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Polyamines and insulin production in isolated mouse pancreatic islets

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The aim of the present study was to evaluate the role of polyamines in the metabolism and insulin production of pancreatic-islet cells. For this purpose islets were prepared from adult mice and used either immediately or after tissue culture. There was a significant decrease in the islet content of spermidine during culture, although the effect was less pronounced in a high glucose concentration. Furthermore, a stimulatory effect of a high glucose concentration, as compared with low glucose, on the content of spermine was observed. To elucidate further the role of polyamines in β-cell physiology, the ornithine decarboxylase inhibitors difluoromethylornithine (DFMO) and methylacetylenic putrescine (MAP) and the *S*-adenosylmethionine decarboxylase inhibitor ethylglyoxal bis(guanylhydrazone) (EGBG) were added to the culture media. Addition of DFMO together with MAP decreased the cellular contents of putrescine and spermidine, whereas the content of spermine was unaffected. When EGBG was added in combination with DFMO and MAP, there was a decrease in the content of spermine also. Cell viability in the islets depleted of their polyamine contents was not impaired, as assessed by determinations of oxygen-uptake rates and ATP contents. Depletion of putrescine plus spermidine by addition of DFMO + MAP was associated with decreased biosynthesis of (pro)insulin and total protein. When the content of spermine was decreased also by the further addition of EGBG, the decrease in (pro)insulin biosynthesis was more pronounced and was paralleled by a decrease in the insulin-mRNA content. Under these conditions, the glucose-stimulated insulin release, the insulin content and the rates of islet DNA synthesis were also decreased. It is concluded that putrescine and spermidine are necessary for the maintenance of normal insulin and protein biosynthesis, whereas spermine may exert a role in some other cellular processes, such as DNA replication, RNA transcription and glucose-stimulated insulin release.

INTRODUCTION

Polyamines are ubiquitous aliphatic cations intimately related to cell differentiation, macromolecular synthesis and growth in numerous cell systems (reviewed by Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1986). Whereas a wealth of knowledge about the biological significance of polyamines in general has accumulated, information on their putative role in the insulin-producing β-cell is still largely lacking. In the investigation by Hougaard *et al.* (1986), localization and biosynthesis of polyamines were studied. It was found that the polyamines were largely restricted to the insulin-producing β-cell and that glucose stimulated the biosynthesis of polyamines. It was therefore speculated that polyamines may stimulate insulin release either by acting as substrates for transglutaminases or by functioning as second messengers. Contrary to this, studies by Thams *et al.* (1986*a*) suggested an inhibitory role of polyamines on insulin secretion. It was proposed that polyamines inhibit protein kinase C and that this inhibitory role of polyamines could be counteracted by stimulus-induced Ca^{2+} mobilization. In spite of these investigations, little is known about the metabolism of the different polyamines during culture *in vitro* and their relation to islet β-cell function.

The first and rate-limiting enzyme in the polyamine-biosynthetic pathway is ornithine decarboxylase (EC 4.1.1.17), which catalyses the formation of putrescine from ornithine (Pegg & McCann, 1982). Ornithine decarboxylase activity is subject to a rapid and powerful stimulation by a vast array of trophic stimuli (Goyns, 1982), and is specifically and irreversibly inhibited by difluoromethylornithine (DFMO) (Metcalf *et al.*, 1978) and methylacetylenic putrescine (MAP) (Mamont *et al.*, 1984). *S*-Adenosylmethionine (AdoMet) decarboxylase (EC 4.1.1.50) brings about the decarboxylation of AdoMet, yielding a substrate for the synthesis of spermidine and spermine. Ethylglyoxal bis(guanylhydrazone) (EGBG) is widely used as a highly selective and potent inhibitor of AdoMet decarboxylase (Seppänen *et al.*, 1984), but it is neither irreversible nor absolutely specific.

In the present investigation, the significance of polyamines for pancreatic islet function has been explored, with particular attention to their role in insulin production. It is shown that glucose regulates polyamine contents *in vitro*. Furthermore, by using the inhibitors of polyamine biosynthesis in different combinations, it was possible to differentiate partially between the effects of the three polyamines and to demonstrate that polyamines are involved in the regulation of insulin-mRNA contents, insulin and protein biosynthesis and glucose-sensitive insulin release.

