Localization and biosynthesis of polyamines in insulin-producing cells

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Two recently developed fluorescence cytochemical methods, specific for spermidine and spermine, were used to localize polyamines in the endocrine pancreas. The polyamines were restricted to the insulin-producing β -cells and were mainly associated with the secretory granules. Chemical polyamine determinations carried out on isolated rat and mouse pancreatic islets revealed large amounts of polyamines. Compared with extracts of whole pancreas, the islets contained very high concentrations of spermine relative to spermidine. Biosynthesis of polyamines from [3 H]ornithine or from [3 H]putrescine in isolated islets was significantly stimulated at high glucose concentrations. Moreover, significant incorporation of label from [3 H]putrescine was also detected in γ -aminobutyric acid. This incorporation, however, was not stimulated by high glucose. Possible roles for polyamines associated with the secretory granules in insulin-producing cells are discussed.

INTRODUCTION

Polyamines are ubiquitous organic cations that are synthesized in large amounts by rapidly growing and dividing cells, including cancer cells (for reviews see Russell & Durie, 1978; Heby, 1981; Pegg & McCann, 1982). They have been suggested to play several intracellular roles and have been shown to have important effects on synthesis of cellular nucleic acid and protein and on nucleic acid stabilization (for reviews see Cohen, 1978; Algranati & Goldemberg, 1977). Furthermore, it has been demonstrated that polyamines can act as substrates for transglutaminases and as modulators of protein kinases. Thus it is possible that polyamines may participate in post-translational modifications of proteins (Folk *et al.*, 1980; Cochet & Chambaz, 1983).

During homogenization and fractionation of tissue, polyamines may be redistributed (Goyns, 1979), and until recently no methods for demonstrating polyamines cytochemically have been available. This has made interpretations of their physiological and cellular roles difficult.

We have developed two fluorescence cytochemical methods, the formaldehyde–fluorescamine and *o*-phthalaldehyde methods, which, although differing in chemical principles, selectively demonstrate the two main polyamines spermidine and spermine (Hougaard & Larsson, 1982; Larsson *et al.*, 1982). These methods reveal identical distributions of polyamines in the body, and this distribution closely corresponds to that obtained by chemical determinations (Hougaard & Larsson, 1982; Larsson *et al.*, 1982). The cytochemical distribution of polyamines in the prostate gland agrees not only with chemical determinations but also with the autoradiographic distribution of [3 H]spermidine and [3 H]spermine, formed after injections of the polyamine precursor [3 H]putrescine (Hougaard *et al.*, 1986). Numerous model experiments, chemical data and experiments with polyamine-biosynthesis inhibitors also corroborate the

specificity of these methods, as detailed elsewhere (Hougaard & Larsson, 1982; Larsson *et al.*, 1982).

When studying the distribution of polyamines in the normal body, we have been struck by the frequent occurrence of spermidine and spermine in endocrine and neuronal cell systems (Hougaard & Larsson, 1982; Larsson *et al.*, 1982). We now present data on the localization of polyamines in endocrine pancreatic islet cells, including chemical and cytochemical evidence and observations on glucose-sensitive polyamine biosynthesis in isolated pancreatic islets.

MATERIALS AND METHODS

Tissue material

Adult NMRI mice and Wistar rats were starved overnight and killed by decapitation under light diethyl ether anaesthesia. For cytochemistry, pancreatic specimens were frozen in melting Freon 22, freeze-dried, vapour-fixed in paraformaldehyde vapour at 80 °C for 1 h (Hougaard & Larsson, 1982; Larsson *et al.*, 1982) and embedded in paraffin *in vacuo*. Tissue specimens for chemical determination of polyamines and γ -aminobutyric acid were immediately frozen in liquid N₂ and stored at –80 °C.

Islet isolation and culture

The excised pancreases were rinsed in Hanks' balanced salt solution (Flow Laboratories, Irvine, Scotland, U.K.), adjusted to pH 7.4 with 25 mM-Hepes (HBSS), and treated with 1.5 mg of collagenase (type I; Sigma, St. Louis, MO, U.S.A.)/ml in HBSS for 15 min at 37 °C in a solid thermoregulator (Grants, Barrington, Cambridge, U.K.), mounted on a shaking table operated at 150 cycles/min. The islets were isolated in a Percoll (Pharmacia, Uppsala, Sweden) gradient and collected under a stereo-microscope with a braking pipette (Nielsen, 1982; Brunstedt *et al.*, 1984). About 50–100

islets were isolated from each adult mouse or rat pancreas. The islets were either frozen in liquid N₂ for chemical determination or cultured for 1 day in plastic Petri dishes in RPMI 1640 medium (Flow Laboratories) containing 11 mM-glucose, 20 mM-Hepes, 4 mM-NaHCO₃ and 10% (v/v) newborn-calf serum (Gibco, Paisley, Scotland, U.K.).

