a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 100 μM), augmented insulin secretion at 11.2 mM glucose in both culture conditions. However, the response was again significantly smaller (by 78%) in the cells cultured in SFM (Fig. 9A). Similar results were obtained when PMA, an activator of PKC, was present during the final incubation period. At 2.8 mM glucose, PMA (100 nM) increased insulin secretion by 1.48 ± 0.08 and 0.98 ± 0.61 ng/μgDNA in CM- and SFM-cultured cells (n=4), respectively. Corresponding values at 11.2 mM glucose were 5.87 ± 0.51 and 4.18 ± 0.34 ng/μgDNA (n=4).

Fig. 9 (next page). Effect of SFM and of serum replenishment on glucose-induced insulin secretion. Insulin secretion was measured after culture of INS-1 cells, either for 3 days in CM or SFM (A), or after 6 days of culture with a medium changed at 3 days, as indicated (B). Cells were stimulated by 2.8 (G2.8) or 11.2 (G11.2) mM glucose in the absence or presence of 1 μM forskolin plus 100 μM IBMX. Values are mean ± SE from seventeen independent experiments (A) and three (B) performed in quadruplicate. Statistical analysis by ANOVA: *$p<0.05$ vs. G2.8 in CM; †$p<0.05$ vs. G11.2 in SFM; §$p<0.05$ vs. G2.8 plus forskolin/IBMX in SFM, #$p<0.05$ vs. G2.8 plus forskolin/IBMX in CM, $$p<0.05$ vs. G11.2 plus forskolin/IBMX in SFM.


A
Insulin secretion (ng/μgDNA)
SFM
CM
*†
§
#$
G2.8
G11.2
G2.8
G11.2
+
1 μmol/l forskolin
/100 μmol/l IBMX


B
Insulin secretion (ng/μgDNA)
CM-CM
SFM-SFM
SFM-CM
*
#
G2.8
G11.2
G2.8
G11.2
+
1 μmol/l forskolin
/100 μmol/l IBMX

Insulin secretion stimulated by other secretagogues was also examined and the results are summarized in Table 5. In the cells cultured in SFM, insulin secretion was again markedly blunted in response to two other metabolizable secretagogues of INS-1 cells, pyruvate (2 mM) and leucine (20 mM) [32]. In marked contrast, when cells were stimulated with a depolarizing concentration (30 mM) of K^+, only slight, non-significant, reduction was observed.

Table 5. Effect of various stimuli on insulin secretion and MTT reduction in INS-1 cells cultured in either SFM or CM.

Stimulus	SFM	CM	%decrease
	ΔInsulin secretion (ng/μg DNA)		
Glucose (11.2 mM)	0.88 ± 0.16 (5)*	3.48 ± 0.43 (5)	-78%
Pyruvate (2 mM)	1.17 ± 0.38 (5)*	3.72 ± 0.83 (5)	-68%
Leucine (20 mM)	2.13 ± 0.33 (7)*	4.85 ± 1.28 (6)	-56%
K^+ (30 mM)	2.77 ± 0.49 (6)	3.72 ± 0.59 (6)	-26%
	ΔMTT reduction (10^3xOD/μg DNA)		
Glucose (11.2 mM)	74 ± 14 (8)	74 ± 9 (8)	0%
Pyruvate (2 mM)	51 ± 8 (7)*	78 ± 8 (7)	-34%
Leucine (20 mM)	37 ± 4 (8)†	69 ± 5 (8)	-47%

Values are mean ± SE of incremental insulin secretion (ΔInsulin secretion) and of incremental MTT reduction (ΔMTT reduction) from the basal values (at 2.8 m glucose) for the number of separate experiments given in parentheses. *$p < 0.05$, †$p < 0.01$ vs CM by unpaired Student's unpaired *t* test

I-4-b. Effects of various factors on insulin secretion of INS-1 cells cultured in SFM

In order to examine whether the impaired insulin secretion in SFM is reversible by replenishment of serum, INS-1 cells were cultured for 3 days in SFM followed by a further 3 day-culture in CM. Under these conditions, the cells resumed the morphological appearance of cells cultured in CM throughout. Moreover, insulin secretion in response to glucose was almost completely restored after 3 days of the serum replenishment (Fig. 9B). This effect was not obvious after 3 h or 24 h of serum replenishment (data not shown).

Increased cAMP levels are known not only to potentiate insulin secretion acutely [115], but also to affect other functions of the β-cells including gene expression [116-118]. Therefore, the cells were exposed to various agents that raise cAMP levels

during the 3-day culture in SFM. However, neither glucagon (100 nM) nor GLP-1 (1 μM) restored insulin secretory function in response to glucose (Table 6), although INS-cells have been shown to respond acutely at least to GLP-1. Similarly, forskolin combined with IBMX did not restore glucose-induced insulin secretion (Table 6), while this treatment increased both basal and stimulated secretory rates resulting in the same incremental secretion as after culture in SFM alone. Furthermore, CPT-cAMP was without effect (data not shown). Another hormone produced by islet cells, somatostatin, did not affect the insulin secretory function (Table 6).

In addition, other growth and differentiation factors were supplemented to examine whether could restore glucose-stimulated insulin secretion of INS-1 cells cultured in SFM. NGF (100 ng/ml) and the PKC activator PMA (100 nM) did not alter glucose-induced insulin secretion following a 3-day exposure (Table 6). bFGF (1 pM), which, like NGF, causes differentiation of PC12 cells [119], was also without effect under these conditions, while increasing [^{3}H]thymidine incorporation by 25% (data not shown). Replacement of prolactin by 5 nM GH in SFM failed to affect the insulin secretory function (Table 6).

In the original report on the defined medium by Clark and Chick, ECM extracted from Engelbreth-Holm-Swarm tumors was used to establish islet cell monolayers [94]. This approach maintained glucose-induced insulin secretion. Therefore, the effect of Matrigel, a similar matrix, was also tested. The cells propagated on Matrigel in SFM, however, showed again impairment of the insulin secretory response to glucose both in the absence and presence of cAMP-raising agents compared to the control cells (Table 6). There was no significant difference in either insulin content or DNA content between the cells cultured on poly-L-ornithine and those on Matrigel (data not shown). The effect was also tested for ECM derived from bovine corneal endothelium. Although treatment of culture plates with this substance resulted in better insulin secretory response to glucose in CM, the effect of glucose was not restored in SFM (Table 6). Phase contrast microscopy showed that there was a tendency for the cells cultured on ECM to be more flattened out in both culture media than on poly-L-ornithine and Matrigel.

Table 6. Effects of various factors added to SFM on glucose-induced insulin secretion in INS-1 cells.

	ΔInsulin Secretion (ng/μg DNA)			
	SFM		CM	
Supplement	Glucose alone	Glucose + For/IBMX*	Glucose alone	Glucose + For/IBMX*
Control	1.07 ± 0.18 (17)	6.29 ± 0.88 (17)	3.68 ± 0.37 (17)†	19.55 ± 3.07 (10)†
For/IBMX*§	0.71 ± 0.85 (4)	7.29 ± 0.45 (4)		
Glucagon	0.92 ± 0.28 (4)	6.44 ± 1.12 (4)		
GLP-1	0.78 ± 0.18 (4)	7.98 ± 0.72 (4)		
Somatostatin	0.80 ± 0.38 (3)	7.68 ± 2.32 (3)		
NGF	0.88 ± 0.28 (9)	5.43 ± 1.07 (9)		
PMA	2.37 ± 1.13 (4)	5.27 ± 1.17 (8)		
bFGF	0.52 ± 0.35 (9)	4.78 ± 0.88 (9)		
GH‡	1.66 ± 0.46 (8)	7.24 ± 1.71 (8)		
Matrigel	0.81 ± 0.12 (3)	5.21 ± 1.14 (3)	2.32 ± 1.00 (3)	21.33 ± 0.32 (3)
Corneal ECM	1.68 ± 0.63 (3)	5.70 ± 0.73 (3)	6.95 ± 0.88 (3)	21.77 ± 1.86 (3)

Values are mean ± SE of incremental insulin secretion from 2.8 to 11.2 mM glucose for the number of separate experiments given in parentheses. The concentrations used are 10 μM for glucagon, 1 μM for GLP-1, 100 ng/ml for NGF, 100 nM for PMA, 100 pM for bFGF, 5 nM for GH. For details, see *Materials and Methods*. †$p < 0.001$ vs SFM by unpaired Student's t test. *For, 1 μmol/l forskolin; IBMX, 100 μmol/l IBMX. ‡Prolactin in SFM was replaced by GH §Cellular insulin contents were not altered by the treatment of factors tested except forskolin/IBMX that increased insulin contents by 78%.

Finally, to exclude the possibility that certain components in SFM inhibit insulin secretion in INS-1 cells, we performed experiments after culture in SFM supplemented with 10% FCS. No significant difference was observed in insulin secretory function compared to that in CM (data not shown), indicating that none of the components in SFM is inhibitory to glucose-induced insulin secretion in INS-1 cells. Moreover, the low concentration of albumin (0.1%) in SFM cannot explain the blunted insulin secretion, since supplementation of 1% human serum albumin to SFM failed to improve insulin secretory function (data not shown).

I-4-c. Mechanisms involved in the impaired insulin secretion of INS-1 cells cultured in SFM

In the following series of experiments, mechanisms involved in the impaired insulin secretion of INS-1 cells cultured in SFM were investigated, with respect to cellular nutrient metabolism, changes in $[Ca^{2+}]_i$, and the membrane potential.

Nutrient metabolism in INS-1 cells cultured in SFM. Cellular nutrient metabolism was assessed by two methods, the MTT assay and the mesurement of oxidation of ^{14}C-labeled nutrients. MTT reduction stimulated by 11.2 mM glucose was not significantly altered in the cells cultured in SFM compared to those cultured in CM (Table 5). In contrast, MTT reduction in response to pyruvate and leucine was significantly inhibited in SFM (Table 5). Since these nutrients are mainly metabolized in the mitochondria, it is probable that the inhibition of MTT reduction was due to impaired metabolism in this organelle. Therefore, oxidative metabolism was examined by measuring $^{14}CO_2$ production from [3,4-^{14}C]glucose, and [1-^{14}C]pyruvate, the metabolism of which reflects mitochondrial reactions. Oxidation of these substrates was significantly decreased in the cells cultured in SFM (Table 7).

Table 7. $^{14}CO_2$ production from [3,4-^{14}C]glucose, and [1-^{14}C]pyruvate in INS-1 cells cultured in either SFM or CM.

Labeled nutrients	SFM	CM
[3,4-^{14}C]glucose* (pmol/h/μgDNA)	372 ± 27 (5)†	478 ± 20 (5)
[1-^{14}C]pyruvate‡ (nmol/h/μgDNA)	1.48 ± 0.13 (5)†	2.18 ± 0.22 (5)

After preincubation in glucose-free KRBH for 30 min, cells were incubated with radiolabeled substrates for 2 h. For details, see Material and Methods. Values are mean ± SE for the number of observations indicated in parentheses. †$p < 0.05$ vs CM by unpaired Student's t test. *in the presence of 11.2 mM glucose. ‡in the presence of 2.8 mM glucose plus 2 mM pyruvate

Cytosolic Ca^{2+} response to glucose and high K^+ in INS-1 cells cultured in SFM. $[Ca^{2+}]_i$ rises in response to glucose or high K^+ were examined in fura-2/AM-loaded cells by video imaging. In the preparation cultured in CM, 70% of the cells examined responded to 11.2 mM glucose, whereas only 25% of cells responded after cultures in SFM (Fig. 10A-E). Some cells in CM displayed oscillations of $[Ca^{2+}]_i$ even at the substimulatory concentration of glucose (2.8 mM) in CM, while this phenomenon was rarely observed in the cells cultured in SFM (data not shown). In contrast, when the cells were stimulated with 30 mM K^+, all the cells, including those cultured in SFM, which did not respond to glucose stimulation, displayed marked transient $[Ca^{2+}]_i$ rises (Fig. 10A-E).

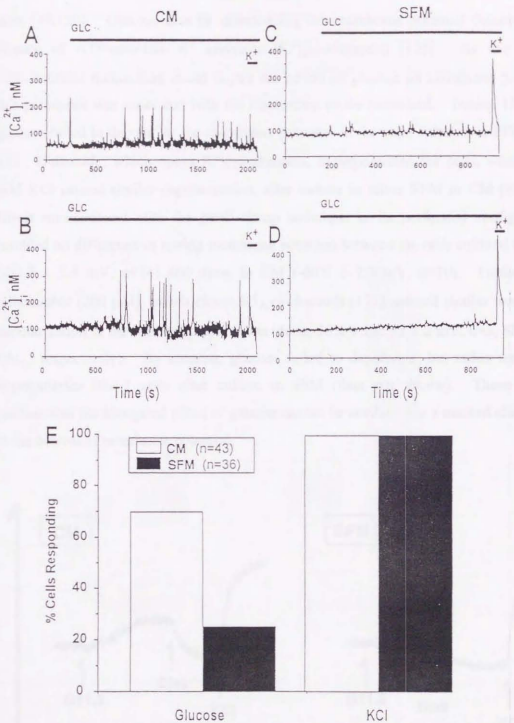


Fig. 10. Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in response to stimulation with glucose or KCl in INS-1 cells cultured in either CM or SFM. Representative traces of $[Ca^{2+}]_i$ examined by video imaging are shown for cells cultured in CM (A, B) and SFM (C, D). E: Percentage of cells responding to either glucose (GLC, 11.2 mM) or KCl (K^+, 30 mM). The n refers to number of cells analyzed in 3 independent experiments.

Membrane potential in INS-1 cells cultured in SFM. The glucose-evoked rise in $[Ca^{2+}]_i$ is due to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) in β-cells [98,120]. Glucose acts by depolarizing the membrane potential following the closure of ATP-sensitive K^+ channels (K^+_{ATP}-channels) [121]. As the altered mitochondrial metabolism could impair the action of glucose on membrane potential, this parameter was measured with the fluorescent probe bisoxonol. Indeed 11.2 mM glucose failed to depolarize the membrane potential in the cells cultured in SFM (Fig. 11). Diazoxide, which opens K^+_{ATP}-channels, hyperpolarized the cells, whereas 30 mM KCl caused similar depolarization, after culture in either SFM or CM (Fig. 11). Direct measurement with the patch-clamp technique in its perforated configuration revealed no difference in resting membrane potential between the cells cultured in SFM (-62.7 ± 2.4 mV, n=14) and those in CM (-60.6 ± 2.3 mV, n=10). Furthermore, tolbutamide (200 μM), which closes K^+_{ATP}-channels [121], caused similar membrane depolarization in the two cell preparations (delta, 36 ± 6 and 32 ± 2 mV, n=3, SFM and CM, respectively). By contrast, glucose failed to depolarize, but rather tended to hyperpolarize INS-1 cells after culture in SFM (data not shown). These results indicate that the abrogated effect of glucose cannot be attributed to a marked alterations in the control of membrane potential.