Abbreviations used: AdoMet, *S*-adenosylmethionine; DFMO, difluoromethylornithine; EGBG, ethylglyoxal bis(guanylhydrazone); MAP, methylacetylenic putrescine; PBS, phosphate-buffered saline (154 mM-Na^+/4 mM-K^+/140 mM-Cl^-/10 mM-HPO_4^{2-}, pH 7.4).

MATERIALS AND METHODS

Chemicals

The chemicals were obtained from the following sources. Boehringer-Mannheim, Mannheim, Germany: collagenase, type CLS (EC 3.4.24.3) and ATP. Miles Laboratories, Slough, U.K.: bovine albumin (fraction V). Statens Bakteriologiska Laboratorium, Stockholm, Sweden: Hanks' balanced salt solution and calf serum. Astra Läkemedel, Södertälje, Sweden: benzylpenicillin. Glaxo Laboratories, Greenford, Middx., U.K.: streptomycin. Flow Laboratories, Irvine, Ayrshire, U.K.: culture medium RPMI 1640 and L-glutamine. Miles–Yeda, Rehovot, Israel: anti-(bovine insulin) serum. Novo, Copenhagen, Denmark: crystalline mouse insulin and ^{125}I-labelled insulin. Amersham International, Amersham, Bucks., U.K.: L-[4,5-^{3}H]leucine (sp. radioactivity 40 Ci/mmol) and [*methyl*-^{3}H]thymidine (sp. radioactivity 5 Ci/mmol). New England Nuclear, Boston, MA, U.S.A.: Econofluor. Pharmacia Fine Chemicals, Uppsala, Sweden: CNBr-activated Sepharose 4B and Ficoll 400. Packard Instruments, Downers Grove, IL, U.S.A.: Soluene. Sigma Chemical Co., St. Louis, MO, U.S.A.: putrescine, spermidine, spermine, dansyl chloride, L-proline, Hepes. All other chemicals of analytical grade were obtained from E. Merck, Darmstadt, Germany.

Preparation and culture of isolated islets

Islets were isolated from the pancreata of male NMRI mice (Anticimex, Stockholm, Sweden) by a collagenase digestion technique and separated from exocrine pancreatic cells by Ficoll-gradient centrifugation (Howell & Taylor, 1968; Lernmark *et al.*, 1976). The islets were subsequently picked free from exocrine tissue by means of a braking pipette and either used immediately or maintained free-floating in tissue culture at 37 °C in a humidified atmosphere of air/5% CO_2. The culture medium was RPMI 1640 containing 10% (v/v) calf serum, 2 mM-L-glutamine, benzylpenicillin (100 units/ml) and streptomycin (0.1 mg/ml) and supplemented with glucose and the inhibitors of polyamine synthesis (DFMO, MAP and EGBG) at the concentrations given below.

Polyamine contents

The islet putrescine, spermidine and spermine contents were measured essentially as described previously (Seiler, 1983). Briefly, groups of 150–200 islets were washed with phosphate-buffered saline (PBS) and homogenized in a sonication water-bath in 20 μl of 0.3 M-$HClO_4$ at 0–4 °C. After centrifugation (5 min, 11000 *g*), the supernatant was neutralized by addition of 7 μl of 1 M-Na_2CO_3. Each homogenate was supplemented with 100 μl of dansyl chloride in acetone (10 mg/ml). After overnight incubation in the dark at room temperature, the remaining dansyl chloride was removed by addition of 5 μl of L-proline (250 mg/ml), followed by sonication for 2 min. Dansylated conjugates were extracted from the water phase in 100 μl of toluene. The toluene solutions were evaporated and redissolved in 5 μl of toluene for application on t.l.c. plates (HPTLC-Fertigplatten, Kieselgel 60 F_{254}; E. Merck). The dansylated polyamines were separated by one run with ethyl acetate/cyclohexane (1:1, v/v), followed by two runs with diethyl ether/cyclohexane (2:3, v/v). The spots were quickly detected under u.v. light, scraped off the plates and eluted in 400 μl of methanol. The silica gel was removed by centrifugation, and the fluorescence intensities of the supernatants were measured in a Farrand model 2 ratio fluorimeter at an excitation of 360 nm and an emission of 510 nm.

