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Regulation of Insulinoma Cell Proliferation and Insulin Accumulation by Peptides and Second Messengers

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The regulation of clonal rat insulinoma (RINm5F) cell proliferation and hormone accumulation was investigated with the aim of identifying putative compounds capable of inducing differentiation, *i.e.* decreased growth and increased insulin accumulation, by the tumor cells. In particular, interest was focused on the role of a number of peptides as well as pharmacological probes modulating various signal transduction systems and which have been shown to regulate normal ß-cell proliferation and insulin accumulation. Growth hormone stimulated insulin accumulation and inhibited DNA synthesis, whereas galanin and insulin-like growth factor I caused a moderate suppression of insulin accumulation but did not affect proliferation, while epidermal growth factor, transforming growth factor ß, platelet-derived growth factor, acidic and basic fibroblast growth factor, bradykinin and somatostatin were virtually inactive on all parameters tested. Exogenous prostaglandins E_2 and $F_{1\alpha}$ were inactive, while the cycloxygenase inhibitor indomethacin slightly suppressed insulin accumulation. The cytokine IL-1ß caused a significant decrease in both ß-cell mitogenesis and insulin accumulation, effects that were mediated through nitric oxide generation. The vitamin A derivative retinyl acetate slightly inhibited serum-stimulated DNA synthesis, but did not affect insulin accumulation. The vitamin E α-tocopherol significantly enhanced insulin release but did not affect mitogenesis. By contrast, γ-tocopherol was inactive on both these parameters. The α-adrenergic agonist clonidine evoked a slight inhibition of serum-stimulated DNA synthesis, without influencing insulin accumulation, whereas phenylephrine did not affect any of these parameters. Carbamylcholine increased insulin accumulation, but not cell proliferation, whereas the adenylyl cyclase activator forskolin suppressed mitogenesis but did not affect insulin accumulation. Inhibition of protein kinase C with staurosporine or prolonged treatment with phorbol ester suppressed DNA synthesis, as did the tyrosine kinase inhibitor genistein. Stimulating Ca^{2+} influx by closing ATP-dependent K^+ channels with glibenclamide enhanced DNA synthesis, while opening of these channels with diazoxide suppressed cell growth. Conversely, preventing Ca^{2+} influx by the Ca^{2+} channel antagonist D-600, chelating intracellular Ca^{2+} by fura-2 AM or inhibiting the Ca^{2+}/calmodulin-dependent protein kinase by calmidazol resulted in a decreased DNA synthesis. On the other hand, uncontrolled influx or mobilization of Ca^{2+} by ionomycin or thapsigargin resulted in an arrested DNA synthesis.
The present paper shows that RINm5F insulinoma cell proliferation and insulin accumulation can be modulated by various peptidergic and pharmacological agents regulating certain signal transduction pathways. However, mitogenesis in the insulinoma cells seemingly is controlled in a vastly different manner in comparison to that in normal ß-cells. The most spectacular finding in this screening study, *i.e.* that growth hormone, contrarily to its effect on normal ß-cells, suppresses insulinoma cell growth, merits further elucidation of the underlying mechanisms. Possibly the hormone might become of utility in a clinical setting in the treatment of patients with insulin-producing tumors.

INTRODUCTION

The clonal insulin-producing cell line RINm5F has frequently been used as a model for studies concerning regulation of insulin accumulation (24,48,55). The usefulness of the cell line is, however, severely limited by its tumor-like properties; in particular the rapid proliferation rate, low insulin content and lack of a normal glucose-sensitive insulin release (48). In this paper we aimed at identifying putative compounds capable of inducing differentiation, *i.e.* decreased growth and increased insulin accumulation, by the tumor cells. In particular, interest was focused on the role of a number of peptides as well as pharmacological probes modulating various signal transduction systems. Possible therapeutic strategies in treatment of insulinoma will be discussed in the light of differences in the regulation of cell proliferation and insulin accumulation between the tumoral RINm5F cells and native pancreatic ß-cells.