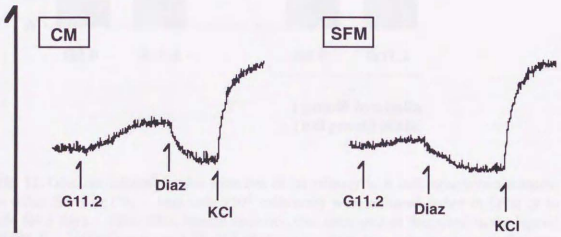


Fig. 11. Effects of glucose, diazoxide, and KCl on membrane potential in INS-1 cells cultured in either CM or SFM. Cells were cultured for 3 days in either CM or SFM. Effects of 11.2 mM glucose (G11.2), 200 μM diazoxide (Diaz), and 30 mM (final) KCl on membrane potential was assessed using 100 nM bisoxonol. The traces are representative for three independent experiments.

Insulin secretory function of INS-1 cells cultured in the medium conditioned by islet cells. The above findings obtained in INS-1 cells appear at variance with the previous report that insulin-secreting cells remain glucose-responsive in SFM [94]. Therefore, the effect of SFM was also examined on rat islet cells in monolayer culture. Insulin secretion in response to 16.7 mM glucose in the islet cells cultured in SFM was comparable to that observed in the cells cultured in the serum-containing medium (Fig. 12), confirming the results of Clark and Chick [94]. Insulin secretion in response to glucose of rat islets was also well preserved when tested after 15 days of culture in SFM (data not shown).

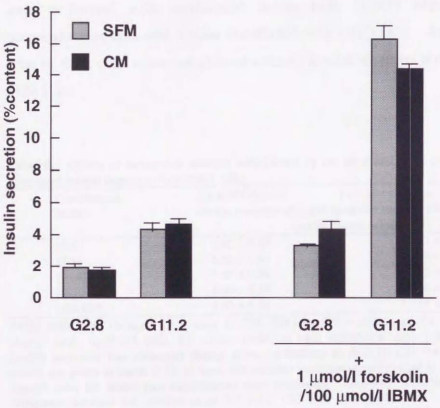


Fig. 12. Glucose-induced insulin secretion in rat primary islet cell monolayers cultured in either SFM or CM. Islet cells (10^5 cells/well) were cultured either in SFM or in CM for 3 days. Thereafter, insulin secretion was measured as described in the legend of Fig. 9. Values are mean ± SE of 6 observations from two independent experiments.

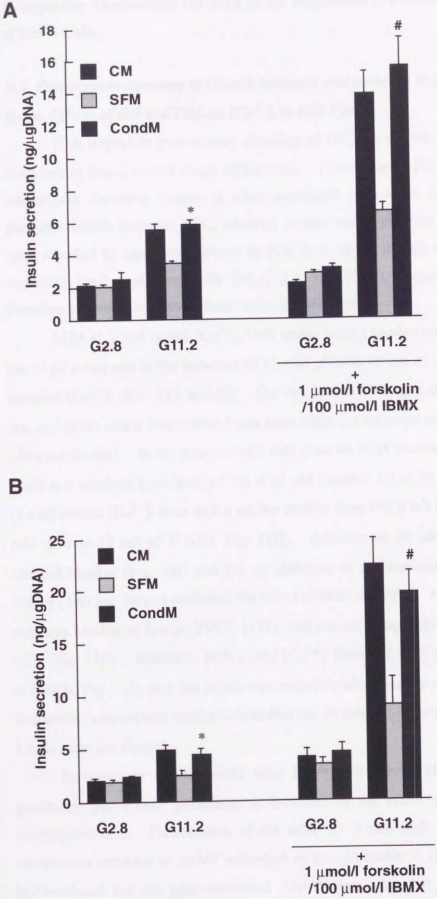
The finding that β-cells can maintain insulin secretory function in SFM suggested that β-cells or other islet cells present in the preparation might produce some factors required for the preservation of differentiated function of INS-1 cells. Therefore, INS-1 cells were cultured in SFM which had been conditioned by islet cells during 3 days. The results of such experiments using medium from islet monolayer culture are shown in Fig. 13A. It can be seen that glucose-induced insulin secretion was no longer impaired following culture in the conditioned medium. Identical results were obtained in experiments performed with conditioned media from whole islets cultured free-floating (Fig. 13B). To elucidate whether other cells than islet cells could restore glucose-stimulated insulin secretion in INS-1 cells, further experiments were performed with conditioned media form HepG2 hepatoma cells, PC12 neuroendocrine cells and human endothelial cells (HUVEC). As shown in Table 8, none of these cells enhanced glucose-induced insulin secretion above the level seen in SFM alone.

Table 8. Effects of serum-free medium conditioned by rat islets and other cultured cells on glucose-stimulated insulin secretion from INS-1 cells.

Conditioned media	2.8 mM Glucose (in the presence of 1 μM forskolin and 100 μM IBMX) Insulin secretion (ng/well)	11.2 mM Glucose (in the presence of 1 μM forskolin and 100 μM IBMX) Insulin secretion (ng/well)
CM	2.73 ± 0.65	15.93 ± 1.97
SFM	4.68 ± 0.62	8.29 ± 0.64
HepG2	5.20 ± 0.99	8.12 ± 0.54
PC12	4.99 ± 0.89	8.46 ± 0.89
Islet CM	5.63 ± 0.91	13.16 ± 1.28

INS-1 cells were cultured for 3 days in CM, SFM or SFM-conditioned for three days by hepatoma HepG2 cells, by PC12 cells, by human umbilical vein endothelial cells (HUVEC) or by rat islets. Insulin secretion was measured during short incubations as described in the legend of Fig. 9. The results are given as mean ± SE of three independent experiments performed in quadruplicate, except for HepG2 cells for which two experiments were performed. One-way ANOVA showed no significant difference between the conditions at 2.8 mM, CM and islet conditioned medium (islet CM) were significantly higher ($p<0.05$) than the other groups, but there was no difference between these two conditions.

Fig. 13 (next page). Effects of the serum-free medium conditioned by rat islet cell monolayers or by free-floating islets on glucose-induced insulin secretion in INS-1 cells. Rat islet cell monolayers (A) or free-floating islets (B) were cultured in SFM for 3 days. The conditioned medium (CondM) was collected and used to culture INS-1 cells for 3 days before the static incubation. Thereafter, insulin secretion was measured as described in the legend of Fig. 9. Values are mean ± SE of eight observations from two independent experiments (A), and of four independent experiments (B). Statistical analysis by ANOVA; *$p<0.05$ vs. G11.2 in SFM; #$p<0.01$ vs. G11.2 plus forskolin/IBMX in SFM.


A
Insulin secretion (ng/μgDNA)
CM
SFM
CondM
G2.8
G11.2
G2.8
G11.2
+
1 μmol/l forskolin
/100 μmol/l IBMX
B
Insulin secretion (ng/μgDNA)
CM
SFM
CondM
G2.8
G11.2
G2.8
G11.2
+
1 μmol/l forskolin
/100 μmol/l IBMX

II. Signaling Mechanisms Involved in the Regulation of Growth and the Function of INS-1 Cells

II-1. Postreceptor signaling of Growth hormone and prolactin in INS-1 cells

II-1-a. Effects of GH and PRL on $[Ca^{2+}]_i$ in INS-1 cells

With respect to postreceptor signaling of GH, the effects on $[Ca^{2+}]_i$ were first examined in fura-2-loaded single INS-1 cells. The change in $[Ca^{2+}]_i$, one of the major intracellular signaling events, is often associated with other mechanisms, such as phosphoinositide turnover, PKC, adenylyl cyclase and G-proteins [122]. GH has also been reported to cause an increase in $[Ca^{2+}]_i$ in some, though not all, systems [42] including insulin-secreting cells [46,123]. The $[Ca^{2+}]_i$-raising effect of GH was therefore examined to further characterize in INS-1 cells.

hGH at 5 nM raised $[Ca^{2+}]_i$ both under resting conditions (3 mM glucose; 35 out of 40 cells) and in the presence of 15 mM glucose (9 out of 11 cells), which itself elevated $[Ca^{2+}]_i$ (Fig. 14A and B). The rise in $[Ca^{2+}]_i$ began after a delay of 30-45 sec, and never lasted longer than 5 min even when the hormone was present for 10 min (data not shown). In the presence of 3 mM glucose, hGH increased $[Ca^{2+}]_i$ from 97 ± 6 nM to a maximal peak level of 321 ± 33 nM (mean ± SE of 35 observations). PRL (5 nM) caused $[Ca^{2+}]_i$ rises with a similar pattern from 100 ± 6.2 to 211 ± 27.7 nM at 3 mM glucose (8 out of 9 cells, Fig. 14D). Addition of 20 μM verapamil, a Ca^{2+}-channel blocker (Fig. 14C and D), or chelation of extracellular Ca^{2+} with 2.5 mM EGTA (data not shown) abolished the effect of hGH and PRL. Calciseptine (1 μM), a selective blocker of L-type VDCC [124], also almost completely blocked the effect of hGH (Fig. 14E). Similarly, bGH raised $[Ca^{2+}]_i$ from 125 ± 16 to 495 ± 50 nM (8 out of 8 cells, Fig. 14I), and this effect was reversibly abolished by calciseptine (Fig. 14J). In addition, calciseptine markedly inhibited the 30 mM K^+-induced $[Ca^{2+}]_i$ rise in INS-1 cells (data not shown).

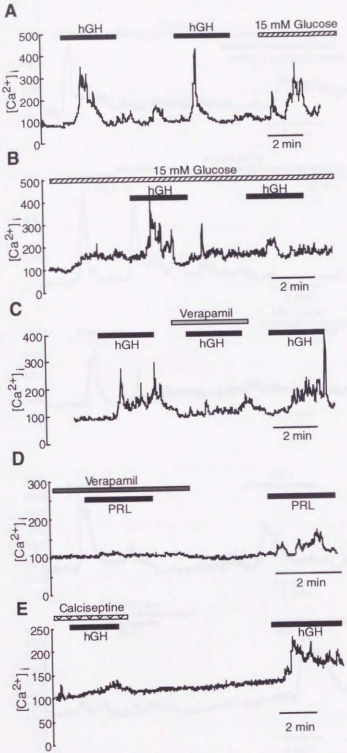
Increases in cAMP levels raise $[Ca^{2+}]_i$ in β-cells [125]. Therefore, the possibility that cAMP generation is involved in the effect of the hormones was investigated next. Pretreatment of the cells for 5 min with 1 mM Rp-cAMPS, a competitive inhibitor of cAMP activation of protein kinase A (PKA) [126], abolished hGH-induced but not glucose-evoked $[Ca^{2+}]_i$ rises (Fig. 14F). Rp-cAMPS alone,

however, raised $[Ca^{2+}]_i$ to a similar extent as GH. It was therefore important to perform control experiments with a hormone known to act through the generation of cAMP levels. The insulin-secretagogue, GLP-1, belongs to this category of hormones [127]. GLP-1 (1 nM) caused a transient increase in $[Ca^{2+}]_i$, which was blocked by Rp-cAMPS (Fig. 14G). Since verapamil blocked the action of hGH, suggesting involvement of VDCC, the hyperpolarizing agent diazoxide, which acts by opening K^+_{ATP}-channels [84], was also tested. In the presence of 200 µM diazoxide, the Ca^{2+}-raising effect of hGH was no longer observed (Fig. 14H).

II-1-b. Effects of GH on cAMP levels in INS-1 cells

The above results of $[Ca^{2+}]_i$ measurements suggested that cAMP played a role in the effect of GH in INS-1 cells. Therefore cellular cAMP levels were measured by RIA in cells exposed to GH. hGH (5 nM) did not significantly alter cellular cAMP levels after 5-min incubation at 3 mM glucose (control, 26.2 ± 1.2 vs. hGH, 27.9 ± 1.2 pmol/10^6 cells). Incubation with 15 mM glucose did not affect cAMP levels (29.1 ± 1.0 pmol/10^6 cells), whereas 1 µM forskolin raised cAMP levels 3.7-fold (96.2 ± 4.9 pmol/10^6 cells).

Fig. 14 (next two pages). Effects of hGH, bGH and PRL on cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in single INS-1 cells. Changes in $[Ca^{2+}]_i$ were investigated in fura-2-loaded cells stimulated with 5 nM hGH at 3 mM glucose except for (B) (at 15 mM glucose) in the presence of 20 µM verapamil (C), 1 µM calciseptine (E), 1 mM Rp-cAMPS (F) or 200 µM diazoxide (H), with 5 nM PRL at 3 mM glucose in the absence and presence of 20 µM verapamil (D), and with 5 nM bGH at 3 mM glucose in the absence (I) or presence (J) of 1 µM calciseptine. The effects of 1 mM Rp-cAMPS on glucose- (F) or GLP-1- (G) induced $[Ca^{2+}]_i$ are shown for control purposes. Cells were attached on glass coverslips and loaded with 1 µM fura-2/AM for 30 min. $[Ca^{2+}]_i$ was monitored by the measurement of 340 nm/380 nm ratio during continuous perifusion with KRBH containing 3 mM glucose as a basal condition. Stimuli were given through large orifice pipettes placed in the vicinity of the cell examined. Representative traces of those obtained in at least 5 cells are shown.