Oxygen uptake

The islet respiratory rates were measured by the Cartesian-diver technique as modified by Hellerström (1967). Islets in groups of 5–10 were allowed to respire at 37 °C in Krebs–Ringer phosphate buffer, pH 7.4, with a gas phase of ambient air. Changes in the concentrations of test substances in the incubation medium were made by addition to the medium of a side-drop containing the test substance (see below). Rates of O_2 consumption were expressed in nl/h per islet at 1.67 mM- or 16.7 mM-glucose.

ATP content

After the culture period, islets in groups of 20 were washed and transferred to 100 μl of ice-cold 0.89 M-$HClO_4$ containing 2 mM-EDTA. The samples were briefly sonicated, neutralized by the addition of 100 μl of 0.89 M-NaOH + 2 mM-EDTA and diluted 100-fold with 0.1 M-Tris acetate buffer, pH 7.75, supplemented with 2 mM-EDTA. The ATP contents of the samples were subsequently measured by a bioluminescence procedure, by adding 20 μl of an ATP reagent (LKB-Wallac, Turku, Finland) to 80 μl of the sample, which provided a constant light signal. The light signal was quantified in a LKB–Wallac 1250 luminometer, and the measurements were performed at room temperature.

Insulin-mRNA content

For insulin-mRNA quantification, as determined by dot-blot analysis (Thomas, 1980; White & Bancroft, 1982), duplicate groups of 50 islets each were washed in phosphate-buffered saline immediately after the culture period and briefly sonicated in 200 μl of 0.1 M-Tris (pH 7.4)/10 mM-EDTA/1% SDS. The sonicates were extracted with 2 × 200 μl of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). Nucleic acids were precipitated with 20 μl of 2 M-potassium acetate and 500 μl of ethanol overnight at −20 °C. The precipitates were washed with 70% (v/v) ethanol and subsequently treated with 1 M-glyoxal for 1 h at 50 °C, diluted with 4 vol. of 3 M-NaCl/0.3 M-sodium citrate and bound to Genescreen membranes (New England Nuclear) at 80 °C for 2 h. After prehybridization (for 1 h at 42 °C), the samples were hybridized at 42 °C with the appropriate probe (pRI-7) (Chan *et al.*, 1979), which had been labelled by using an oligonucleotide-labelling kit (Pharmacia). After hybridization, the filters were washed and then exposed at −70 °C to Hyperfilm-MP (Amersham) with an intensifying screen. The intensities of the spots thus obtained were determined by densitometry and expressed as optical density (o.d.). The dot-blot assay for insulin mRNA was linear over the range of concentrations of insulin mRNA tested, without detectable background hybridization.

(Pro)insulin and total protein biosynthesis

Cultured islets in duplicate groups of 20 each were transferred to multiwell plates (well capacity 0.5 ml; Linbro Scientific, Hamden, CT, U.S.A.) containing

Table 1. Effects of glucose on polyamine contents in isolated islets

Isolated pancreatic islets were used fresh or after culture for 2 days in RPMI 1640 plus 10% calf serum supplemented with 3.3 mM- or 16.7 mM-glucose. The islets were homogenized in $HClO_4$. The $HClO_4$-insoluble material was collected after centrifugation, neutralized and exposed to dansyl chloride overnight. Dansylated compounds were extracted with toluene, evaporated and separated by t.l.c. Spots co-migrating with standard polyamines were scraped off and extracted with methanol. The fluorescence intensities of the methanol solutions were quantified fluorophotometrically and converted into nmol. Values are means ± S.E.M., with the numbers of experiments in parentheses: * and *** denote $P < 0.05$ and $P < 0.001$ for chance differences versus freshly isolated islets; † and †† denote $P < 0.05$ and $P < 0.01$ for chance differences when islets cultured in 16.7 mM-glucose are compared with islets cultured in 3.3 mM-glucose (Student's paired t test).