Islet cell preparation

Cell suspensions of isolated rat islets were prepared by treatment of the islets with 100 µg of trypsin (Sigma)/ml and 2 µg of deoxyribonuclease (Sigma)/ml and 2 mM-EDTA in RPMI 1640 for 15 min at 37 °C. The cells were dispersed by aspiration and centrifuged through 4% (w/v) bovine serum albumin (Lernmark *et al.*, 1984).

Incorporation of [³H]ornithine and [³H]putrescine

Islets were incubated for 24 or 48 h with 2–10 µCi of [2,3-³H]ornithine (NEN; 30 Ci/mmol)/ml in 'supplemented RPMI' (RPMI 1640 medium supplemented with 20 mM-Hepes, 4 mM-NaHCO₃ and 1 mg of human serum albumin/ml) containing 3.3 mM-, 11 mM- and 20 mM-glucose, washed twice in HBSS and frozen in liquid N₂. In addition, islets were incubated for 24 h with 10 µCi of [³H]ornithine/ml in supplemented RPMI containing 11 mM-glucose with or without 2 mM- α -difluoromethylornithine (generously given by Merrell International, Strasbourg, France), whereafter the samples (300 islets) were washed twice in HBSS before freezing.

Islets were also pulse-labelled for 2 h with 50 µCi of [2,3-³H]putrescine (NEN; 30 Ci/mmol)/ml in supplemented RPMI containing 11 mM-glucose and subsequently chased for 1, 2, 4, 24, 48 and 72 h in 2 ml of non-radioactive supplemented RPMI containing either 3.3 mM-glucose or 20 mM-glucose. The chase media were stored at -20 °C, and samples (100 islets) were washed twice in HBSS before freezing.

Polyamine and γ -aminobutyric acid separation and polyamine quantification

Tissue specimens, isolated islets and labelled islets were homogenized at 4 °C in 0.2 M-HClO₄ containing a known amount of internal standard, 1,6-diaminohexane (Sigma). The chase media were freeze-dried after addition of 1,6-diaminohexane, hydrolysed in 6 M-HCl at 110 °C for 16 h, Millipore-filtered, evaporated in vacuum, redissolved in 2 M-NaOH and shaken for 30 min with 4 vol. of butan-1-ol. The organic phases were washed with 0.25 vol. of 1 M-NaOH, evaporated in vacuum and the residues redissolved in 0.1 M-HCl. Calibration solutions were prepared by dissolving γ -aminobutyric acid (Sigma), putrescine dihydrochloride (Sigma), spermidine trihydrochloride (Fluka), spermine tetrahydrochloride (Sigma) and 1,6-diaminohexane in 0.2 M-HClO₄. The samples were dansylated (Seiler, 1975; Seiler *et al.*, 1978), and the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) derivatives (in toluene) were dried in vacuum.

For t.l.c. the evaporated residues were redissolved in toluene and spotted on pre-activated silica-gel plates. The polyamines were developed with ethyl acetate/cyclohexane (3:2, v/v) as solvent twice in the same direction. γ -Aminobutyric acid was separated from interfering spots by using benzene/cyclohexane/methanol (170:30:3, by vol.) in the first dimension and ethyl acetate/cyclohexane (3:2, v/v) in the second dimension

(Seiler, 1975). Spots corresponding to the positions of dansylated putrescine, spermidine, spermine and γ -aminobutyric acid were subjected to liquid-scintillation counting (Seiler, 1975).