A
hGH
hGH
15 mM Glucose
500
400
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200
100
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[Ca2+]i
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B
15 mM Glucose
hGH
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100
0
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2 min
C
Verapamil
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hGH
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400
300
200
100
0
[Ca2+]i
2 min
D
Verapamil
PRL
PRL
300
200
100
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2 min
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hGH
hGH
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150
100
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0
[Ca2+]i
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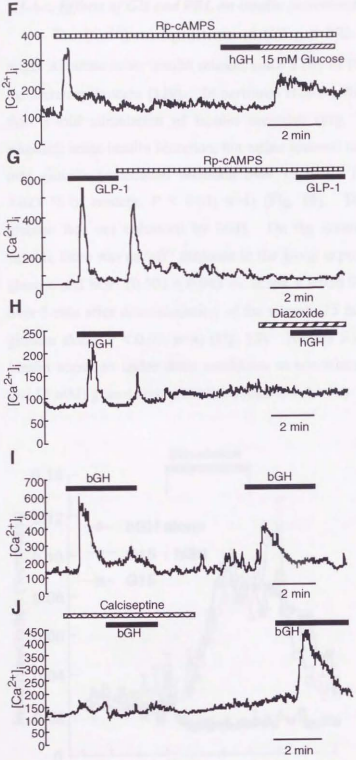
F
Rp-cAMPS
hGH 15 mM Glucose
[Ca2+]i
2 min
G
Rp-cAMPS
GLP-1
GLP-1
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2 min
H
Diazoxide
hGH
hGH
[Ca2+]i
2 min
I
bGH
bGH
[Ca2+]i
2 min
J
Calciseptine
bGH
bGH
[Ca2+]i
2 min

II-1-c. Effects of GH and PRL on insulin secretion from INS-1 cells

The $[Ca^{2+}]_i$-raising actions of GH and PRL suggested that these hormones might stimulate acute insulin release, since a rise of $[Ca^{2+}]_i$ is a key signaling event in the secretory process [128]. In perifused INS-1 cells, 15 mM glucose caused a more than 3-fold stimulation of insulin secretion (Fig. 15). However, hGH failed to stimulate acute insulin secretion, but rather lowered the basal level in the presence of 3 mM glucose (cumulated secretion over 15 min, from 0.365 ± 0.040 to 0.237 ± 0.023 % of content, $P < 0.01$; n=4) (Fig. 15). The stimulatory effect of 15 mM glucose was not enhanced by hGH. On the contrary, after discontinuation of the stimuli, there was an 'off' response in the group exposed to the combination of 15 mM glucose and hGH (0.504 ± 0.043 vs. 0.364 ± 0.036 % of content, cumulated secretion over 5 min after discontinuation of the stimuli, 15 mM glucose plus hGH vs. 15 mM glucose alone, $P < 0.05$; n=4) (Fig. 15). Neither 5 nM hGH nor 5 nM PRL affected insulin secretion under static conditions at non-stimulatory (3 mM) or stimulatory (7 and 15 mM) glucose concentrations (data not shown).

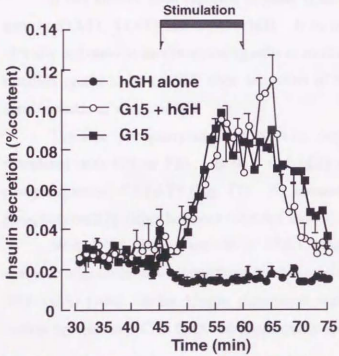


Fig. 15. Effect of hGH on insulin secretion from perifused INS-1 cells. Cells were mounted into a small chamber and perifused in KRBH containing 3 mM glucose for 45 min as basal condition. Cells were stimulated for 15 min with 5 nM hGH at 3 mM (*closed circles*) or 15 mM (*open circles*) glucose, and with 15 mM glucose alone (*closed squares*). Error bars indicate SE of four perifusions.

II-1-d. Effects of GH and PRL on tyrosine phosphorylation of JAK2

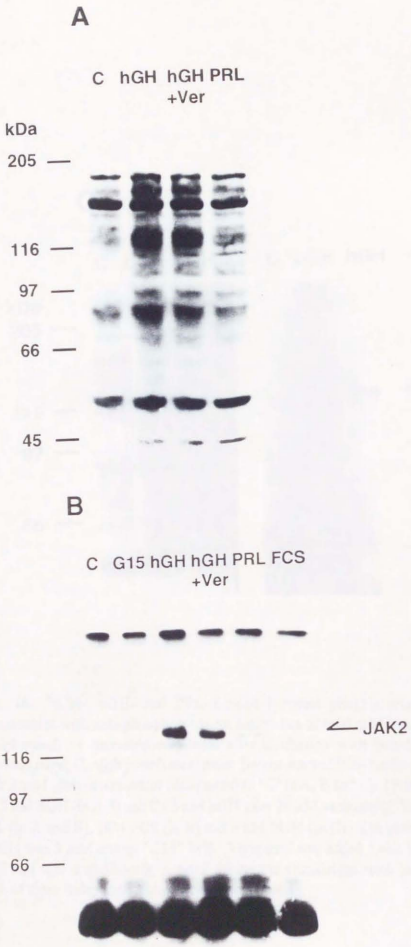
Immunoblotting with anti-phosphotyrosine antibodies revealed that several proteins were tyrosine phosphorylated in INS-1 cells stimulated for 5 min with 5 nM hGH (Fig. 16A and C, *left panel*). Among these, proteins of 120-130 kDa, ~90 kDa and ~85 kDa were consistently phosphorylated. A similar pattern of protein tyrosine phosphorylation was observed with 5 nM PRL (Fig. 16A) as well as 5 nM bGH (Fig. 16C, *left panel*). To know whether the 120-130 kDa protein is the tyrosine kinase, JAK2, total lysates from stimulated cells were immunoprecipitated with anti-JAK2 antiserum and immunoblotted with anti-phosphotyrosine antibodies. The results demonstrate that JAK2 is tyrosine phosphorylated in the cells treated with hGH (Fig. 16B and C, *right panel*), bGH (Fig. 16C, *right panel*) or PRL (Fig, 16B), although the effect was more pronounced with hGH than with PRL at equimolar concentrations (Fig. 16B). By contrast, neither 15 mM glucose nor 10% FCS stimulated JAK2 tyrosine phosphorylation (Fig. 16B).

II-1-e. Activation of STAT proteins by GH and PRL in INS-1 cells

It has already been reported in other systems that GH activates three STATs, namely STAT1, STAT3 and STAT5 [42]. It is, however, not known which STAT is virtually activated in insulin-secreting cells to mediate the effects of GH or PRL. This question seems to be crucial, since activation of STATs might determine the tissue-specific mode of action of GH.

Tyrosine phosphorylation of STATs were first examined in INS-1 cells stimulated with GH or PRL. Both 5 nM bGH and 5 nM PRL promoted tyrosine phosphorylation of STAT5 (Fig. 17). By contrast, neither STAT1 nor STAT3 was phosphorylated by either hormone (data not shown).

Next, DNA-binding activity of STAT5 were investigated by EMSA using a synthetic oligonucleotide representing its binding site in the β-casein gene promotor (β-CAS GAS) [104]. After 15-min stimulation with bGH or PRL, DNA binding of nuclear proteins to β-CAS GAS was significantly increased (Fig. 18).


A
C hGH hGH PRL
+Ver
kDa
205
116
97
66
45
B
C G15 hGH hGH PRL FCS
+Ver
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97
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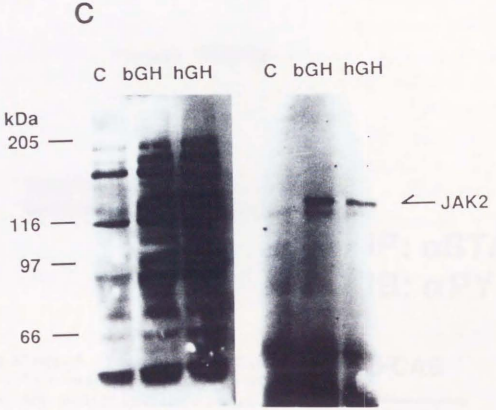


Fig. 16. hGH-, bGH- and PRL-induced tyrosine phosphorylation in INS-1 cells. Immunoblot with anti-phosphotyrosine antibodies of total cell lysates (A, *previous page*, C, *left panel*) or immunoprecipitates after incubation with anti-JAK2 antibodies (B, *previous page*; C, *right panel*) are shown. Serum-starved INS-1 cells were treated for 5 min with 3 mM glucose as control (designated as "C" in A, B and C), 15 mM glucose ("G15" in B), 5 nM hGH (in A, B and C), 5 nM hGH plus 20 µM verapamil ("Ver" in A and B), 5 nM PRL (in A and B), 10% FCS (in B) and 5 nM bGH (in C). The glucose concentration of KRBH was 3 mM except "G15" in B. Verapamil was added 1 min before the addition of hGH and was continuously present during the stimulation with hGH. Representative blots of three independent experiments are shown.

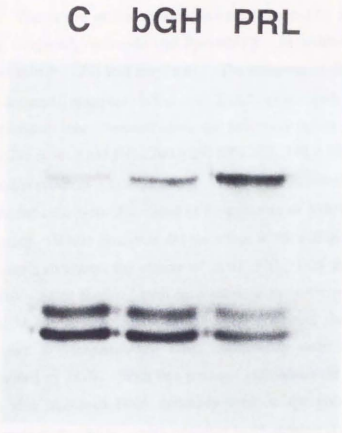


Fig. 17 (upper). Tyrosine phosphorylation of STAT5 promoted by bGH and PRL in INS-1 cells. After overnight incubation in a serum-free medium, cells were preincubated for 30 min without stimuli, and stimulated for 15 min with 5 nM bGH or 5 nM PRL. Total cell lysates were immunoprecipitated (IP) with the anti-STAT5 antibody (αSTAT5) and immunoblotted (IB) with antibodies against phosphotyrosine (αPY).

Fig. 18 (right). DNA binding of nuclear proteins to β-CAS GAS promoted by bGH and PRL in INS-1 cells. After overnight incubation in a serum-free medium, cells were preincubated for 30 min without stimuli, and stimulated for 15 min with 5 nM bGH or 5 nM PRL. DNA-binding of nuclear extracts to β-CAS GAS were examined by EMSA.

II-1-f. Effects of various pharmacological agents on hGH- and PRL-stimulated DNA synthesis and JAK2 tyrosine phosphorylation

The nature of the signaling events triggered by hGH and PRL was examined using verapamil, diazoxide and Rp-cAMPS. In addition, a tyrosine kinase inhibitor lavendustin A [129] was employed. The presence of 20 μM verapamil did not affect hGH-induced phosphorylation of JAK2 (Fig. 16A and B). [^{3}H]Thymidine incorporation was increased when the following agents were present for 24 h: 5 nM hGH, 256 ± 36; 5 nM PRL, 200 ± 29; 10% FCS, 148 ± 29; 15 mM glucose, 173 ± 22 (% of incorporation at 3 mM glucose). To test the involvement of voltage-sensitive Ca^{2+} influx, the cells were stimulated in the presence of either 20 μM verapamil or 200 μM diazoxide. While diazoxide did not affect hGH- and glucose-induced DNA synthesis, verapamil abolished the effects of hGH, PRL, FCS and 15 mM glucose (data not shown). Since the long-term incubation of the cells with verapamil, however, could result in non-specific inhibition of DNA synthesis, the effect of a short-term (6 h) exposure to verapamil and other compounds were examined on DNA synthesis stimulated by hGH. With this protocol (see *Materials and Methods*), both hGH and PRL also increased DNA synthesis even in the presence of verapamil (Fig. 19). Exposure to diazoxide, which inhibited hGH-induced $[Ca^{2+}]_i$ rises, did not affect DNA synthesis stimulated by hGH during the 6 h-incubation (Fig. 19). On the other hand, Rp-cAMPS, which also blocked the $[Ca^{2+}]_i$ response, inhibited the mitogenic action of hGH (Fig. 19). Lavendustin A (1 μM) abolished hGH-induced DNA synthesis over 6h (Fig. 19) and attenuated the effect by 67% over 24 h (data not shown). By contrast, glucose-stimulated DNA synthesis was not altered by lavendustin A (measured over 24 h, 173 ± 22 vs. 159 ± 17 % of the incorporation at 3 mM glucose, in the absence and presence of the inhibitor, respectively).

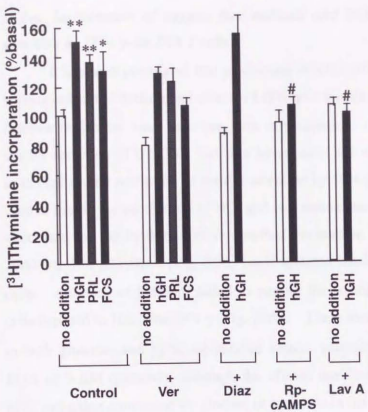


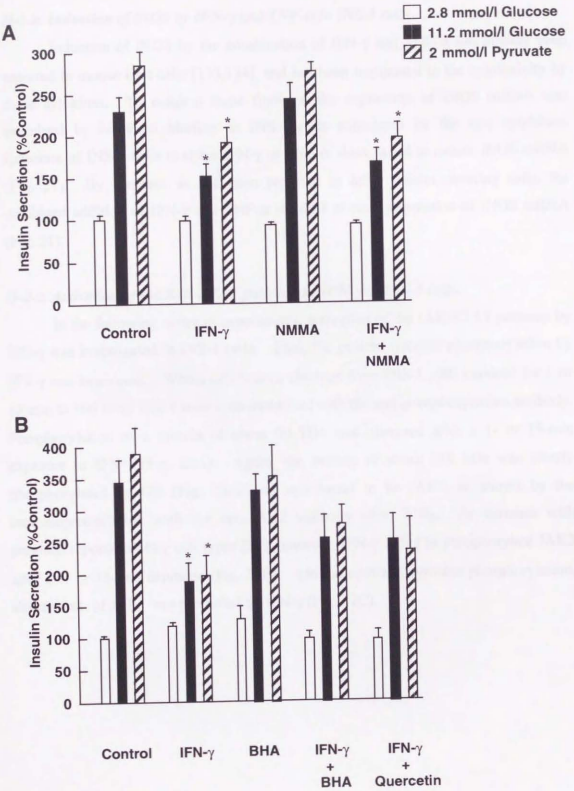
Fig. 19. Effects of various pharmacological agents on hGH- and PRL-stimulated DNA synthesis in INS-1 cells. Cells were stimulated by 5 nM hGH, 5 nM PRL and 10% FCS in the absence or presence of the agents as indicated. Stimuli and inhibitors were present during 6 h, whereafter the cells were washed and incubated at 3 mM glucose for an additional 18-h period. [^{3}H]Thymidine was present during the last 4 h of the latter period. The results are given as % basal, i.e. the rate of [^{3}H]thymidine incorporation at 3 mM glucose throughout, corresponding to 1391 ± 128 cpm. The agents are: Ver, 20 µM verapamil; Diaz, 200 µM diazoxide, Lav A, 1 µM lavendustin A, 1 mM Rp-cAMPS. Data are mean ± SE of three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. "no addition" in the control group; $\#P < 0.05$ vs. hGH alone, analyzed by Mann-Whitney *U* test.

II-2. Intracellular events mediating the effects of IFN-γ in INS-1 cells

II-2-a. Involvement of oxygen free radicals and NO in the inhibition of insulin secretion by IFN-γ on INS-1 cells.