Islet culture	Content (nmol/200 islets)		
	Putrescine	Spermidine	Spermine
Freshly isolated islets	0.02 ± 0.01 (7)	0.56 ± 0.05 (6)	0.49 ± 0.04 (6)
3.3 mM-glucose (2 days)	0.05 ± 0.01 (4)	0.19 ± 0.05 (6)***	0.39 ± 0.04 (6)
16.7 mM-glucose (2 days)	0.04 ± 0.01 (4)	0.35 ± 0.06 (6)*††	0.56 ± 0.07 (6)†

Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) supplemented with 10 mM-Hepes, 2 mg of albumin/ml, 16.7 mM-glucose, [^{3}H]leucine (5 μCi/ml) and inhibitors of polyamine synthesis as shown in Table 3, and incubated at pH 7.4 in air/5% CO_2 for 2 h at 37 °C. The islets were then washed with Hanks' solution and subsequently sonicated in redistilled water. The homogenates were incubated with anti-(bovine insulin) serum coupled to Sepharose 4B beads to separate labelled (pro)insulin from other labelled islet proteins (Berne, 1975). The total protein biosynthesis was estimated by measurement of the radioactivity in the trichloroacetic acid-precipitable fraction of the homogenate.

Insulin release and insulin content

Groups of 10 cultured islets were incubated in triplicates at 37 °C and pH 7.4 in an atmosphere of air/5% CO_2. The incubation medium consisted of 250 μl of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 10 mM-Hepes, 2 mg of albumin/ml, 1.67 mM-glucose and the inhibitors of polyamine synthesis as shown in Table 3. After 1 h the medium was carefully removed and replaced by 250 μl of a similar medium supplemented with 16.7 mM-glucose, and the incubation was continued for another 60 min. The insulin concentrations in samples of the incubation media were determined by radioimmunoassay, with crystalline mouse insulin as standard and ^{125}I-insulin as a tracer (Heding, 1972). After the culture period, groups of 10 islets were extracted overnight at 4 °C in 500 μl of acid/ethanol (0.18 M-HCl in 70% ethanol) for measurement of their insulin content.

Islet [^{3}H]thymidine incorporation, DNA content and protein content

Islets in groups of 150–200 were cultured for 2 days as described above. During the last 5 h of the culture period, 1 μCi of [*methyl*-^{3}H]thymidine/ml was present in the culture media. After the end of the labelling period, the islets were washed with PBS, homogenized in 0.3 M-$HClO_4$, and the acid-insoluble material was collected by centrifugation. The pellet thus obtained was solubilized in redistilled water. Samples were taken for measurements of DNA and protein contents (Kissane & Robins, 1958; Hinegardner, 1971; Lowry *et al.*, 1951). The remaining volume was precipitated with ice-cold 10% trichloroacetic acid. The precipitate was washed twice in trichloroacetic acid and dissolved in 100 μl of Soluene. The incorporated radioactivity was then measured by liquid-scintillation counting after addition of 1.5 ml of Econofluor.

Statistical analysis

Means ± S.E.M. were calculated, and groups of data were compared by means of Student's unpaired or paired t test.

RESULTS

Effects of glucose on islet polyamine contents

The contents of the polyamines putrescine, spermidine and spermine in freshly isolated islets or islets cultured for 2 days in 3.3 mM- or 16.7 mM-glucose are shown in Table 1. As compared with freshly isolated islets, there was a decrease in the contents of spermidine in islets cultured in both low and high glucose concentrations. The content of spermidine in islets cultured at 16.7 mM-glucose was, however, less decreased as compared with culture in 3.3 mM-glucose. The content of spermine was significantly higher in islets cultured in 16.7 mM-glucose as compared with 3.3 mM-glucose. There were no differences in the putrescine contents betweeen the different groups.

Effects of inhibitors of polyamine synthesis on islet polyamine contents

No effects of MAP alone on the contents of the different polyamines were observed (Table 2). The contents of putrescine and spermidine in islets cultured for 2 days with DFMO alone or with DFMO + MAP + EGBG were decreased as compared with control islets. On the other hand, islets cultured with EGBG alone exhibited a 15-fold increase in the putrescine content. This increase was partially attenuated by the addition of MAP. No effects were exerted by EGBG and MAP + EGBG on the spermidine contents. The spermine contents were diminished only in the groups containing EGBG (EGBG, MAP + EGBG, DFMO + MAP + EGBG).