MATERIALS AND METHODS

Materials

Human recombinant growth hormone (GH) was kindly given by Dr. Anna Skottner, Pharmacia Corp., Stockholm, Sweden. Prostaglandins were generously supplied by the Upjohn Company, Kalamazoo, MI. D-600 was a generous gift from Knoll A.G., Ludwigshafen-am-Rhein, Germany, while glibenclamide was kindly given by Hoechst A.G., Frankfurt, Germany. Diazoxide was graciously donated by Schering Corp., Kenilworth, NJ. Upstate Biotechnology, Lake Placid, NY, supplied acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor ß (TGF-ß) and bradykinin. L-arginine, thrombin, indomethacin, clonidine, phenylephrine, carbamylcholine, heparin, somatostatin, N^G-methyl-L-arginine (NAME), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), ionomycin, fura-2 AM, calmidazol, retinyl acetate, tocopherols, adenosine trisphosphate (ATP), cytidine trisphosphate (CTP) and guanosine trisphosphate (GTP) were from Sigma Chemical Co, St Louis, MO. Culture media BME (basal medium Eagle) and DME (Dulbecco's modified Eagle's medium) were delivered by Sigma or Gibco (Grand Island, NY), the latter also supplying recombinant murine interleukin-1ß (IL-1ß), while fetal calf serum (FCS) was purchased from Hyclone Laboratories, Logan, UT. KCl and $CaCl_2$ were from Fisher Scientific, Pittsburgh, PA, while forskolin, genistein and staurosporine were purchased from Calbiochem, La Jolla, CA.

Culture of RINm5F cells

RINm5F cells (48,55) were cultured at 37 °C in medium BME or DME containing 25 µg/ml gentamycin sulfate and 10 % fetal calf serum. For subcultivation, cells were

dislodged from culture dishes by a brief incubation with trypsin/EDTA in sterile PBS. For experiments involving test compounds, we utilized the same protocol as previously reported (43,54), *i.e.* an initial 2-day culture period of newly trypsinized cells in BME or DME containing 10 % fetal calf serum without test compounds. The medium was then changed to BME or DME containing 1 % or 10 % fetal calf serum and the desired additions of test compounds, and cultures extended for another 24 h. This procedure resembles that we usually employ for normal islet ß-cells; furthermore, the different test substances used herein were added at the same concentrations previosly used in experiments involving normal ß-cells.

RINm5F cell proliferation and hormone accumulation

RINm5F cells were cultured for 2 days in medium DME containing 10 % FCS without additions. New media containing 1 % or 10 % FCS and supplemented as above were then added and cultures extended for another 24 h. During the final 60 min, 0.1 µCi/ml of [^{3}H]thymidine was present in media and DNA synthesis assessed by measuring incorporation of radioactivity into DNA. The amount of insulin accumulating in the culture media during the last 24 h of culture was determined radioimmunologically (43). Since no quantitation was made regarding the number of remaining and viable cells after the 24 h culture period, the amount of insulin detected needs not necessarily reflect solely exocytotically released hormone. On the other hand, since RINm5F cellular insulin content is merely ≈ 1 % of that of native ß-cells (48), and the fractional release (*i.e.* insulin released related to cellular hormone content) of the RINm5F cells is approximately 10 % per hour (as opposed to 1 % in normal ß-cells), it seems likely that uncontrolled release of insulin would contribute comparably little to the total amount of accumulating hormone. Additionally, DNA synthesis rates represent transients that ultimately will lead to corresponding changes in cell number; however, ideally cell number would be quantitated directly or more accurately assessed by measurements of the DNA content of the well.

RESULTS

The effects of various test compounds on RINm5F cell DNA synthesis are shown in Tables 1 & 3, while effects on insulin accumulation are shown in Tables 2 & 4. It was found that GH stimulated insulin accumulation and inhibited DNA synthesis in 1 % serum, but was inactive in 10 % serum. Of other peptides screened, galanin and IGF-I caused a moderate suppression of insulin accumulation in 10 % serum but did not affect proliferation, whereas EGF, TGF-ß, PDGF, aFGF, bFGF, bradykinin and somatostatin were inactive on all parameters tested at both serum concentrations, except for a slight stimulation of insulin accumulation in 1 % serum by a heroic

concentration of PDGF.