It has been postulated that production of NO [130] or oxygen free radicals [131] is involved in the detrimental effects of IFN-γ in insulin-secreting cells. These highly reactive molecules may interfere with mitochondrial metabolism, thereby impairing insulin secretion [132]. To test this hypothesis, the effects of their inhibitors were examined on the inhibition of insulin secretion by IFN-γ in INS-1 cells. NMMA was used to inhibit the production of NO, and two antioxidants, namely BHA and quercetin, were used for the inhibition of free radical generation. Neither 1 mM NMMA (Fig. 20A) nor 100 μM BHA (Fig. 20B) itself affected insulin secretory function of INS-1 cells. Addition of NMMA failed to restore the insulin secretory function of INS-1 cells exposed to 100 U/ml IFN-γ (Fig. 20A). There seemed to be a slight improvement in both glucose- and pyruvate-induced insulin secretion by the addition of 100 μM BHA or 5 μM quercetin, although the effects were not significant (Fig. 20B). The MTT reduction stimulated by glucose or pyruvate in the cells exposed to IFN-γ was not restored by all the three agents (data not shown).

Fig. 20 (next page). Effects of NMMA, BHA, and quercetin on insulin secretion in INS-1 cells exposed to IFN-γ. INS-1 cells were cultured for 24 h in the absence or presence of 100 U/ml IFN-γ. During the incubation period, NMMA (A) or two antioxidants 100 μM BHA and 5 μM quercetin (B) were added in the culture medium as indicated. After a 30-min preincubation in KRBH, cells were stimulated with 11.2 mM glucose or 2 mM pyruvate. Values are mean ± SE with the value at 2.8 mM glucose in *Control* as 100% from three independent experiments performed in quadruplicate. Statistical analysis by ANOVA: $*P < 0.05$ vs. Control.


A
2.8 mmol/l Glucose
11.2 mmol/l Glucose
2 mmol/l Pyruvate
Insulin Secretion (%Control)
300
250
200
150
100
50
0
Control
IFN-γ
NMMA
IFN-γ + NMMA
B
Insulin Secretion (%Control)
400
350
300
250
200
150
100
50
0
Control
IFN-γ
BHA
IFN-γ + BHA
IFN-γ + Quercetin

II-2-b. Induction of iNOS by IFN-γ and TNF-α in INS-1 cells.

Induction of iNOS by the combination of IFN-γ and TNF-α has already been reported in mouse islet cells [133,134], and has been implicated in the cytotoxicity by these cytokines. To confirm these findings, the expression of iNOS mRNA was examined by Northern blotting in INS-1 cells stimulated by the two cytokines. Exposure of INS-1 cells to either IFN-γ or TNF-α alone failed to induce iNOS mRNA (Fig. 21). By contrast, as has been reported in other insulin-secreting cells, the combined addition of IFN-γ and TNF-α resulted in clear expression of iNOS mRNA (Fig. 21).

II-2-c. Activation of the JAK-STAT pathway by IFN-γ in INS-1 cells.

In the following series of experiments, activation of the JAK-STAT pathway by IFN-γ was investigated in INS-1 cells. First, the protein tyrosine phosphorylation by IFN-γ was examined. Whole cell lysates obtained from INS-1 cells exposed for 1 or 15 min to 100 U/ml IFN-γ were immunoblotted with the anti-phosphotyrosine antibody. Phosphorylation of a protein of about 90 kDa was observed after a 1- or 15-min exposure to IFN-γ (Fig. 22A). Again, the protein of about 130 kDa was clearly phosphorylated by GH (Fig. 22A) and was found to be JAK2, as shown by the immunoprecipitation with the anti-JAK2 antibody (Fig. 22B). At variance with previous reports in other cell types [37], however, IFN-γ failed to phosphorylate JAK2 after a 1- or 15-min exposure (Fig. 22B). On the contrary, tyrosine phosphorylation, albeit weak, of JAK1 was promoted by IFN-γ (Fig. 22C).

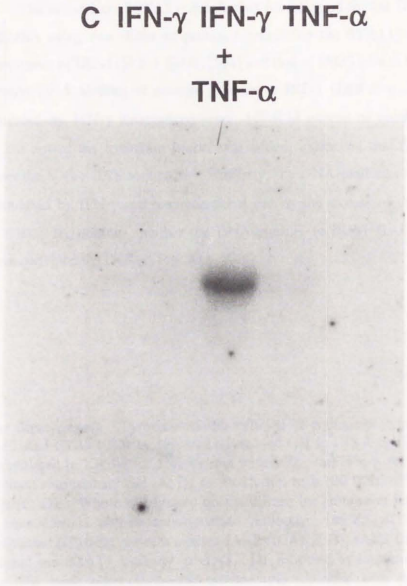
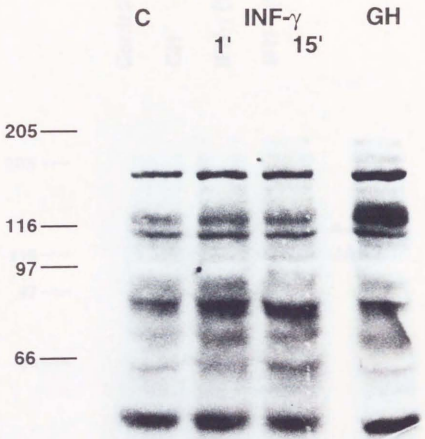


Fig. 21. Expression of iNOS mRNA induced by IFN-γ and TNF-α in INS-1 cells. Cells were incubated in CM in the absence or presence of 100 U/ml IFN-γ and/or 50 ng/ml TNF-α for 6 h. Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method for the detection of iNOS mRNA by Northern blotting using a specific cDNA probe of the mouse macrophage iNOS.

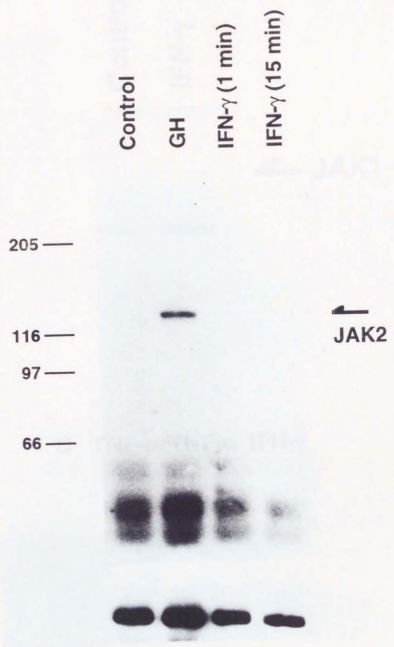
Next, activation of STAT proteins by IFN-γ was investigated in INS-1 cells. After a 15-min exposure of INS-1 cells to IFN-γ, STAT1 was found to be phosphorylated on tyrosine residue (Fig. 22D). The protein phosphorylated by IFN-γ in the whole cell lysates (Fig. 22A) was thus assumed to be STAT1. By contrast, TNF-α did not affect STAT1 phosphorylation either in the presence or absence of IFN-γ (Fig. 22D). The activation of STAT1 was further investigated for the DNA-binding activity by EMSA using two different probes representing the STAT1 binding site: GAS in the promoter of IRF-1 (IRF-1 GAS) [104] and that of iNOS (iNOS GAS) [105]. IFN-γ stimulated DNA-binding of nuclear proteins to IRF-1 GAS (Fig. 23A). The binding stimulated by IFN-γ disappeared when 100-fold excess of unlabeled IRF-1 GAS probe, but not of the irrelevant probe, was added, indicating that this effect of IFN-γ was specific to the GAS sequence. Similarly, the DNA-binding to iNOS GAS was also stimulated by IFN-γ and was competed out by the excess of the unlabeled probe (Fig. 23B). By contrast, neither the DNA-binding to IRF-1 GAS nor that to iNOS GAS was affected by TNF-α (Fig. 23A, B).

Fig. 22 (next three pages). Tyrosine phosphorylation of cellular proteins (A), JAK2 (B), JAK1 (C), and STAT1 (D) by IFN-γ, TNF-α, and GH in INS-1 cells. Quiescent cells were stimulated in KRBH for 1 or 15 min with 100 U/ml IFN-γ, for 15 min with 100 ng/ml human recombinant GH (A, B), or for 15 min with 100 U/ml IFN-γ and/or 50 ng/ml TNF-α (C, D). Whole cell lysates obtained from the cells were immunoblotted with the monoclonal anti-phosphotyrosine antibody (αPY, 4G10, A), or immunoprecipitated (IP) with antisera against JAK2 (αJAK2, B), JAK1 (αJAK1, C) or with monoclonal anti-STAT1 antibody (αSTAT1, D), followed by immunoblotting (IB) with 4G10. The same nitrocellulose membrane was reblotted with the anti-JAK2 antibody in the lower panel (B). C, Control.

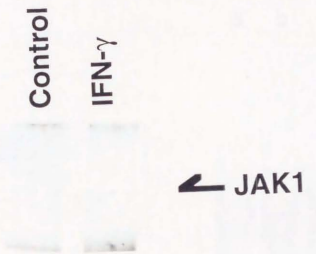
A



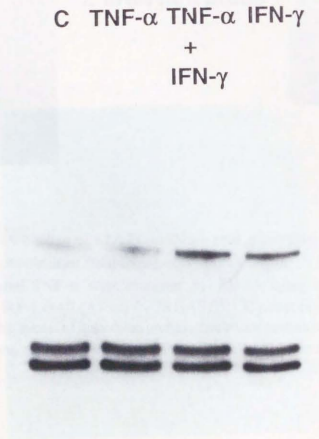
B



C



D



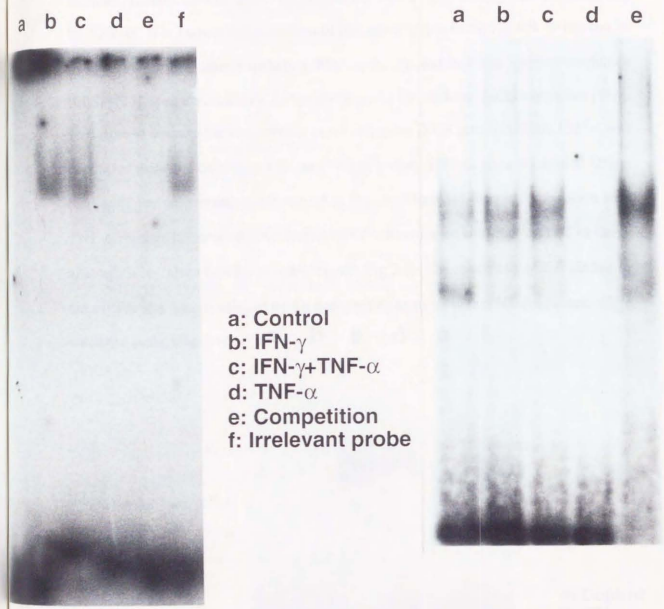


Fig. 23. DNA binding of STAT1 in INS-1 cells stimulated with IFN-γ and TNF-α. Nuclear extracts obtained from INS-1 cells stimulated for 15 min with 100 U/ml IFN-γ and/or 50 ng/ml TNF-α were examined by EMSA using synthetic oligonucleotides representing IRF-1 GAS (A) and iNOS GAS (B). Competition analysis was performed with a 100-fold excess of unlabelled probes. Irrelevant probes used as negative control for the competition analysis were κB site in A and IRF-1 GAS in B.

II-2-d. Synergistic activation of NF-κB by IFN-γ and TNF-α in INS-1 cells.

The above findings indicated that the activation of STAT1 by IFN-γ alone was not sufficient to induce iNOS mRNA expression and that STAT1 binding was not modulated by TNF-α. It is known that activation of the transcription factor NF-κB, which can be induced by various stimuli including TNF-α, IL-1β, and bacterial lipopolysaccharide (LPS) [135], is an essential step for the cytokine- or LPS-induced iNOS expression [136]. Therefore to examine the involvement of NF-κB in the iNOS gene induction, EMSA was conducted using the consensus κB site [106] as probe. TNF-α alone stimulated DNA-binding of nuclear proteins to κB sites (Fig. 24). Addition of IFN-γ in combination with TNF-α resulted in increased DNA-biding of NF-κB compared with that by TNF-α alone, although IFN-γ alone failed to activate NF-κB (Fig. 24). The specificity of this binding to the κB site was again confirmed by the competition analysis with a 100-fold excess of the unlabeled probe (Fig. 24).

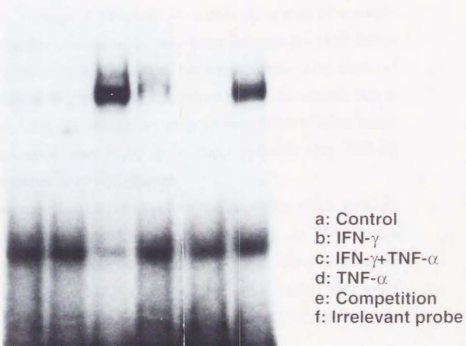


Fig. 24. DNA binding of NF-kB in INS-1 cells stimulated with IFN-γ and TNF-α. Nuclear extracts obtained from INS-1 cells stimulated for 15 min with 100 U/ml IFN-γ and/or 50 ng/ml TNF-α were examined by EMSA using synthetic oligonucleotides representing the consensus NF-κB binding sequence. Competition analysis was performed with a 100-fold excess of unlabeled probes. Irrelevant probe used as negative control for the competition analysis was IRF-1 GAS.

II-3. Role of the MAP kinase cascade in the regulation of growth and the function of INS-1 cells

II-3-a. Secretagogues of insulin activate MAP kinase in INS-1 cells

Effects of glucose and various secretagogues of insulin on the activity of 44-kDa MAP kinase was first investigated in INS-1 cells. Exposure of INS-1 cells to 15 mM glucose led to a small 2.5-fold increase in the activity of MAP kinase (Fig. 25A). The effect of glucose was augmented 3-4-fold by an addition of CPT-cAMP. In the absence of glucose, however, CPT-cAMP had little stimulatory effect. Similar enhancement of glucose-induced activation of MAP kinase was observed with forskolin (data not shown). The activation of MAP kinase by glucose, whether in the absence or presence of CPT-cAMP, was sustained from 12 to 30 min (and remained at the same level for up to 60 min; not shown). To compare activation of MAP kinase with insulin secretion, the amount of insulin which accumulated in the incubation medium during the experiment was measured by RIA. As shown in Fig. 25B, the 2.5-fold stimulation of MAP kinase by glucose was associated with a 2.5-fold increase in secreted insulin as measured after 30 min of incubation. Likewise, the potentiation of glucose-induced activation of MAP kinase by CPT-cAMP was associated with a large increase in insulin secretion, whereas CPT-cAMP alone induced no detectable insulin secretion (Fig. 25B). To further investigate the correlation between the MAP kinase activation and the stimulation of insulin secretion, the concentration dependence of these two processes with respect to glucose was determined both in the absence and in the presence of CPT-cAMP. The dose-response curves for stimulation of MAP kinase and for that of insulin secretion were found to be superimposable (Fig. 26A,B). Maximal stimulation was obtained at 10 mM glucose.