Table 2. Polyamine contents of islets cultured for 2 days with inhibitors of polyamine biosynthesis

Pancreatic islets were cultured for 2 days in medium RPMI 1640 containing 11.1 mM-glucose plus 10% calf serum. The inhibitors of polyamine synthesis were added to the culture medium at the concentrations given in the Table. Polyamines were extracted by precipitation with $HClO_4$ and subsequently dansylated and separated by t.l.c. After separation, the spots were scraped off and the fluorescence intensities were quantified fluorophotometrically. The values thus obtained were converted into nmol by using a standard curve. Values are means ± S.E.M. for six experiments: *, ** and *** denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively for chance differences versus control islets (Student's paired t test).

Islet culture	Content (nmol/200 islets)		
	Putrescine	Spermidine	Spermine
Control	0.06±0.03	0.24±0.02	0.36±0.06
MAP (200 μM)	0.04±0.01	0.21±0.05	0.35±0.04
DFMO (5 mM)	0.02±0.004*	0.12±0.01**	0.35±0.05
MAP (200 μM)+DFMO (5 mM)	0.01±0.001**	0.14±0.01**	0.40±0.02
EGBG (100 μM)	0.88±0.09***	0.26±0.03	0.26±0.02*
MAP (200 μM)+EGBG (100 μM)	0.28±0.04**	0.26±0.03	0.27±0.05*
DFMO (5 mM)+MAP (200 μM) +EGBG (100 μM)	0.02±0.002*	0.12±0.01**	0.23±0.02*

Islet oxygen uptake and ATP contents

Islets cultured in media containing DFMO+MAP+EGBG displayed higher respiration in 1.67 mM-glucose than did control islets, whereas the stimulated respiration at 16.7 mM-glucose was not affected by any of the inhibitors (Table 3). DFMO+MAP affected the respiration at neither 1.67 mM- nor 16.7 mM-glucose. All groups of islets responded promptly to glucose stimulation with an increased respiratory rate. There was no difference in the magnitude of this response between islets cultured with different additives. The ATP content was higher in EGBG-treated islets than in control islets, whereas DFMO+MAP did not affect this parameter (Table 3).

Islet insulin and insulin-mRNA contents

The insulin content and the insulin-mRNA content of islets cultured for 2 days in the presence of MAP+DFMO+EGBG were lower than in control islets (Table 3). DFMO+MAP did not affect the insulin content.

Islet (pro)insulin and total protein biosynthesis

(Pro)insulin biosynthesis was significantly lower in iselts cultured with DFMO+MAP and DFMO+MAP+EGBG as compared with control islets (Table 3). In addition, the inhibitors exerted similar effects on the total protein biosynthesis. However, when expressed as a percentage of the total protein biosynthesis, the (pro)-insulin biosynthesis was not affected by DFMO+MAP. This percentage was decreased only when EGBG was present, indicating a more pronounced inhibition by this compound of (pro)insulin biosynthesis than of total protein biosynthesis.

Islet insulin release

Both control islets and islets cultured with the inhibitors responded to glucose with increased insulin release (Table 3). Islets cultured with DFMO+MAP+EGBG exhibited a lower insulin release at 16.7 mM-glucose than did control islets, whereas no differences were observed at 1.67 mM-glucose. Islet insulin release was not affected by DFMO+MAP.

Effects of exposure to inhibitors of polyamine synthesis on islet DNA and protein contents and [^{3}H]thymidine incorporation

No effects were observed of the inhibitors on the DNA or protein contents (Table 4). However, [^{3}H]thymidine-incorporation rates were decreased in all groups containing EGBG (EGBG, MAP+EGBG, DFMO+MAP+EGBG). MAP or DFMO alone did not affect [^{3}H]thymidine-incorporation rates.