Exogenous prostaglandins E_2 and $F_{1\alpha}$ were inactive, while the cycloxygenase inhibitor indomethacin slightly suppressed insulin accumulation in 10 % serum.

TABLE 1. Modulation of RINm5F cell proliferation.

	DNA synthesis (% of control)	
Cell culture	1 % FCS	10 % FCS
Control	100	100
Ionomycin (1 μM)	18.1 ±5.8*	53.0 ±0.8**
Thapsigargin (2 μM)	4.6 ±2.3*	7.4 ±3.9*
Fura 2-AM (5 μM)	91.9 ±14	83.2 ±1.1***
IL-1ß (25 U/ml)	51.2 ±7.1**	63.8 ±7.4*
NAME (1 mM)	103 ±16	95.6 ±3.1
IL-1ß + NAME	97.1 ±12	105 ±8.1
α-tocopherol (30 μM)	99.0 ±12	88.8 ±4.8
γ-tocopherol (30 μM)	108 ±12	92.0 ±5.9
Retinyl acetate (1 μM)	96.5 ±13	90.0 ±2.3*
PDGF (10 ng/ml)	96.2 ±1.9	99.7 ±1.9
IGF-I (100 ng/ml)	97.5 ±4.8	96.8 ±1.6
PDGF + IGF-I	95.1 ±1.1	97.2 ±2.0
aFGF (40 ng/ml)	97.5 ±2.5	92.1 ±3.6
bFGF (40 ng/ml)	94.7 ±4.0	90.5 ±4.2
Bradykinin (5 μM)	108 ±11	95.2 ±3.5

RINm5F cells were cultured for 2 days in medium DME containing 10 % FCS without additions. New media containing 1 % or 10 % FCS and supplemented as above were then added and cultures extended for another 24 h. During the final 60 min, 0.1 μCi/ml of [^{3}H]thymidine was present in media and DNA synthesis assessed by measuring incorporation of radioactivity into DNA. Values are mean percent of controls ±SEM for 4 experiments. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ for chance differences *vs.* controls using Student's paired *t*-test. Control values were for 1 % FCS 44,974 ±15,835 cpm/well, and for 10 % FCS 71,095 ±9,843 cpm/well. Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FCS, fetal calf serum; IGF-I, insulin-like growth factor I; IL-1ß, interleukin-1ß; NAME, N^G-methyl-L-arginine; PDGF, platelet-derived growth factor.

Extracellularly applied ATP and CTP decreased, whereas GTP increased, DNA synthesis. CTP also caused a small decrease in serum-stimulated insulin

accumulation. The α-adrenergic agonist clonidine evoked a slight inhibition of serum-stimulated DNA synthesis, without influencing insulin accumulation, whereas phenylephrine did not affect any of these parameters. The muscarinic receptor agonist carbamylcholine increased insulin accumulation, but not cell proliferation, whereas the adenylyl cyclase activator forskolin suppressed mitogenesis but did not affect insulin accumulation at any serum concentration. Inhibition of protein kinase C with staurosporine or prolonged treatment with phorbol ester suppressed DNA synthesis, as did the tyrosine kinase inhibitor genistein.

TABLE 2. Modulation of RINm5F cell insulin accumulation.

	Insulin accumulation (% of control)	
Cell culture	1 % FCS	10 % FCS
Control	100	100
Ionomycin (1 μM)	105 ±25	90 ±20
Thapsigargin (2 μM)	30 ±0.1***	35 ±5*
Fura 2-AM (5 μM)	122 ±25	102 ±20
α-tocopherol (30 μM)	143 ±4.8**	97 ±11
γ-tocopherol (30 μM)	110 ±23	100 ±20
Retinyl acetate (1 μM)	160 ±45	92 ±6
IL-1ß (25 U/ml)	42 ±6**	62 ±8**
NAME (1 mM)	98 ±12	98 ±27
IL-1ß + NAME	102 ±13	106 ±21
PDGF (10 ng/ml)	105 ±25	110 ±20
IGF-I (100 ng/ml)	107 ±21	72 ±7.5*
PDGF + IGF-I	120 ±10	95 ±5
aFGF (40 ng/ml)	112 ±18	78 ±19
bFGF (40 ng/ml)	123 ±50	117 ±19
Bradykinin (5 μM)	135 ±40	130 ±14