Next, effects of two hormonal/neurotransmitter secretagogues which stimulate cAMP synthesis in β-cells, GLP-1 [137] and PACAP38 [138], on MAP kinase activation were examined. Both factors increased by 2-5-fold, depending on the experiment, the effect of glucose on MAP kinase activity, while having little effect when added alone (Fig. 27). PACAP27, an alternatively processed PACAP form, also stimulated MAP kinase in the presence of glucose (data not shown).

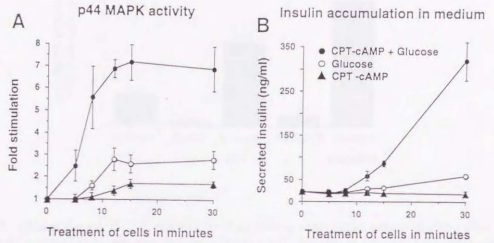


Fig. 25. Effect of glucose and CPT-cAMP on 44-kDa MAP kinase activity (A) and insulin secretion (B) by INS-1 cells. Cells were incubated with glucose (15 mM) or CPT-cAMP (1 mM) in combination as indicated and for the periods of time shown. Thereafter, the medium was collected, and the cells were solubilized. A: 44-kDa MAP kinase was immunopurified from the cell extracts, and its kinase activity was measured *in vitro* using myelin basic protein as a substrate as described in *Materials and Methods*. MAP kinase activity is expressed as -fold stimulation compared with its activity in untreated cells. B: the amount of insulin accumulated in the incubation medium was determined by RIA. Data are means ± S.D. of triplicate determinations. The experiment was performed twice with similar results.

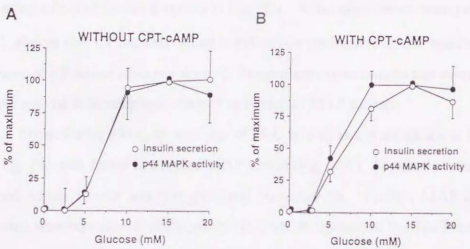


Fig. 26. Dose-response relationship of glucose stimulation of 44-kDa MAP kinase and insulin secretion by INS-1 cells. INS-1 cells were incubated with glucose at the concentration indicated in the absence (A) or presence of 1 mM CPT-cAMP (B). After 30 min of incubation, the medium was collected, and the cells were solubilized. The activity of 44-kDa MAP kinase immunopurified from the cell extracts and the insulin content of the incubation media were measured and expressed in percent of the value obtained with 15 mM glucose. Data are means ± S.D. of three experiments performed in triplicates.

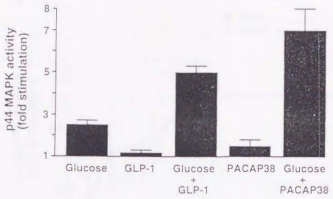


Fig. 27. Effect of glucose, GLP-1, and PACAP38 on 44-kDa MAP kinase activity in INS-1 cells. INS-1 cells were incubated for 15 min with glucose (15 mM), GLP-1 (50 nM) or PACAP38 (10 nM) as indicated and thereafter solubilized. The activity of immunopurified 44-kDa MAP kinase was measured and expressed as -fold stimulation compared with its activity in untreated cells. Data are means ± S.D. of triplicate determinations. The experiment was performed twice with similar results.

Activation of the insulin receptor leads to stimulation of MAP kinase cascade in the physiological target tissues of insulin. Addition of insulin at concentrations ranging from 10 nM to 5 μM, however, did not significantly stimulate MAP kinase in INS-1 cells. The effect of 5 μM insulin is shown in Fig. 28A. When experiments were performed at 20°C, glucose plus CPT-cAMP failed to induce any measurable insulin secretion but still activated MAP kinase (data not shown). These experiments suggest that secreted insulin is not involved in secretagogue-induced activation of MAP kinase.

Phorbol ester PMA, an activator of PKC as well as a secretagogue in INS-1 cells (see Fig. 30), was found to activate MAP kinase (Fig. 28A). Maximal stimulation was reached within 15 min and was sustained thereafter for. Finally, MAP kinase was activated after exposure of INS-1 cells to KCl, which depolarizes the membrane potential leading to influx of extracellular Ca^{2+} through VDCC (Fig. 28B). A swift peak of MAP kinase activity was observed at 2.5 min of exposure to KCl, followed by a rapid decline to a low level sustained for up to 30 min. Chelation of extracellular Ca^{2+} with EGTA abolished the activation of MAP kinase by KCl, suggesting that the effect of KCl was mediated by Ca^{2+}-influx.

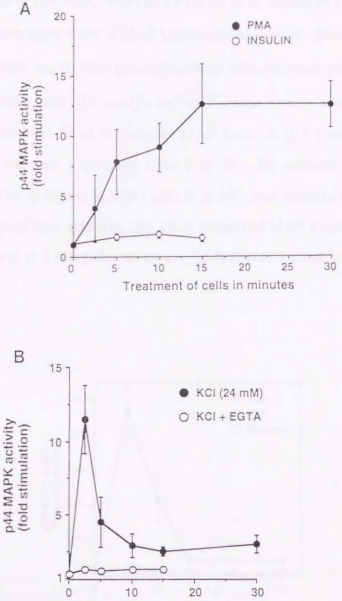


Fig. 28. Effect of phorbol ester, insulin (A), and KCl (B) on 44-kDa MAP kinase in INS-1 cells. INS-1 cells were exposed to 1 μM PMA or 5 μM insulin (A) and 24 mM KCl (B) in the absence or presence of 5 mM EGTA added 2 min before the KCl. After incubation for the periods of time shown, the cells were solubilized, and the activity of immunopurified 44-kDa MAP kinase was measured and expressed as -fold stimulation compared with its activity in untreated cells. Data are means ± S.D. of three experiments performed in triplicates.

II-3-b. Effect of GH, PRL, NGF, and FCS on MAP kinase in INS-1 cells

To investigate a role of MAP kinase activation in the stimulation of β-cell growth, the effect of PRL and GH (the latter not shown), which promote proliferation of β-cells [7] as well as INS-1 cells (this study), on MAP kinase activity was first examined. Both hormones, however, failed to stimulate MAP kinase in INS-1 cells, whereas FCS had a modest and transient stimulatory effect (Fig. 29). By contrast, NGF, which does not stimulate DNA synthesis in INS-1 cells (Fig. 6B), was found to be a strong activator of MAP kinase in INS-1 cells (Fig. 29). NGF stimulated MAP kinase in a transient manner, showing a peak at 5 min followed by a rapid decline to a lower level sustained for 15-30 min.

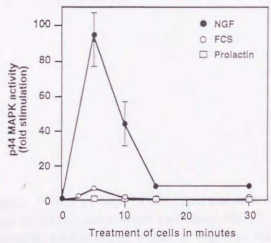
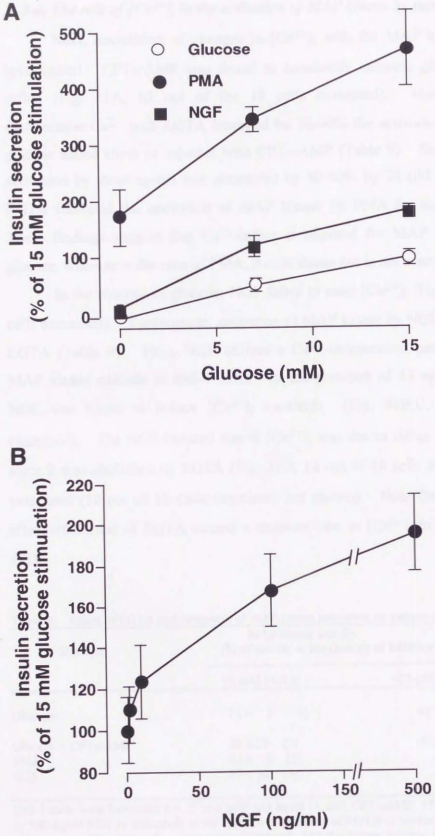


Fig. 29. Effect of PRL, NGF, and FCS on 44-kDa MAP kinase in INS-1 cells. INS-1 cells were incubated with PRL (1 nM), NGF (100 ng/ml), or FCS (10%) for the periods of time shown. Thereafter, the cells were solubilized, and the activity of immunopurified 44-kDa MAP kinase was measured and expressed as -fold stimulation compared with its activity in untreated cells. Data are means ± range of two experiments performed in triplicates.

II-3-c. NGF stimulates insulin secretion by INS-1 cells in the presence of glucose

It was of interest to investigate whether NGF stimulates insulin secretion from INS-1 cells, considering the strong activation of MAP kinase by NGF despite the absence of its effect on DNA synthesis. In the absence of glucose (the experimental condition of Fig. 29), NGF failed to stimulate insulin secretion (Fig. 30A). In the presence of glucose, however, NGF enhanced by two-fold the secretion of insulin (Fig. 30A). A dose-response analysis of NGF-stimulated insulin secretion showed that the factor was near optimal at 100-500 ng/ml (Fig. 30B). In contrast to NGF, PMA was found to induce insulin secretion when added alone, in addition to enhancing glucose-induced secretion by INS-1 cells (Fig. 30A).

Fig. 30 (next page). Effect of NGF, PMA, and glucose on insulin secretion by INS-1 cells. Monolayers of INS-1 cells were incubated for 30 min with glucose (A) at the concentration indicated, added either alone or together with 100 ng/ml NGF or 1 μM PMA, or with NGF (B), at the concentration indicated, together with glucose (15 mM). Thereafter, the medium was collected, and its insulin content was measured and expressed as percent of insulin secretion in response to 15 mM glucose. Data are means ± S.D. of four to eight observations.


A
Insulin secretion
(% of 15 mM glucose stimulation)
500
400
300
200
100
0
Glucose
PMA
NGF
0
5
10
15
Glucose (mM)
B
Insulin secretion
(% of 15 mM glucose stimulation)
220
200
180
160
140
120
100
80
0
50
100
150
500
NGF (ng/ml)

II-3-d. The role of $[Ca^{2+}]_i$ in the activation of MAP kinase by various stimuli

Next, association of changes in $[Ca^{2+}]_i$ with the MAP kinase activation was investigated. CPT-cAMP was found to transiently enhance glucose-induced Ca^{2+}-influx (Fig. 31A, 14 out of the 18 cells examined). Moreover, chelation of extracellular Ca^{2+} with EGTA inhibited by 70-90% the activation of MAP kinase by glucose added alone or together with CPT-cAMP (Table 9). Similarly, MAP kinase activation by these agents was attenuated by 30-60% by 20 μM verapamil (Table 9). EGTA inhibited the activation of MAP kinase by PMA by some 40 % (Table 9). These findings suggest that Ca^{2+}-influx is required for MAP kinase activation by glucose, whereas in the case of PMA, it contributes but is not essential for activation.

In the absence of glucose, NGF failed to raise $[Ca^{2+}]_i$ (Fig. 31C, 5 out of the 5 cells examined). Furthermore, activation of MAP kinase by NGF was not inhibited by EGTA (Table 9). Thus, NGF utilizes a Ca^{2+}-independent pathway to activate the MAP kinase cascade in INS-1 cells. In the presence of 15 mM glucose, however, NGF was found to induce $[Ca^{2+}]_i$ transients (Fig. 31B,C, 15 out of 17 cells examined). The NGF-induced rise in $[Ca^{2+}]_i$ was due to influx of extracellular Ca^{2+}, since it was abolished by EGTA (Fig. 31D, 18 out of 18 cells examined) and 20 μM verapamil (10 out of 10 cells examined, not shown). Note that reexposure to Ca^{2+} after withdrawal of EGTA caused a transient rise in $[Ca^{2+}]_i$ in the INS-1 cells (Fig. 31D).

Table 9. Effect of EGTA and verapamil of MAP kinase activation by various agents in INS-1 cells.

Stimulus	MAP kinase activity (% of activity in the absence of inhibitor)	
	+5 mM EGTA	+20 μM Verapamil
Glucose	11 ± 5 (5)	41 ± 10 (3)
Glucose + CPT-cAMP	26 ± 28 (3)	72 ± 11 (3)
PMA	63 ± 9 (3)	
NGF	97 ± 30 (3)	

INS-1 cells were incubated for 15 min with test agent (1 mM CPT-cAMP, 15 mM glucose, 1 μM PMA, or 500 ng/ml NGF as indicated) in the absence or presence of EGTA or verapamil added 2 min before the test agents. Thereafter, the cells were solubilized, and the kinase activity of immunopurified 44-kDa MAP kinase was measured and expressed as percent of the activity found in the absence of inhibitor of Ca^{2+} influx (=100%). The number of experiments (*n*), performed in triplicate, is given in parenthesis. Data are mean ± S.D.

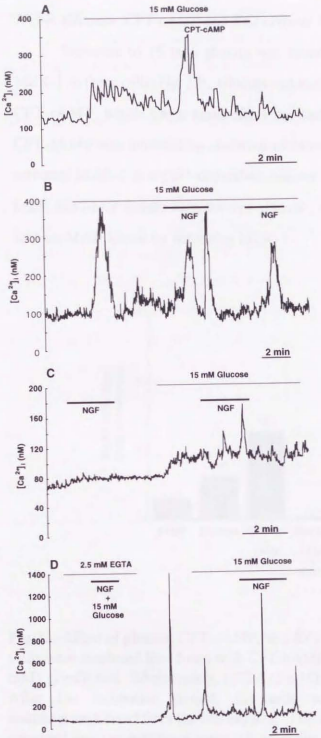


Fig. 31. Effect of glucose, CPT-cAMP, and NGF on $[Ca^{2+}]_i$, measured in single fura-2-loaded INS-1 cells. The basal superfusion medium contained 2.8 mM glucose in A, B, and D, but contained no glucose in C. As indicated by *bars*, glucose (at the concentration shown), CPT-cAMP (1 mM), NGF (100 ng/ml), or EGTA (2.5 mM) were added in a square-wave manner from a pipette close to the cell. Each trace was reproduced from 5 to 18 times with similar results as described in the text.