DISCUSSION

Attempts to evaluate the importance of polyamines for β-cell function are associated with certain difficulties. The addition of exogenous spermidine and spermine during culture *in vitro* leads to cell destruction. This is probably due to a combination of direct interactions between the polyamines and phospholipids of the plasma membrane and the conversion of polyamines into toxic aldehydes by enzymes present in serum (Caldarera *et al.*, 1975; Yung & Green, 1986; Tabor *et al.*, 1964). However, by utilizing specific inhibitors of polyamine biosynthesis, the putative role of polyamines may nevertheless be elucidated. The present study shows that the inhibitor of ornithine decarboxylase, DFMO, depletes the β-cell of its putrescine and spermidine contents. The spermine content was not affected, a finding not surprising, since slowly proliferating cells are not readily depleted of their spermine contents by using only an ornithine decarboxylase inhibitor (Pegg, 1986; Bolkenius & Seiler, 1987). MAP was shown to exert only modest inhibitory effects on the contents of putrescine when added together with EGBG, but the drug was, however, included in the different experiments, since it has been reported to be taken up more rapidly than DFMO (Pegg, 1986; Mamont *et al.*, 1984). EGBG, the inhibitor of AdoMet decarboxylase, inhibits the synthesis of spermidine and spermine by decreasing the cellular contents of decarboxylated AdoMet, the aminopropyl donor to putrescine and spermidine. When EGBG was used alone there was, besides a decrease in the spermine content, a marked increase in the putrescine content, which would suggest

Table 3. Oxygen uptake, ATP content, insulin-mRNA content, (pro)insulin biosynthesis, total protein biosynthesis, insulin content and insulin release in islets cultured for 2 days with inhibitors of polyamine biosynthesis

Pancreatic islets were cultured for 2 days in medium RPMI 1640 containing 11.1 mM-glucose plus 10% calf serum, supplemented with the inhibitors of polyamine synthesis at the concentrations given in the Table. O_2 uptake was measured by the Cartesian-diver technique and the islet ATP content was determined by a bioluminescence technique. Insulin-mRNA contents were measured by dot-blot analyses using a ^{32}P-labelled cDNA probe and quantified by densitometry (o.d., optical density). Rates of (pro)insulin and total protein biosynthesis were estimated by measuring the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable proteins. Labelled (pro)insulin was separated from other labelled proteins by an immunoabsorption technique. The islet insulin content extracted by acid/ethanol and the insulin release to the incubation buffer were determined radioimmunologically. Values are means ± S.E.M., with numbers of experiments in parentheses: *, ** and *** denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, for chance differences versus control islets by Student's unpaired t test (respiratory rate at 1.67 mM-glucose) or Student's paired t test [ATP content, insulin-mRNA content, (pro)insulin and total protein biosynthesis, insulin content and insulin release]; †† and ††† denote $P < 0.01$ and $P < 0.001$, respectively, for chance differences versus the respiratory rate and insulin release of islets incubated at 1.67 mM-glucose, by Student's paired t test. N.D., not determined.

	Islet culture		
	Control	DFMO (5 mM) + MAP (200 μM)	DFMO (5 mM) + MAP (200 μM) + EGBG (100 μM)
Respiration in 1.67 mM-glucose (nl of O_2/h per islet)	3.1 ± 0.3 (4)	2.9 ± 0.4 (4)	4.3 ± 0.4 (5)*
Respiration in 16.7 mM-glucose (nl of O_2/h per islet)	6.8 ± 0.6 (4)††	7.1 ± 1.0 (4)††	8.7 ± 0.6 (5)††
ATP content (pmol/islet)	4.8 ± 0.3 (9)	5.2 ± 0.3 (9)	5.6 ± 0.3 (9)**
Insulin mRNA content (o.d./50 islets)	150 ± 20 (6)	N.D.	77 ± 20 (6)*
10^{-3} × (Pro)insulin biosynthesis (d.p.m./2 h per 20 islets)	43 ± 8 (7)	27 ± 5 (7)**	18 ± 4 (7)**
10^{-3} × Total protein biosynthesis (d.p.m./2 h per 20 islets)	120 ± 21 (7)	86 ± 11 (7)*	76 ± 11 (7)**
(Pro)insulin biosynthesis (% of total protein biosynthesis)	35 ± 2 (7)	32 ± 2 (7)	22 ± 2 (7)***
Insulin content (ng/10 islets)	240 ± 28 (5)	250 ± 14 (5)	170 ± 24 (5)*
Insulin release in 1.67 mM-glucose (ng/h per 10 islets)	1.5 ± 0.2 (6)	1.4 ± 0.2 (6)	1.6 ± 0.4 (6)
Insulin release in 16.7 mM-glucose (ng/h per 10 islets)	23 ± 4 (6)†††	28 ± 7 (6)†††	16 ± 3 (6)†††