RINm5F cells were cultured for 2 days in medium DME containing 10 % FCS without additions. New media containing 1 % or 10 % FCS and supplemented as above were then added and cultures extended for another 24 h. The amount of insulin accumulation in culture media over the last 24 h of culture was analyzed radioimmunologically. Values are mean percent of controls ±SEM for 4 experiments. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ *vs.* controls using Student's paired *t*-test. Control values were for 1 % FCS 5,402 ±781 μU/ml, and for 10 % FCS 10,326 ±1,664 μU/ml. Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FCS, fetal calf serum; IGF-I, insulin-like growth factor I; IL-1ß, interleukin-1ß; NAME, N^G-methyl-L-arginine; PDGF, platelet-derived growth factor.

Stimulating Ca^{2+} influx by closing ATP-dependent K^+ channels with glibenclamide enhanced DNA synthesis, while opening of these channels with diazoxide suppressed cell growth. Conversely, preventing Ca^{2+} influx by the Ca^{2+} channel blocker D-600, chelating intracellular Ca^{2+} by fura-2 AM or inhibiting the Ca^{2+}/calmodulin-dependent protein kinase by calmidazol resulted in a decreased DNA synthesis. On the other hand, sustained, uncontrolled influx or mobilization of Ca^{2+} by ionomycin or thapsigargin resulted in an arrested DNA synthesis.

The cytokine IL-1ß caused a significant decrease in both ß-cell mitogenesis and insulin accumulation at both serum concentrations. When the nitric oxide synthase inhibitor NAME was added along with IL-1ß, the inhibitory actions of the cytokine were reversed, indicating that IL-1ß functions specifically through nitric oxide generation. By contrast, NAME itself was inactive.

The vitamin A derivative retinyl acetate slightly inhibited serum-stimulated DNA synthesis, but did not affect insulin accumulation. The vitamin E α-tocopherol significantly enhanced insulin release at low serum concentrations but did not affect mitogenesis. By contrast, γ-tocopherol was inactive on both these parameters at any serum concentration. Thrombin inhibited, while heparin stimulated, DNA synthesis in 1 % serum but neither of the agents affected insulin accumulation.

DISCUSSION

Compelling evidence now indicates that insulin accumulation is closely regulated by different intracellular signaling systems, *i.e.* phosphoinositide hydrolysis, increases in cytosolic Ca^{2+} and cyclic AMP generation (14,17,27,66,67). The mechanisms controlling ß-cell proliferation remain less well understood; however, they in many aspects resemble those that control insulin accumulation (reviewed in [15,16,51,53,65]).

Influx of Ca^{2+} through ion channels is crucial in glucose-stimulated insulin release (39). Similar Ca^{2+} fluxes have described early in mitogenic signaling (31,40,63). In the presently studied insulinoma cells, a number of agents were used to mimick or block such Ca^{2+} transients. Thus, stimulating Ca^{2+} influx by closing ATP-dependent K^+ channels with the hypoglycemic sulfonylurea glibenclamide (39) enhanced DNA synthesis, while opening of these channels with diazoxide suppressed cell growth. Conversely, preventing Ca^{2+} influx by the Ca^{2+} channel antagonist D-600, chelating intracellular Ca^{2+} by fura-2 or inhibiting the Ca^{2+}/calmodulin-dependent protein kinase by calmidazol resulted in a decreased DNA synthesis, Taken together, these data would favor an important stimulatory role for Ca^{2+} in RINm5F cell growth. On the other hand, uncontrolled Ca^{2+} elevation by ionomycin or thapsigargin resulted in an arrested DNA synthesis.

TABLE 3. Regulation of DNA synthesis by RINm5F cells.