II-3-e. Glucose, CPT-cAMP and KCl activate MEK1 in INS1 cells

Exposure to 15 mM glucose was found to activate the MAP kinase activator MEK-1 in these cells (Fig. 32). Glucose-induced stimulation of MEK-1 was increased by CPT-cAMP, which had a small effect by itself. MEK-1 activation by glucose plus CPT-cAMP was inhibited by chelation of extracellular Ca^{2+} with EGTA. Finally, KCl activated MEK-1 in a Ca^{2+}-dependent manner. Thus, the similar activation pattern of MEK and MAP kinase suggests that glucose-, cAMP-, and Ca^{2+}-stimulated pathways activate MAP kinase by activating MEK-1.

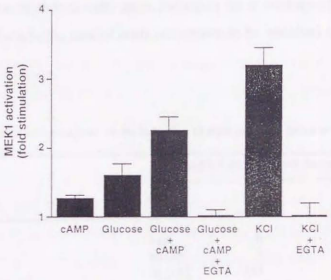


Fig 32. Effect of glucose, CPT-cAMP, and KCl activate MEK-1 activity in INS-1 cells. Cells were incubated for 15 min with CPT-cAMP (1 mM), glucose (15 mM), or KCl (24 mM) as indicated. When present, EGTA (5 mM) was added 2 min before the other agents. After the incubation period, the cells were solubilized, and MEK-1 was immunoprecipitated from the cell extracts. The activity of immunopurified MEK-1 was measured in a reconstitution assay by its ability to activate recombinant 44-kDa MAP kinase, the activity of which was measured using myelin basic protein as a substrate. The activity of MEK-1 is expressed as -fold stimulation compared with its activity in untreated INS-1 cells. Data are means ± S.D. of triplicate determinations. The experiment was performed three times with similar results.

II-3-f. Induction of early response genes in INS-1 cells

Finally, the induction of early response genes that code for transcription factors, *jun*B, *nur*77, and *zif*268, was investigated using the MAP kinase activators in INS-1 cells. Glucose caused no or very weak induction of either of the genes (Table 10). CPT-cAMP had a small effect on *jun*B and *nur*77. Glucose and CPT-cAMP added together, however, caused synergistic induction of all three genes, which in the case of *jun*B was markedly attenuated by EGTA, thus showing a correlation with MAP kinase activation by these agents. KCl, on the other hand, had only little effect on the expression of the early response genes, possibly due to the swift and transient action of KCl on INS-1 cells (confer Fig. 29), whereas gene induction was measured after 1 h of stimulation. NGF caused a small induction of the genes, most markedly of *zif*268. For comparison is shown early gene induction by a mixture of PMA, forskolin, and ionomycin, which in the case of *jun*B was shown to be inhibited by EGTA.

Table 10. Effect of various agents on the induction of early response genes in INS-1.

Condition	mRNA level (% over control)		
	*jun*B	*nur*77	*zif*268
Control	0 (0)	0	0
Glucose	40 (50)	0	0
CPT-cAMP	100 (120)	240	40
Glucose+CPT-cAMP	400 (130)	800	210
KCl	100 (60)	70	100
NGF	100 (80)	100	200
PMA+Forskolin+Ionomycin	600 (60)	900	240

Cells were incubated for 60 min with test agent (15 mM glucose, 1 mM CPT-cAMP, 40 mM KCl, 500 ng/ml NGF, 1 µM PMA, 5 µM forskolin, or 2 µM ionomycin as indicated. Data of *jun*B expression in the presence of 5 mM EGTA are given in parentheses. After the incubation period, cells were lysed, whereafter RNA was isolated and analyzed by Northern blotting. Blotted membranes were hybridized with *jun*B, *nur*77, *zif*268 and 18 S ribosomal ^{32}P-labeled RNA probes. Autoradiographs of probed membranes were quantitated using an Image Quant v3D densitometer. mRNA levels of *jun*B, *nur*77, and *zif*268 were normalized to those of the invariant 18 S ribosomal transcript and expressed as percent induction over that of untreated cells (control). Data are mean of two experiments.

DISCUSSION

I. Feasibility of INS-1 cells as a β-cell model

INS-1 cells could provide a useful experimental system from various aspects. This cell line retains many differentiated features of native β-cells. They secrete insulin in response to glucose in the physiological range with a parallel increase in cellular metabolism [32,84,89]. Their differentiated features, especially with respect to insulin secretory function, have been shown to be associated with the enzymatic profiles involved in cellular nutrient metabolism [32]. INS-1 cells, as observed in native β-cells, possess high mitochondrial glycerophosphate dehydrogenase activity and low lactate dehydrogenase activity, both of which could contribute to promote mitochondrial metabolism, thereby stimulating insulin secretion efficiently [32]. In addition, this study shows that this model system is useful, not only for studying the function, but also for investigating physiological mechanisms of the regulation of β-cell growth. It might be argued that the findings obtained in these transformed cells are not necessarily applicable to native β-cells. Nonetheless, the fact that INS-1 cells show DNA synthesis in response to various factors in a quite similar manner to that of native β-cells [7,34] indicates that identical signaling mechanisms are operative in this cell line for the physiological β-cell mitogens.

To specify the mode of action of individual factors, it is necessary to examine their effects in a defined condition. Serum replacement with various components has been tried for the maintenance of insulin secretory function of β-cells [94,139,140], and of insulinoma cell lines [108,109], although complete removal of serum from the culture media results in loss of insulin responsiveness to glucose [141,142]. Serum substitution has been found to preserve glucose-induced insulin secretion in islet cells [94,140]. The present study indicates that INS-1 cells can be propagated in SFM which maintains growth and the function of islet cells [94]. Cell growth as well as DNA synthesis of INS-1 cells cultured in SFM were comparable to those in CM. Among the components in SFM, PRL was found to be the most potent mitogen, because its elimination resulted in marked decrease in [^{3}H]thymidine incorporation. By contrast, DNA synthesis was not affected by the elimination of IGF-I, which is also a mitogen for β-cells [110]. The possibility of autocrine secretion of IGF-I by INS-1 cells can be excluded, since they do not express IGF-I mRNA [143]. Insulin secreted

into the culture medium could however elicit growth-promoting actions through IGF-I receptors [144], thereby masking the effect of IGF-I present in SFM. The final insulin concentration in SFM after a 3 day-culture was 62 ± 15 nmol/l (n=3), a concentration approaching those exerting IGF-I-like mitogenic effects [144]. Alternatively, IGF-II, which is expressed by INS-1 cells [143], might substitute for IGF-I as an autocrine growth factor.

Glucose also promotes growth of INS-1 cells, as is the case with native β-cells [7], although little information is available as to how glucose stimulates β-cell growth [145]. The possibility that secreted insulin elicited mitogenic effects cannot be excluded in this study. The effect of cAMP on DNA synthesis is complex [15,146]. The fact that metabolites of cAMP analogues could affect mitogenesis in variable ways makes interpretation difficult [15]. CPT-cAMP had no effect on DNA synthesis in low (3 mM) glucose medium, but inhibited high (15 mM) glucose-induced INS-1 cell DNA synthesis, whereas this inhibition was not seen with forskolin, raising the possibility that the effect of CPT-cAMP was not cAMP specific.

It has been shown that several growth factors which act through receptor tyrosine kinases stimulate β-cell replication [7]. In contrast to those previous reports [110,147], however, DNA synthesis in INS-1 cells was not affected by NGF, PDGF, and EGF. These findings could be explained by the difference in expression of their membrane-bound receptors. INS-1 cells have indeed shown to express little EGF receptors [148]. Only bFGF, which has been reported to stimulate the formation of islet-like cell clusters by human fetal pancreatic cells [149], had small stimulatory effect on DNA synthesis.

In conclusion, the serum-free culture of INS-1 cells is feasible for studying the effects of β-cell mitogens. Considering the advantage of using clonal cell line especially for signaling events, this experimental system is useful and convenient to investigate the mechanisms regulating growth and the differentiated function of insulin-secreting cells.

II. Factors maintaining insulin secretory function of INS-1 cells cultured in SFM

Despite the maintenance of growth and survival of INS-1 cells in SFM, insulin secretion in response to nutrient secretagogues, such as glucose, pyruvate, and leucine, was markedly impaired after a 3-day culture in this medium. By contrast, membrane depolarization with 30 mM K^+, which leads to Ca^{2+} influx through VDCC followed by stimulation of exocytosis of insulin granules, was still able to induce insulin release comparable to that of controls. It is now well established that nutrient secretagogues need to be metabolized to generate the signals that trigger insulin secretion [33,150]. The impaired insulin secretion could therefore be due to decreased cellular metabolism. Indeed, the MTT reduction rate was decreased during stimulation with pyruvate and leucine which are exclusively metabolized in the mitochondria. This suggests that oxidative metabolism is impaired in INS-1 cells after culture in SFM. This idea is strengthened by the finding that $^{14}CO_2$ production from both [3,4-^{14}C]glucose and [1-^{14}C]pyruvate was significantly diminished. It should be noted that MTT reduction stimulated by glucose was not altered in these cells. This can be explained by the enhanced generation of reducing equivalents in the cytosol [114], which might mask a reduction of oxidative metabolism in the mitochondria. Taken together, these results suggest that impaired mitochondrial metabolism contributes to the blunted glucose-induced insulin secretion following culture in SFM.

As expected from the attenuation of oxidative metabolism, the signaling events downstream from the generation of metabolic coupling factors [150,151] were also affected. Glucose thus failed to depolarize the plasma membrane potential, an action mediated by closure of K_{ATP}-channels [121]. However, the regulation of the K_{ATP}-channels was preserved, as revealed by: i) identical resting membrane potentials in control and SFM-cultured cells; ii) unaltered effects of diazoxide and tolbutamide, which respectively open and close K_{ATP}-channels [121]. $[Ca^{2+}]_i$ imaging revealed that the number of cells responding to glucose was markedly decreased by culture in SFM, whereas the response to high K^+ was unaffected. These results are consistent with the data on insulin secretion. Moreover, stimulation of PKA by forskolin plus IBMX as well as that of PKC by the phorbol ester PMA were less affected after treatment with SFM. Since a rise in $[Ca^{2+}]_i$ as well as activation of both the PKA and PKC pathways are known to act directly on exocytosis of insulin [125,152], these results indicate that this process is not impaired after culture in SFM.

Which are the factors that maintain the differentiated function of INS-1 cells? Serum contains component(s) that sustain the insulin secretory function, since the blunted glucose-induced insulin secretion was almost completely restored by a 3-day replenishment period. This phenomenon was not observed after 3 h or even 24 h of serum replenishment, suggesting that the underlying mechanism may be mediated by induction of genes involved in nutrient metabolism, most probably expressed in the mitochondria. The fact that the insulin secretory function of native islet cells is preserved in SFM, which has been reported by Clark and Chick [94] and confirmed in this study, indicates that the active principles are synthesized by islet cells. This contention is borne out by the restoration of glucose-stimulated insulin secretion in INS-1 cells treated with the media conditioned by islet cell monolayers or free-floating islets. This effect was not reproduced by HepG2, PC12 or endothelial cells, supporting the notion that the beneficial factor is of islet origin.

In an attempt to restore the insulin secretory function of INS-1 cells cultured in SFM, various factors were tested in the present study. Agents raising cAMP levels were of interest in this context, especially since glucagon secreted by α-cells has been shown to be required for glucose-induced insulin secretion from purified β-cells [116]. However, neither glucagon nor GLP-1 altered the insulin secretory function after long-term exposure of the cells. The combination of forskolin and IBMX augmented both basal and glucose-stimulated insulin release, most probably as a consequence of the increased insulin transcription rates [117]. In contrast to native β-cells [116], raising cAMP levels is not sufficient to keep INS-1 cells glucose-responsive. Supplementation of SFM with somatostatin, which is produced by δ-cells in islets, was also ineffective. The involvement of another islet derived inhibitory neurohormone, neuropeptide-Y [153], is also unlikely, in view of its expression in INS-1 cells [154].

NGF is present in fetal and neonatal mouse islets [155], and has been reported to induce neuron-like differentiation [156] and to increase expression of insulin and secretogranin II in RINm5F cells [157]. NGF, as well as PMA, but not GH, activate the MAP kinase pathway in INS-1 cells. However, none of these agents affected the insulin secretory function of INS-1 cells cultured in SFM, not even GH and bFGF which stimulate INS-1 cell proliferation. The results obtained with PMA should be interpreted with caution, since down-regulation of PKC may occur by the long-term treatment with PMA [158].

The morphological changes in SFM and the tendency to detach from could be overcome by coating the dish with attachment factors, such as poly-L-ornithine, poly-L-lysine, or ECM. These findings suggest that factors on the cell surface may become deficient after culture in SFM. Importance of attachment factors in maintaining secretory function has been claimed by several reports [94,159,160]. It has been shown in islet cells [160,161] and in human islets [159] that cell replication and insulin secretory function are better preserved by culturing on ECM derived from bovine corneal endothelial cells. Therefore in the present study, both Matrigel and cornea-derived ECM were tested instead of poly-L-ornithine. Matrigel failed to improve the insulin secretory function of INS-1 cells cultured either in SFM or in CM. On the other hand, the response was better when the cells were cultured on ECM only in CM, confirming the results of others in native β-cells [159-161]. Cell-to-cell communication via gap junctions has been shown to be important for normal glucose-induced insulin secretion in pancreatic islets [162]. The loss of glucose-stimulated insulin release after culture of INS-1 cells in SFM is, however, not due to a loss of cell-to-cell communication, as INS-1 cells lack gap junctions already when cultured in CM [163].

In conclusion, culture of the β-cell line INS-1 in the chemically defined serum substitute preserves proliferation and insulin biosynthesis as well as insulin secretion in response to K^+ depolarization. Therefore, the impairment of insulin secretion in response to glucose and other metabolizable secretagogues can not be explained by defective exocytosis of insulin, but rather by reduced mitochondrial metabolism. It appears that islet cells produce factors permissive to insulin secretion, as medium conditioned by such cells restored glucose-stimulated insulin release. Whether these components are the same as the permissive factors present in FCS remains to be established.