Table 4. DNA content, protein content and [^{3}H]thymidine incorporation of islets cultured for 2 days with inhibitors of polyamine biosynthesis

Pancreatic islets were cultured for 2 days in medium RPMI 1640 plus 10% calf serum. The inhibitors of polyamine synthesis were added to the culture medium at the concentrations given in the Table. The islet DNA and protein contents were determined by standard methods. For estimation of DNA synthesis, 1 μCi of [*methyl*-^{3}H]thymidine/ml was added to the media during the last 5 h of culture, and the radioactivity incorporated into trichloroacetic acid-precipitable material was determined by liquid-scintillation counting. Values are means ± S.E.M., with numbers of experiments in parentheses: *denotes $P < 0.05$ for a chance difference versus control islets, by Student's paired t test. N.D., not determined.

Islet culture	DNA content (μg/100 islets)	Protein content (μg/100 islets)	[^{3}H]Thymidine incorporation (c.p.m./100 islets)
Control	1.3 ± 0.1 (7)	11 ± 1.5 (6)	370 ± 60 (5)
MAP (200 μM)	1.3 ± 0.2 (7)	13 ± 1.2 (6)	340 ± 80 (5)
DFMO (5 mM)	1.4 ± 0.2 (4)	N.D.	420 ± 40 (6)
EGBG (100 μM)	1.2 ± 0.2 (7)	14 ± 0.8 (6)	270 ± 60 (5)*
MAP (200 μM) + EGBG (100 μM)	1.5 ± 0.3 (7)	13 ± 2.0 (6)	260 ± 40 (5)*
DFMO (5 mM) + MAP (200 μM) + EGBG (100 μM)	1.5 ± 0.2 (6)	N.D.	230 ± 30 (5)*

that spermine exerts an inhibitory action on the activity of ornithine decarboxylase. This feedback mechanism is considered to be mediated by casein kinase II, a polyamine-dependent protein kinase which phosphorylates ornithine decarboxylase (Meggio *et al.*, 1984). In agreement with this notion, the increase in the putrescine content was attenuated by the addition also of DFMO + MAP. Thus islets cultured in the presence of all three inhibitors of polyamine biosynthesis were depleted of their putrescine, spermidine and spermine contents. Since EGBG is considered not to be a completely specific inhibitor of AdoMet decarboxylase, and to exclude the possibility that its actions are generally cytotoxic to the β-cell, O_2 uptake was measured in islets cultured in the presence of the inhibitors of polyamine biosynthesis. It was found that such islets responded well to glucose with an increased respiratory rate. Furthermore, the islets cultured in the presence of EGBG showed elevated O_2 uptake and ATP contents, which rules out the possibility that EGBG acts as an uncoupler in the islet cells. A previous study reported that polyamine depletion resulted in increased ribonucleotide pools, including the ATP and ADP contents (Oredsson *et al.*, 1986). The possibility that the effects of EGBG might be mediated via some additional but unrecognized specific mechanism, rather than the depletion of spermine, cannot, however, completely be ruled out. The mechanisms by which polyamine depletion causes these effects are unknown. However, the oxidative-metabolism data in conjunction with the unaltered contents of protein and DNA suggest that EGBG does not exert a cytotoxic effect on the β-cell.