	DNA synthesis (% of control)	
Cell culture	1 % FCS	10 % FCS
Control	100	100
GH (1 μg/ml)	55.4 ±2.6 (8)***	96.5 ±3.1 (8)
EGF (100 ng/ml)	105 ±4.0 (8)	100 ±2.8 (8)
PDGF (100 ng/ml)	113 ±3.8 (8)*	98.8 ±2.7 (8)
TGF-ß (500 pM)	105 ±3.7 (7)	95.4 ±2.6 (7)
Somatostatin (100 nM)	108 ±4.2 (8)	100 ±1.7 (8)
Galanin (100 nM)	104 ±2.4 (8)	104 ±4.4 (8)
ATP (200 μM)	84.3 ±3.0 (7)**	99.1 ±9.1 (7)
GTP (200 μM)	122 ±7.2 (7)*	110 ±5.8 (6)
CTP (200 μM)	89.3 ±4.5 (6)	77.8 ±2.0 (6)***
Carbamylcholine (100 μM)	98.1 ±3.1 (7)	93.1 ±3.0 (7)
TPA (25 ng/ml)	85.3 ±4.0 (8)**	84.4 ±4.9 (7)*
Staurosporine (10 nM)	56.1 ±1.0 (4)***	ND
Genistein (10 μM)	70.1 ±3.9 (4)**	ND
Forskolin (10 μM)	74.3 ±2.6 (8)***	60.6 ±2.8 (8)***
Clonidine (10 μM)	107 ±3.3 (8)	95.9 ±1.3 (8)*
Phenylephrine (10 μM)	101 ±2.6 (8)	93.2 ±3.3 (8)
Indomethacin (10 μM)	106 ±3.3 (7)	95.6 ±2.7 (7)
Prostaglandin E_2 (10 μM)	103 ±6.9 (3)	103 ±2.0 (4)
6-keto-prostaglandin $F_{1\alpha}$ (10 μM)	112 ±9.6 (3)	100 ±3.0 (4)
Thrombin (10 mU/ml)	90.0 ±1.0 (3)*	108 ±5.8 (3)
Heparin (50 μg/ml)	108 ±2.1 (8)**	96.5 ±1.9 (7)
Glibenclamide (3 μM)	112 ±4.8 (8)*	98.8 ±1.8 (8)
Diazoxide (40 μM)	103 ±5.2 (6)	91.2 ±3.1 (6)*
D-600 (50 μM)	5.7 ±0.8 (3)***	30.8 ±6.4 (4)**
Calmidazol (5 μM)	11.7 ±2.6 (7)***	14.3 ±3.8 (7)**

RINm5F cells were cultured for 2 days in medium BME containing 10 % calf serum. Fresh media supplemented with the different test substances were then added and cultures extended for 24 h. During the final 60 min, 1 μCi/ml of [^{3}H]thymidine was present in media and DNA synthesis assessed by measuring incorporation of radioactivity into DNA. Values are mean percent of controls ±SEM for the number of observations in parentheses. *, **, and *** denote $P<0.05$, $P<0.01$, and $P<0.001$, respectively, for chance differences *vs.* control cells using Student's paired *t*-test. Control values were for 1 % FCS 10,113 ±735 cpm/well, and for 10 % FCS 14,321 ±855 cpm/well. Abbreviations: ATP, adenosine trisphosphate; CTP, cytidine trisphosphate; EGF, epidermal growth factor; FCS, fetal calf serum; GTP, guanosine trisphosphate; ND, not determined; PDGF, platelet-derived growth factor; TGF-ß, transforming growth factor ß; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Thus, the role of Ca^{2+} in mitogenesis appears somewhat complex, inasmuch as the ion apparently both is necessary and sufficient to promote growth, but that unrestricted inlet of Ca^{2+} seems to exert toxic effects. This latter finding is by no means surprising since intracellular Ca^{2+} concentrations are tightly controlled by a number of mechanisms (17,66).