III. Effects and signaling mechanisms of GH and PRL in INS-1 cells

Among the factors tested in this study, GH and PRL are the most potent mitogenic factors of INS-1 cells. Moreover, these hormones maintained viability as well as insulin content of INS-1 cells cultured in SFM. It should be noted that both GH and PRL increased MTT reduction, which reflects the generation of reducing equivalents during nutrient metabolism [32] in agreement with the reported stimulation of glucose oxidation in neonatal islets by PRL [20]. Thus INS-1 cells respond like normal β-cells following exposure to these hormones [21,22,24,30].

INS-1 cells have been reported to express a high number of receptors with ligand affinity for human GH (hGH) comparable to that of rat hepatocytes [90]. hGH, however, binds to both somatogenic and lactogenic receptors [112], although the majority of the hGH binding sites are of lactogenic specificity [90]. Therefore, the effect of bGH, which specifically binds to somatogenic receptors, was also examined. The dose-response characteristics for hGH-, bGH- and PRL-stimulated [^{3}H]thymidine incorporation in INS-1 cells were comparable and show that these actions are exerted in the physiological hormone concentration range [164]. bGH and PRL appear to share common post-receptor signaling events in mitogenesis, as their effects were not additive.

Little is known about the post-receptor signaling mechanisms of GH and PRL in insulin-secreting cells. hGH, bGH and PRL all caused a marked but transient $[Ca^{2+}]_i$ rise in single INS-1 cells. With respect to hGH, the findings in this study are consistent with those reported recently in Chinese hamster ovary (CHO) cells expressing wild-type GH receptors and in the insulin-secreting cell line RIN 5AH [46], and with preliminary results in fetal rat islet cells [123]. The $[Ca^{2+}]_i$ rise elicited by hGH is due to Ca^{2+} influx, since it requires extracellular Ca^{2+}, and is abolished by treatment with verapamil. L-type VDCC gating is involved, since calciseptine, which blocks these channels with high selectivity, inhibited the hGH and bGH effects. This idea is further supported by the use of diazoxide which abolished the $[Ca^{2+}]_i$ response.

Considering that the onset of the rise in $[Ca^{2+}]_i$ is relatively slow (30-45 sec lag), it is unlikely that receptor-mediated activation of phospholipase C is involved. The strict dependence on Ca^{2+}-influx also indicates that GH and PRL do not generate the Ca^{2+}-mobilizing messenger inositol 1,4,5-trisphosphate. An increase in cAMP levels could explain the $[Ca^{2+}]_i$ rise, since GLP-1, forskolin and CPT-cAMP all raise

$[Ca^{2+}]_i$ with similar kinetics in INS-1 cells. cAMP, by activation of PKA, could cause phosphorylation of the α_1 and β subunits of L-type VDCC, as demonstrated in other cell types [165]. Alternatively, cAMP generation could promote membrane depolarization [166]. After pretreatment with Rp-cAMPS, the effects of hGH and GLP-1 on $[Ca^{2+}]_i$ were no longer observed, suggesting the involvement of cAMP in the $[Ca^{2+}]_i$ rise. The inhibition by Rp-cAMPS is not due to a non-specific effect, since Rp-cAMPS did not block the glucose-induced $[Ca^{2+}]_i$ rise. However, measurement of intracellular cAMP levels did not reveal any increase in response to hGH. The involvement of cAMP in hGH action can thus not be substantiated. The rise in cAMP levels might only be transient and escape detection under the experimental conditions in this study. It is of interest in this context that, in Leydig cells, activation of a cAMP-mediated pathway can occur after stimulation with luteinizing hormone and human chorionic gonadotropin without detectable changes in intracellular cAMP levels [167]. Further studies are required to clarify the role of cAMP in GH signaling. Taken together, the results in the present study are compatible with an effect of hGH on the gating of L-type VDCC, requiring permissive membrane potential levels, but not necessarily involving membrane depolarization. Finally, it remains to be clarified whether GH-induced Ca^{2+}-influx is secondary to other signaling events, and whether this event is associated with, or independent of JAK2 activation.

The evidence that GH is capable of raising $[Ca^{2+}]_i$ and, possibly, activating PKA suggested that GH might stimulate insulin secretion, since these two signaling events are known to augment exocytosis of insulin [125,168]. However, hGH failed to stimulate both basal and glucose-induced insulin secretion in perifused INS-1 cells. Rather, the presence of an inhibitory influence on insulin secretion is suggested, as the basal secretion was lowered by hGH, and an 'off ' response was observed by the simultaneous removal of hGH and high glucose. It may be speculated that hGH exerts two opposing (stimulatory and inhibitory) effects in INS-1 cells.

Much evidence has been obtained for the crucial role of Ca^{2+} in the mitogenesis of many cell types [169]. However, in INS-1 cells both verapamil and diazoxide, which blocked the hGH-induced $[Ca^{2+}]_i$ rise, failed to inhibit DNA synthesis stimulated by hGH during the 6-h incubation. These observations indicate that the $[Ca^{2+}]_i$ rise is not a prerequisite for the mitogenic effects of GH and PRL. The non-specific inhibition of GH- and PRL-induced DNA synthesis by verapamil has also been reported

in rat lymphoma cells, in which the PRL stimulation is indeed independent from extracellular Ca^{2+} [170]. Nonetheless, the rise in $[Ca^{2+}]_i$ has been implicated in some other effects of GH and PRL. For example, it has recently been shown that, in CHO cells expressing mutant GH receptors lacking the C-terminal domains, which are distinct from those required for JAK2 activation, GH promotes neither $[Ca^{2+}]_i$ elevation nor Spi 2.1 gene transcription [46]. In the same report, verapamil (at a concentration 10 times that used in this study) is shown to inhibit both $[Ca^{2+}]_i$ rise and insulin transcription induced by GH in RIN 5AH cells. These findings suggest that GH stimulates a Ca^{2+}-dependent pathway which leads to transcription of some genes including insulin [46]. It is thus possible that the effects of GH and PRL on the differentiated function of insulin-secreting cells, such as the regulation of insulin biosynthesis, require a $[Ca^{2+}]_i$ rise.

Tyrosine phosphorylation is a well-known mechanism implicated in signaling events of many hormones, growth factors [171] and cytokines [36], but information on its role in β-cell growth is limited [172,173]. Immunoblotting with anti-phosphotyrosine antibodies revealed that hGH, bGH and PRL promoted tyrosine phosphorylation of several proteins in INS-1 cells. One of these was identified as JAK2 tyrosine kinase by immunoprecipitation, which confirms previous reports in other cell types [39,40,53]. Although activation of JAK2 has already been shown in RIN-5AH cells transfected with an exogenous liver-type GH receptor [53], the finding obtained in INS-1 cells which express abundant intrinsic GH receptors [90] strongly suggests that the JAK2 tyrosine kinase pathway is implicated in the mitogenic effects of GH and PRL in native β-cells. Its presence has recently been demonstrated by immunohistochemical techniques in the nucleus of rat β-cells [174]. Moreover, another protein of ~90 kDa, which was also phosphorylated by the stimulation with GH and PRL, was found to be STAT5. Although three different STAT proteins (STAT1, STAT3, and STAT5) have been reported to be involved in GH signaling events [52,175,176], only the activation (i.e. tyrosine phosphorylation as well as the binding to DNA) of STAT5 could be demonstrated in INS-1 cells. Considering that the effects of GH and PRL demonstrated in INS-1 cells are almost identical, it is reasonable to speculate that the same molecules mediate their effects. In this context, it is likely that at least some of their actions in insulin-secreting cells are mediated by the activation of JAK2 followed by STAT5, but neither by STAT1 nor by STAT3, both of which are not

activated by PRL [37]. Lavendustin A abolished hGH-stimulated DNA synthesis, further supporting the idea that tyrosine phosphorylation cascades play a pivotal role in the mitogenic effect of GH.

Studies with mutated GH receptors have shown that the proline-rich juxta-membrane domain is required for both JAK2 and MAP kinase activation [54]. However, the absence of MAP kinase activation by hGH or PRL in INS-1 cells indicates that, at least in insulin-secreting cells, the MAP kinase pathway is not involved in the mitogenic action of the two hormones. A similar dissociation of MAP kinase activation from stimulation of mitogenesis has been reported for the effects of erythropoietin on a bone marrow cell line [177]. Furthermore, lack of MAP kinase activation by GH has been reported in IM9 lymphocytes, in which GH indeed activates JAK2 and STAT5, suggesting that GH stimulation of the MAP kinase pathway is cell type specific [178].

In contrast to the effects of GH or PRL, 10% FCS failed to stimulate JAK2 phosphorylation, suggesting that the mitogenic activity in FCS in this case is due neither to GH nor to PRL. Glucose, which also stimulates DNA synthesis in INS-1 cells, did not promote JAK2 tyrosine phosphorylation. Moreover, treatment with lavendustin A did not affect the glucose-stimulated DNA synthesis. These results suggest that the mechanisms underlying the mitogenic effects of glucose and FCS are different from those of GH and PRL. It remains to be clarified whether the mitogenic effects of glucose or FCS, both of which activate MAP kinase in INS-1 cells, are indeed mediated by this pathway.

Finally, the inhibition of hGH-induced DNA synthesis by Rp-cAMPS suggests that the basal activity of the cAMP pathway may be permissive to hGH-mediated mitogenesis, whereas agents that raise cellular cAMP levels have been reported both to increase and to decrease β-cell proliferation [7].

Despite the potent effects of GH and PRL on β-cells, it is not known whether these hormones play an essential role in the physiological regulation of growth and the function of these cells. No precise studies are available on the β-cell growth or development in situations lacking these hormones. On the contrary, expansion of the β-cell mass and the β-cell hyperplasia have been reported in pregnancy and tumoral hypersecretion of GH or PRL [8,9,24], where these hormones as well as placental lactogen may promote β-cell growth. It should be noted that these conditions are

associated with insulin resistance, suggesting that the increase in the insulin secretory capacity (i.e. β-cell mass and insulin biosynthesis) stimulated by the GH-related hormones might contribute, at least in part, to the compensation for the increased demand of insulin.

In conclusion, GH and PRL are able to maintain viability, to promote growth, and to stimulate insulin biosynthesis of INS-1 cells within the physiological concentration range, as noted in primary β-cells. Among the post-receptor signaling cascades of these hormones, protein tyrosine phosphorylation, especially that of JAK2 and STAT5, is considered to be a major event, in contrast to an increase in $[Ca^{2+}]_i$ which is not required for the stimulation of mitogenesis.

IV. Signaling mechanisms mediating the effects of IFN-γ in INS-1 cells

Cytokines such as IL-1β, TNF-α, and IFN-γ have been implicated in the destruction of β-cells in IDDM [73]. These cytokines, being produced by T lymphocytes, macrophages, and natural killer cells, may exert deleterious effects on β-cells as humoral mediators of the immunological process in IDDM [73,179,180,181]. IL-1β alone is able to induce β-cell dysfunction and destruction, of which underlying mechanisms have been extensively studied [132,181,182]. On the contrary, either IFN-γ or TNF-α alone has little effect on β-cell viability, but potentiates the cytotoxic effect of IL-1β [72,73,133,180,181,183]. Moreover, the combination of these two cytokines synergistically elicits cytotoxicity in β-cells [72,74,130,133,184-186]. It is therefore speculated that there may be cross talk among the signaling events activated by these cytokines so as to induce β-cell death.

The effects of IFN-γ in INS-1 cells demonstrated in this study are consistent with those reported in other insulin-secreting cells, especially human islet cells [130,185,186], in that IFN-γ alone inhibits glucose-induced insulin secretion, and, in combination with TNF-α, elicits β-cell cytotoxicity. This study shows that after a 24-h exposure to IFN-γ, INS-1 cells show blunted insulin secretion in response to the nutrient secretagogues, glucose and pyruvate, suggesting that the inhibition of MTT reduction is due to impaired mitochondrial metabolism. This is consistent with the finding that insulin secretion induced by membrane depolarization with high K^+ was unaffected by exposure to IFN-γ, which indicates that the machinery for insulin exocytosis is intact.

The inhibitory effect of IFN-γ appears to require induction of IFN-γ-regulated genes, because the impairment in insulin secretion was observed after 24-h, but not 30-min, of exposure to IFN-γ. It remains elusive whether this effect is associated with decreased activity of mitochondrial enzymes such as aconitase, as reported for IL-1β-induced inhibition of insulin release [16] or with any other mechanisms. Although NO has been implicated in the impaired mitochondrial metabolism induced by IL-1β [73,132], the finding that the iNOS inhibitor NMMA failed to restore the impaired insulin secretion by IFN-γ suggests that NO is not likely to be involved. This is further supported by the finding in this study that expression of iNOS mRNA was not induced by IFN-γ alone. Since neither nitrite production nor iNOS activity was not measured in this study, it cannot be denied that NO production is stimulated by IFN-γ alone, even though the expression of iNOS mRNA is not obvious. It has indeed been shown that 50 or 500 U/ml IFN-γ alone increases iNOS activity in rat β-cells as well as nitrite levels in the culture media [74]. Nevertheless, recent studies rather limit the involvement of NO in the cytokine-induced β-cell dysfunction [74,185,186]. Thus the inhibitory effect of IFN-γ on nutrient-induced insulin secretion cannot totally explained by the production of NO. It seems possible that generation of oxygen free radicals partially mediates the inhibition of insulin secretion by IFN-γ, since the addition of BHA or quercetin appears to slightly restore nutrient-induced insulin secretion. The effect is, however, not significant, indicating that oxygen free radicals play no major role in IFN-γ-induced β-cell dysfunction, as suggested by the previous reports [73].

As has been already shown in other insulin-secreting cells [72,74,185,186], the combination of IFN-γ and TNF-α elicited cytotoxicity in INS-1 cells, although each cytokine alone had no effect. This effect was observed in parallel with the induction of iNOS mRNA, which has been implicated in the cytokine-induced cytotoxicity of β-cells [73,179,132]. Thus, to clarify the underlying mechanisms of the synergistic effect by the two cytokines, signaling events which could be involved in the induction of iNOS were investigated. The expression of iNOS gene may be regulated by the cytokine-activated transcription factors such as STAT1, IRF-1, and NF-κB [77,105,136,187,188]. In fact, the promoter of iNOS gene contains binding-sites for NF-κB, as well as those for IFN-γ-responsive transcription factors [77]. Although activation of NF-κB [189-192] as well as induction of IRF-1 [78,193] have also been shown in insulin-secreting cells, the role of these factors for the synergistic induction of

iNOS by the cytokines remains unclear. Similarly, a requirement of an IFN-γ-activated transcription factor IRF-1 for the synergistic induction of iNOS by IFN-γ and LPS has also been reported [188]. It has been shown in insulin-secreting cells that IL-1β activates NF-κB in insulin-secreting cells [189-192], and that the iNOS expression induced by IL-1β is reduced by pyrrolidine dithiocarbamate, a potent inhibitor of NF-κB [191]. Similarly, induction of IRF-1 mRNA as well as that of iNOS by IL-1β can be prevented by nicotinamide [78], suggesting that IRF-1 is also an essential element for the induction of iNOS in β-cells. The induction of IRF-1 is thought to be regulated by STAT1 [194], which has itself been implicated in the antiproliferative effect of IFN-γ [75].