The only parameters affected of those investigated in islets with diminished putrescine and spermidine contents were (pro)insulin and total protein biosynthesis. The insulin-mRNA contents of islets depleted of putrescine and spermidine were not determined under the conditions of this investigation. In separate experiments, however, DFMO + MAP did not exert any effect on the insulin-mRNA metabolism (results not shown). Interestingly, the degree of inhibition of (pro)insulin biosynthesis under these conditions was of the same magnitude as the inhibition of total protein biosynthesis, leaving the percentage unaltered. It thus appears that putrescine and spermidine are regulatory at some translational level which does not specifically control insulin biosynthesis. Polyamines have been reported to affect translation by interacting with RNA (Karpetsky *et al.*, 1977) and regulating ribosomal assembly (Kakegawa *et al.*, 1986). In addition, spermidine has been shown to supply a butylamino group to the side chain of lysine, thereby forming the unusual amino acid hypusine, which is incorporated into the initiation factor eIF-4D (Park *et al.*, 1981).

The decrease in spermine, in addition to those in putrescine and spermidine, resulted in decreased insulin-mRNA contents, a further pronounced impairment of (pro)insulin biosynthesis, a decreased insulin content and a diminished release of insulin at a high glucose concentration. Possible levels of spermine regulation of the insulin-mRNA content are interactions with DNA and transcriptional factors (Goyns, 1982), activation of RNA polymerases (Blair, 1985), regulation of RNA stability (Karpetsky *et al.*, 1977) and the modulation of the activity of the polyamine-dependent enzyme casein kinase II (Thams *et al.*, 1986*b*). The observed 50% decrease in the insulin-mRNA content readily accounts for the additional decrease in (pro)insulin biosynthesis evoked by spermine depletion.

In agreement with the stimulatory effects of glucose on polyamine synthesis observed previously (Hougaard *et al.*, 1986), glucose partially counteracted the decrease in islet spermidine content which occurred *in vitro* and increased the spermine content during a 2-day culture period. The findings that the contents of polyamines are high in β-cells and that they are associated with secretory granules led Hougaard *et al.* (1986) to suggest that these substances may exert a role in glucose-stimulated insulin release by functioning as intracellular messengers. This is an attractive hypothesis, since it has been shown in lymphocytes that activation of ornithine decarboxylase occurs rapidly upon mitogenic stimulation by the phospholipase-C-dependent release of ornithine decarboxylase covalently bound to phosphatidylinositol (Mustelin *et al.*, 1987). It has also been shown that influx and mobilization of Ca^{2+} in mouse kidney-cortex cells is secondary to increased polyamine contents (Koenig *et al.*, 1983). However, in the present investigation islets depleted of their putrescine and spermidine contents displayed an excellent insulin release in response to glucose, suggesting that these polyamines are without importance for this process. It was also suggested that polyamines may be necessary for glucose-stimulated insulin release by acting as substrates for transglutaminases, enzymes which have been implicated in insulin release (Gomis *et al.*, 1983). This notion is supported by the present observation that islets depleted of spermine released less insulin during incubation at a high glucose concentration than do control islets. However, this permissive or stimulatory role of spermine in glucose-stimulated insulin release appears to operate in the long term rather than the short term, since previous observations indicate that spermine contents were not increased during a 60 min incubation in the presence of a high glucose concentration (Thams *et al.*, 1986*a*). An inhibitory role of polyamines in the stimulus–secretion coupling was, however, suggested by Thams *et al.* (1986*a*), who, by adding high concentrations of polyamines extracellularly to pancreatic islets, showed that this resulted in an inhibition of insulin release and an attenuation of the islet protein kinase C activity. This suggestion is not supported by the present investigation, since insulin release was not higher from polyamine-depleted islets. It cannot be excluded, however, that islet cells synthesize other substances than the polyamines investigated in the present work, which are closely related to these and can in this context substitute for their actions (Bachrach & Shtorch, 1985).

A decrease in the islet spermine content rather than in putrescine or spermidine content caused decreased rates of DNA synthesis. This is in agreement with previous observations (Tabor & Tabor, 1984; Pegg, 1986), suggesting an essential role for spermine in DNA replication.

In summary, glucose regulates the islet contents of spermidine and spermine *in vitro*. Putrescine and spermidine appear to be necessary for biosynthesis of insulin and protein, whereas spermine depletion decreases insulin-mRNA contents, insulin biosynthesis, islet insulin content, glucose-sensitive insulin release and DNA synthesis. These findings support the view that polyamines act either as permissive or as stimulatory factors

at several different sites of the insulin production *in vitro*.

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