Another way of increasing cytosolic Ca^{2+} is via mobilization from intracellular stores, which occurrs during phospholipase C-catalyzed hydrolysis of phosphoinositides by certain agonists, *e.g.* carbamylcholine and ATP (7,13), which also activate protein kinase C (21,22,27,37). The importance of this kinase has been studied by the specific agonists phorbol esters (reviewed in [21,22,37]). However, prolonged phorbol ester treatment will result in down-regulation of protein kinase C. Phorbol esters increase insulin accumulation and ß-cell protein kinase C activity (1,3,27,46,67). The present findings indicate that the phospholipase C and protein kinase C systems appear to operate fairly similarly in RINm5F cells and in normal ß-cells with regard to mitogenesis. Thus, the present findings that carbachol stimulates insulin accumulation without affecting DNA synthesis are in concordance with our own data in normal adult and fetal ß-cells ([6], Å.S., submitted). Conversely, down-regulation of RINm5F cell protein kinase C by prolonged phorbol ester treatment or inhibition of its activity by staurosporine led to a decreased DNA synthesis, findings which are in agreement with our previous reports describing that stimulation of protein kinase C in normal ß-cells promotes DNA replication (46). In contrast, application of nucleotides, which in normal islets promotes ß-cell mitogenesis and insulin accumulation (Å.S., unpublished), to RINm5F cell cultures mainly resulted in inhibitory effects in this system.

Extant data favor the view that cyclic AMP (cAMP) functions as a permissive stimulus in normal ß-cell growth and insulin release (14,44,45,50,51,53). These results are in sharp contrast to our present findings in the tumoral RINm5F cells studied here, indicating that the cAMP-raising agent forskolin not only failed to enhance insulin accumulation but also suppressed DNA synthesis. The pancreatic islets are abundantly innervated and surrounding nerve terminals release neurotransmitter substances acting on ß-cell adrenergic receptors (30,57). In a previous study it was shown that proliferation and insulin accumulation of rat ß-cells could be markedly suppressed by α-adrenergic stimulation, either with the α_1-agonist phenylephrine or the α_2-agonist clonidine (44). However, as opposed to the normal ß-cells, the insulinoma cells obviously respond differently to such a treatment. Thus, our present data show that RINm5F cell DNA synthesis and insulin accumulation were not consistently suppressed by phenylephrine or clonidine, at the same concentrations used in normal islets. Hence, it is conceivable that either the α-adrenergic receptor, the connecting G-protein, or adenylyl cyclase is

TABLE 4. Regulation of long-term insulin accumulation by RINm5F cells.

	Insulin accumulation (μU/ml per 24 h)	
Cell culture	1 % FCS	10 % FCS
Control	5815 ±688 (9)	11089 ±1185 (6)
GH (1 μg/ml)	7884 ±610 (8)**	11310 ±673 (5)
EGF (100 ng/ml)	4848 ±870 (6)	9618 ±548 (7)
PDGF (100 ng/ml)	5211 ±339 (5)	8581 ±857 (6)
TGF-ß (500 pM)	4504 ±807 (7)	9279 ±820 (6)
Somatostatin (100 nM)	5582 ±351 (6)	11028 ±938 (7)
Galanin (100 nM)	5876 ±788 (8)	7914 ±1010 (6)*
ATP (200 μM)	5406 ±962 (5)	11301 ±662 (5)
GTP (200 μM)	4423 ±776 (6)	9171 ±719 (3)
CTP (200 μM)	6774 ±334 (5)	9018 ±840 (5)*
Carbamylcholine (100 μM)	7470 ±873 (7)*	11357 ±1714 (6)
TPA (25 ng/ml)	6128 ±670 (6)	9259 ±1471 (5)
Forskolin (10 μM)	6333 ±726 (7)	9960 ±1075 (6)
Clonidine (10 μM)	5561 ±522 (8)	9378 ±1338 (6)
Phenylephrine (10 μM)	5618 ±389 (6)	10085 ±1442 (6)
Indomethacin (10 μM)	5433 ±656 (6)	9342 ±877 (6)*
Prostaglandin E_2 (10 μM)	7947 ±2091 (3)	11643 ±143 (3)
6-keto-prostaglandin $F_{1\alpha}$ (10 μM)	8178 ±2805 (3)	10439 ±980 (3)
Thrombin (10 mU/ml)	6579 ±682 (4)	10771 ±962 (3)
Heparin (50 μg/ml)	5760 ±504 (8)	8294 ±945 (7)
Glibenclamide (3 μM)	5533 ±315 (8)	9226 ±803 (6)
Diazoxide (40 μM)	5840 ±509 (7)	8377 ±930 (7)
D-600 (50 μM)	2008 ±1141 (4)***	3750 (1)
Calmidazol (5 μM)	1062 ±84 (7)***	1554 ±193 (6)***