The present study shows that IFN-γ promotes tyrosine phosphorylation of STAT1 and the DNA-binding of nuclear proteins to IRF-1 GAS, the specific binding site of STAT1. These results suggest that IFN-γ activates STAT1, which binds to IRF GAS to induce IRF protein in INS-1 cells, and support the notion that IFN-γ-induced expression of IRF-1 is mediated by STAT1 [194]. Moreover, it is shown that IFN-γ increases the DNA-binding of nuclear proteins to iNOS GAS in INS-1 cells. STAT1 may thus contribute to iNOS induction both directly or indirectly (i.e. via induction of IRF-1). This is the first demonstration of STAT1 activation by IFN-γ in insulin-secreting cells. STAT1 is believed to be activated through the activation of both JAK1 and JAK2 by IFN-γ [37,71]. In INS-1 cells, however, only JAK1, but not JAK2, was found to be phosphorylated by IFN-γ. The finding favors the notion that other kinases can substitute for JAK2 in signal transduction of IFN-γ [195], and suggests that IFN-γ might activate JAK kinases in a cell-type-specific, but not in a ligand-specific manner. In this respect, it is noteworthy that GH, which exerts rather beneficial effects on β-cells, activates JAK2 and STAT5 [196 and this study] in insulin-secreting cells. Thus GH and IFN-γ could share the identical signaling molecules, such as JAK2, insulin receptor substrate (IRS)-1, and IRS-2 [50]. This should not necessarily be the case in β-cells, where these two factors elicit opposite effects. In fact, this study using INS-1 cells indicates that the receptor of IFN-γ and that of GH couple distinctive JAK kinases divergently leading to activation of STAT1 and STAT5, respectively, in β-cells.

The activation of STAT1 by IFN-γ alone, however, is not sufficient for iNOS induction in INS-1 cells. Moreover, STAT1 binding is not affected by TNF-α, suggesting that at least another transcription factor is required for iNOS induction.

TNF-α alone is capable of stimulating the DNA-binding of NF-κB, albeit less than that by the combination of IFN-γ plus TNF-α, but fails to induce iNOS. Such discrepancy between NF-κB activation and iNOS induction may be consistent with the finding by Kwon et al. that IL-1β-induced iNOS expression is completely blocked by the inhibitors of NF-κB despite the partial inhibition of NF-κB activation [189]. They speculate that there exists a threshold level of NF-κB required for iNOS induction. This could explain the finding that the expression of iNOS gene was observed in association with the augmentation of TNF-α-induced NF-κB activation by IFN-γ. Thus, one possible mechanism mediating the synergistic induction of iNOS is such a "full" activation of NF-κB by the combination of IFN-γ plus TNF-α. Similar synergism at the level of NF-κB activation has been reported in PC12 cells [197], but not in other cells [197,198], for the combination of IFN-γ plus TNF-α. Thus this phenomenon may be specific to limited types of cells. The synergistic effects of cytokines in β-cells have also been studied using IL-1β and IFN-γ [183,193]. It has been shown that IFN-γ increases the sensitivity of pancreatic β-cells for iNOS expression induced by IL-1β by increasing the stability of iNOS mRNA [183], while IL-1β-induced NF-κB binding is not increased by IFN-γ [193]. It should be noted, however, that the NF-κB binding stimulated by IL-1β alone has been shown to be somewhat less than that by the combination of IL-1β, IFN-γ, and TNF-α in human islets [192]. This suggests that there may be an interplay among these three cytokines to augment NF-κB activation.

The underlying mechanism of the synergistic activation of NF-κB by IFN-γ plus TNF-α is unclear. It remains elusive whether the augmentation of TNF-α-induced NF-κB by IFN-γ is due to the modification (e.g. phosphorylation) of IκB, which binds NF-κB in a latent form [199]. The recent report [197] has shown that cotreatment of PC12 cells with IFN-γ and TNF-α leads to persistent activation of NF-κB via a mechanism that involves the induced degradation of IκB. Another explanation may be that the concurrent binding of STAT1/IRF-1 and NF-κB, which are independently activated by each cytokine, is required to induce iNOS in INS-1 cells. It is unknown whether any other IFN-γ-activated factors than STAT1 and IRF-1 are also required for the induction of iNOS.

In conclusion, this study provides new information in terms of IFN-γ signaling in insulin-secreting cells: 1) IFN-γ activates JAK1 and STAT1, thereby inducing its binding to GAS in the promoter of both IRF-1 gene and iNOS gene, 2) IFN-γ augments TNF-α-induced NF-κB binding. Either of these events may mediate the synergistic induction of iNOS by IFN-γ and TNF-α. Although this study does not substantiate the involvement of NO in the cytotoxic effects of these cytokines, it may be speculated, at least, that similar mechanisms are conducted both for iNOS induction and for the cytotoxicity in β-cells. Finally, it remains to be clarified whether the impairment in insulin-secretion by IFN-γ is mediated by STAT1 or other transcription factors.

V. Implication of the MAP kinase cascade activation in INS-1 cells

The present findings in INS-1 cells suggest that three major signaling pathways employed by nutrient and hormonal secretagogues, *i.e.* the Ca^{2+}-, cAMP-, and PKC-stimulated pathways, converge on the MAP kinase cascade in the β-cell. Because of a high degree of cross-talk among signaling pathways in this cell type, it is difficult to dissect the mechanism by which any given secretagogue activates MAP kinase. For instance, the results of this study indicate that Ca^{2+} influx is a prerequisite for glucose-induced activation of MAP kinase. Subsequent to Ca^{2+} influx, however, divergent pathways, *e.g.* PKC or Ca^{2+}/calmodulin-dependent kinase II [200], may propagate the signal(s) leading to activation of MAP kinase. In the case of PMA, Ca^{2+}-dependent as well as -independent pathways contribute to the activation of MAP kinase. The Ca^{2+}-independent mechanism may involve direct phosphorylation and activation of Raf-1 by PKC [201]. Increased levels of cAMP *per se* have little effect on MAP kinase in INS-1 cells, in contrast to a large potentiating effect on MAP kinase activation by glucose as well as by PMA or NGF [M. Frödin and E. Van Obberghen, personal communication]. The potentiation by cAMP of glucose-induced stimulation of MAP kinase may in part be due to cAMP enhancement of Ca^{2+} influx. However, cAMP was able to potentiate MAP kinase activation by NGF in the presence of EGTA [M. Frödin and E. Van Obberghen, personal communication], suggesting that cAMP acts also at a point distal to Ca^{2+} influx to enhance MAP kinase activation. The results of this study suggest that glucose as well as the cAMP- and Ca^{2+}-stimulated pathways act at least at the level of MEK to activate MAP kinase in INS-1 cells. Upstream of MEK, multiple divergent pathways for activation of MAP kinase may be operating. It will be of particular

interest to determine whether $[Ca^{2+}]_i$ activates the MAP kinase cascade through Ras in INS-1 cells, as has been shown in PC12 cells [202].

In smooth muscle cells [203], adipocytes [204], CHO cells [204], and fibroblasts [205-207], cAMP inhibits the activation of MAP kinase by external stimuli, whereas only in PC12 cells cAMP has so far been shown to stimulate MAP kinase, acting, at least partly, at the level of MEK [208]. cAMP inhibits activation of the MAP kinase cascade by interfering with Ras activation of several MAP kinase kinase kinases, including Raf-1, B-Raf, and 98-kDa MEK kinase, and this inhibitory mechanism(s) seems to operate even in PC12 cells [67,68,70,206], leaving the question, how cAMP activates MEK and MAP kinase, unresolved. The finding that cAMP stimulates MEK and MAP kinase also in INS-1 cells suggests that cAMP activation of the MAP kinase cascade is a more widely occurring response than previously believed.

INS-1 cells and other insulin-secreting cell lines express two types of NGF receptors, the trkA receptor tyrosine kinase and the p75 NGF receptor ($p75^{NGFR}$) [209]. In PC12 cells, trkA-mediated activation of Ras constitutes a major pathway by which NGF activates MAP kinase. The role of $p75^{NGFR}$ in NGF signaling is controversial, but this receptor may stimulate cAMP synthesis in PC12 cells [210], thereby generating additional signaling pathways for activation of MAP kinase in this cell type [208] and possibly also in INS-1 cells. The data of this study show that in INS-1 cells, Ca^{2+} influx is dispensable for MAP kinase activation by NGF. By contrast, the inability of NGF to stimulate insulin secretion by these cells in the absence of glucose probably relates to its failure to stimulate Ca^{2+} influx under these conditions. The mechanism by which NGF potentiates glucose-induced insulin secretion remains to be established. Enhancement of Ca^{2+} influx, activation of MAP kinase, or generation of cAMP are possible mechanisms. The physiological importance of NGF as an insulin secretagogue is unclear, as it was not detected in islets of adult mouse [155]. Furthermore, long-term (3 days) exposure of INS-1 cells to NGF did not alter responsiveness to glucose or insulin production.

The finding that MAP kinase is activated by major secretagogue signaling pathways, some of them acting synergistically, and the close correlation between MAP kinase activation and insulin secretion in response to some secretagogues suggest that MAP kinase may regulate the secretory function of β-cells. MAP kinase activation, however, was clearly insufficient for secretion, since NGF activated MAP kinase

without stimulating secretion in the absence of glucose. cAMP-dependent protein kinase activation, however, is also insufficient for secretion but nevertheless believed to play an important role by potentiating glucose-induced insulin release [125,168]. Similarly, MAP kinase could have a modulatory function in secretion. Alternatively to a role in the stimulus-secretion coupling mechanism, MAP kinase may regulate secretion-related processes, such as glucose metabolism or insulin synthesis, possibly at the transcriptional level. Although secretagogues induce the transcription of genes implicated in β-cell function, many regulatory pathways remain unknown [211,212]. The induction of early response genes *jun*B, *nur*77, and *zif*268 was correlated with activation of MAP kinase by glucose and CPT-cAMP, suggesting that MAP kinase may mediate secretagogue regulation of these genes.

The results with [^{3}H]thymidine incorporation in this study, however, show no good correlation between the MAP kinase activation and DNA synthesis: 1) the effect of glucose and of increased cAMP on DNA synthesis was not well correlated with the effect of these agents on MAP kinase activity; 2) GH and PRL were efficient mitogens, although these factors did not activate MAP kinase; 3) NGF did not stimulate DNA synthesis, despite being a strong activator of MAP kinase in these cells. Thus these results do not support a role of the MAP kinase pathway in INS-1 cell proliferation. Nevertheless, the possibility that MAP kinase mediates glucose- and secretagogue-stimulated proliferation of normal β-cells [7] cannot be totally ruled out. Alternatively, MAP kinase might regulate β-cell differentiation. In this respect, NGF has been proposed to be implicated in the development of the endocrine pancreas [209], where evidence for its presence has been obtained in fetal and neonatal mouse [155]. Moreover, bFGF, which promotes islet development *in vitro* [149], efficiently stimulates (10-20-fold) MAP kinase in INS-1 cells [M. Frödin and E. Van Obberghen, personal communication]. A role of MAP kinase in β-cell differentiation could imply that secretagogues may also regulate this process.

Finally, the observation that glucose stimulates MEK and MAP kinase in INS-1 cells adds a nutrient to the list of extracellular signals that activate the MAP kinase cascade, illustrating further the versatility of this signal transduction pathway in multicellular organisms.

SUMMARY

1.An experimental model system using INS-1 cells was established to investigate mechanisms involved in the regulation of growth and the function of pancreatic β-cells. INS-1 cells not only secrete insulin in response to the physiological secretagogues of insulin, but also respond as observed in native β-cells to various factors affecting their growth and function.

2. INS-1 cells cultured in SFM showed impairment in insulin secretion due to decrease in mitochondrial metabolism, although their growth rate was comparable to that in the serum-containing medium. The impaired insulin secretion in SFM could be restored by (a) unknown factor(s) derived from pancreatic islets.

3. GH and PRL, both of which stimulated growth and insulin biosynthesis of INS-1 cells, increased Ca^{2+} influx through L-type VDCC, and activated JAK2 as well as STAT5. The latter event may be essential, whereas the former is not a prerequisite, for the mitogenic effect of both hormones.

4. IFN-γ inhibited glucose-induced insulin secretion through impairment in mitochondrial metabolism, and, in combination with TNF-α, induced iNOS expression leading to severe cytotoxicity in INS-1 cells. IFN-γ activated STAT1, and potentiated TNF-α-induced activation of NF-κB, which might play a role in the synergistic induction of iNOS by both cytokines.

5. Various insulin secretagogues, which raise $[Ca^{2+}]_i$, increase cAMP levels, or activate PKC, were found to activate MAP kinase in INS-1 cells. The activation of MAP kinase itself is not sufficient for stimulation of insulin secretion, but might modulate intracellular events leading to insulin secretion. No clear relationship was demonstrated between stimulation of growth and the MAP kinase activation in INS-1 cells.

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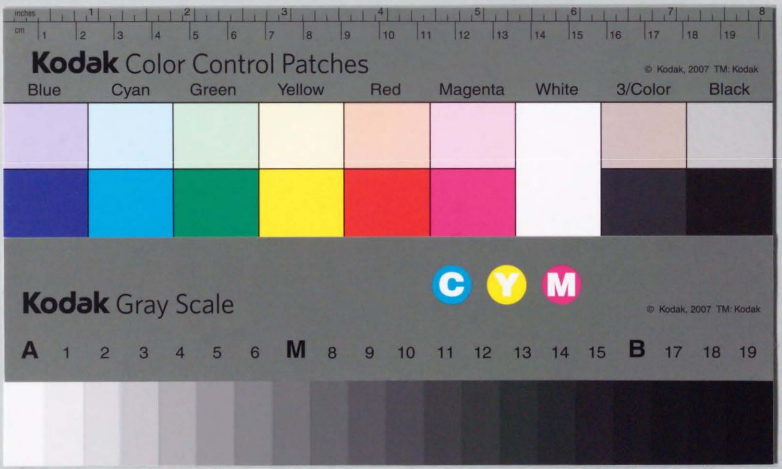
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