RINm5F cells were cultured for 2 days in medium BME containing 10 % calf serum. Fresh media supplemented with the different test substances were then added and insulin accumulation to the medium over a 24-h period measured by radioimmunoassay. Values are means ±SEM for the number of observations in parentheses. *, **, and *** denote $P<0.05$, $P<0.01$, and $P<0.001$, respectively, for chance differences *vs.* control cells using Student's *t*-test. Abbreviations: ATP, adenosine trisphosphate; CTP, cytidine trisphosphate; EGF, epidermal growth factor; FCS, fetal calf serum; GTP, guanosine trisphosphate; PDGF, platelet-derived growth factor; TGF-ß, transforming growth factor ß; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

aberrantly regulated in the tumoral cells.

Recent findings have shown that certain cytokines are able to exert both inhibitory

and cytotoxic actions on pancreatic islet cells *in vitro* (2,5,19). The by far most studied cytokine in this context, IL-1ß, is believed to inhibit insulin accumulation through production of the noxious gas nitric oxide which depletes ß-cells of ATP and causes apoptosis of RINm5F cells (2). In studies with normal ß-cells (45,50), it was found that IL-1ß not only suppressed insulin accumulation but moreover exerted antiproliferative actions on ß-cells. Our present findings in the RINm5F insulinoma cells are in agreement with those observations. Thus, the cytokine repressed both proliferation and hormone accumulation by the insulinoma cells, effects that seemingly occurred through nitric oxide generation since they were reversed by a specific inhibitor of nitric oxide synthase. It is noteworthy that serum from patients with type I diabetes mellitus, whose ß-cells are succumbed by autoimmune assault, not only contains elevated levels of IL-1 (43,45,50) but also causes exaggerated Ca^{2+} influx in RINm5F cells leading to DNA fragmentation characteristic of apoptosis (20), and that IL-1ß activates apoptosis in these cells through nitric oxide generation (2). Our present IL-1ß findings confirm previous data in this system (11,43), which also showed that the vitamin E γ-tocopherol, a major antioxidant in Western diets, could reverse the effects of IL-1ß, probably by detoxifying the nitrogen dioxide formed (10,11). Alluding to this, and to our previous studies on the role of prooxidants in RINm5F cells (12), our present data show that α-tocopherol, but not γ-tocopherol, promoted insulin accumulation whereas none of the E-vitamins affected RINm5F cell growth. In contrast, we detected a small inhibitory effect on insulinoma cell proliferation of the vitamin A derivative retinyl acetate, which has been shown to possess antiproliferative properties in other cell systems (32). Notably, another retinoid was without effect on RINm5F cell growth and function (58).

TGF-ß has been shown to stimulate insulin accumulation and to block glucose-mediated mitogenesis in normal islets (4,26,33,34,38,47). In contrast, no such effects were presently observed in the RINm5F cells in spite of the same concentration of TGF-ß being used. Possibly the discrepancy in mitogenic responses to different growth factors between normal and tumoral ß-cells reflect aberrations in this regulatory component, or in receptor binding.

Our finding (42) and the report by Brelje *et al.* (8) of a lack of effect of EGF and TGF-α on DNA synthesis and insulin accumulation in normal islets, are at variance with two other papers (9,36), which reported a modest mitogenic effect on islet cells of EGF and FGF. The possibility cannot be excluded that these apparent discrepancies reflect differences in islet content of fibroblasts, EGF being a major mitogen for this cell type. The role of TGF-α, which shares some 40 % sequence homology with EGF and functions through the EGF receptor, has been studied in three transgenic mouse models (18,23,41,62). In these studies, TGF-α overexpression in the pancreas

promoted proliferation of both acinar cells and fibroblasts, leading to a massive interstitial fibrosis and florid acinoductular metaplasia, whereas no consistent abnormalities in islets were noted. In the present study EGF affected neither RINm5F cell replication nor their rate of insulin accumulation. Our own findings (42,47,49,50,52), as well as those by others (8,15,35,59), indicate that GH and PDGF + IGF-I are able to induce a significant mitogenic response by the normal ß-cell, whereas EGF/TGF-α, FGF and PDGF alone were inactive (8,25,64). A totally different scenario was found to exist in the RINm5F cells, inasmuch as GH unexpectedly caused an marked suppression of DNA synthesis, while increasing insulin accumulation. Interestingly, these effects vanished when the serum concentration was raised from 1 % to 10 %, confirming findings in normal ß-cells (59). Among other peptides screened, PDGF was only able to elicit a very minor increase in DNA synthesis at the extremely high concentration of 100 ng/ml, while 10 ng/ml PDGF or IGF-I either alone or in combination were inactive, again results that differ from most (8,36,42,64) reports on normal ß-cells. Notably, IGF-I receptors have been identified in normal ß-cells (61) and in RINm5F cells (Q. Zhang, personal communication). Somatostatin, produced by islet α-cells, is a well-known paracrine inhibitor of insulin accumulation, whereas the role of the peptide in normal ß-cell proliferation is unclear. Somatostatin lacked effect on proliferation on the RINm5F cells and in these cells also did not affect insulin accumulation. Galanin is another polypeptide that has been localized to islet cells and which inhibits insulin accumulation, but not DNA synthesis (Å.S., unpublished). In contrast, galanin affected neither DNA synthesis nor insulin accumulation in the presently studied RINm5F cells. As for the fibroblast growth factors, a growth-promoting effect of FGF in normal ß-cells has been reported in one paper (36) and a mitogenic action of PDGF alone was proposed in another study (60). However, in a subsequent work mitogenesis was enhanced only after ß-cells had been transfected with the FGF-1- and PDGF-receptor (25). Similarly, in normal islet cells a mitogenic response to PDGF was conferred only after the cells had been transfected with a cDNA construct to express the PDGF receptor, which usually is not a noteworthy feature in this cell type (64). Both acidic and basic fibroblast growth factors were without effect on RINm5F cell proliferation and insulin accumulation, as was bradykinin, whose effects on normal ß-cells to my best knowledge have not been studied.

Tyrosine kinases are instrumental in the transduction of mitogenic signals (40,65), but not much is known about their role in ß-cells as of yet. In the present study we employed low concentrations of genistein, a general inhibitor of tyrosine kinases, to address this issue. It was found that this maneuver resulted in a significant reduction in DNA synthesis, implying that RINm5F cell proliferation is dependent upon tyrosine phosphorylation. It is evident, however, that this area requires

further investigation.

In an attempt to elucidate the role of prostaglandins, which are involved in regulation of the stimulus-secretion coupling in normal ß-cells (27), in RINm5F cell function, we added exogenous prostaglandins E_2 and $F_{1\alpha}$, which were inactive on both insulin accumulation and DNA synthesis even at high concentrations, while the cycloxygenase inhibitor indomethacin caused a subtle suppression of insulin accumulation. Together, these findings suggest that prostaglandins play a very limited role in regulation of RINm5F cell function.

In conclusion, the present results indicate that RINm5F cell proliferation and insulin accumulation can be modulated by peptides and certain second messengers, including Ca^{2+}, cAMP and protein kinases. However, as we have noted previously (54,56), the tumoral RINm5F cells exhibit many important qualitative and quantitative differences in comparison to their non-transformed counterparts, suggesting that they have acquired perturbations in signal transduction mechanisms that normally regulate, not only insulin accumulation (24), but also cell proliferation. The most spectacular finding in this screening study, *i.e.* that growth hormone contrarily to its effect on normal ß-cells suppresses insulinoma cell growth, certainly warrants further elucidation of the underlying mechanisms. Conceivably, the hormone might become of utility in a clinical setting in the treatment of patients with insulin-producing tumors.

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