Additionally, evaporated residues of the toluene phases were redissolved in methanol and separated and quantified by h.p.l.c. in a Waters h.p.l.c. system consisting of two 510 pumps, an U6K injector, a radial compression system Z-module equipped with a Radialpak C₈ (10 µm) column (internal diameter 8 mm), a 720 system controller, a 420 fluorescence detector (excitation 360 nm; emission 510 nm) and a 730 data module. The mobile phase consisted of (A) methanol and (B) 0.25 M-triethylammonium phosphate buffer, pH 3.5 (Brossat *et al.*, 1983). Polyamines and γ -aminobutyric acid were separated by a linear gradient from 40 to 90% of A over 23 min at a flow rate of 3 ml/min. Retention times were as follows: γ -aminobutyric acid 8.5 min, putrescine 14.3 min, 1,6-diaminohexane 15.6 min, spermidine 18.5 min and spermine 21.2 min. Polyamines were quantified by the internal-standard method. Fractions (1.5 ml) were collected and subjected to liquid-scintillation counting.

Protein determination

The HClO₄ precipitates of tissue specimens and isolated islets were resuspended in 1 M-NaOH and assayed for protein with bovine serum albumin as standard (Lowry *et al.*, 1951).

Cytochemistry

Formaldehyde-fluorescamine staining was performed as described by Hougaard & Larsson (1981). Deparaffinized 5 µm sections or smears of isolated islet cells were exposed to paraformaldehyde vapours at 80 °C for 130 min, rinsed in 0.2 M-borate buffer, pH 9.0, for 3 min, drained and exposed to the fluorescamine reagent (0.2 mg of Fluram/ml of acetone). The reagent was applied for 2 × 1.5 min with a pipette. Subsequently the slides were drained, washed in the borate buffer, dehydrated and mounted in Entellan (Merck) and examined in a Zeiss standard 18 epi-illumination microscope or a Leitz Orthoplan epi-illumination microscope by using excitation at 405 nm. *o*-Phthalaldehyde staining was performed as described by Hougaard & Larsson (1982). The 5 µm sections were mounted on chrome-alum/gelatine-coated slides, deparaffinized, exposed to paraformaldehyde vapours at 80 °C for 60 min and subsequently covered with the *o*-phthalaldehyde reagent (0.08% in water adjusted to pH 8 with 0.05 M-NaOH) and incubated at 60 °C for 60 min. Finally the slides were mounted in glycerine/phosphate buffer, pH 2.4 (1:1 v/v), and examined in the microscope by using excitation at 405 nm.

Immunocytochemistry

Formaldehyde-fluorescamine or *o*-phthalaldehyde-stained sections were occasionally restained for immunocytochemical identification of insulin, glucagon, somatostatin and pancreatic polypeptide cells, by using specific antisera and the peroxidase-antiperoxidase procedure (Sternberger, 1979) as detailed elsewhere (Larsson, 1983). Controls included absorptions of the antisera against the appropriate antigens as well as conventional staining controls as recommended (Larsson, 1983).

Fig. 1. Fluorescence cytochemical localization of polyamines in rat pancreas by the formaldehyde-fluorescamine method

(a) Section of freeze-dried formaldehyde-vapour-fixed pancreas. Note the fluorescence in zymogen granules of exocrine cells as well as in cytoplasm of pancreatic islet cells, which by restaining can be identified as insulin cells. Magnification $\times 190$. (b) Isolated rat pancreatic islet cells stained by the formaldehyde-fluorescamine method. Note the distinct granular localization of the polyamines. Magnification $\times 1500$.

Table 1. Concentrations of spermidine and spermine in whole pancreas and in isolated islets

Polyamines were quantified by h.p.l.c. as described in the Materials and methods section. Results are means $\pm$ s.d. for n experiments.

		n	Spermidine (nmol/mg of protein)	Spermine (nmol/mg of protein)	Spermidine/ spermine ratio
Rat	Islets	4	12.8 ± 1.7	20.5 ± 3.1	0.63 ± 0.09
	Whole pancreas	3	22.6 ± 2.4	6.74 ± 1.6	3.42 ± 0.44
Mouse	Islets	3	28.1 ± 5.6	17.2 ± 6.8	1.75 ± 0.43
	Whole pancreas	3	22.1 ± 4.4	3.24 ± 0.50	7.10 ± 2.5

RESULTS

In the pancreas of mice and rats, strong formaldehyde-fluorescamine- and *o*-phthalaldehyde-induced fluorescence was displayed by islet cells, which by restaining could be shown to correspond to insulin-producing β -cells. Also the acinar cells in the exocrine pancreas displayed strong fluorescence. In both β -cells and acinar cells the fluorescence was localized to cytoplasmic granules, with little or no nuclear fluorescence (Fig. 1).

Chemical polyamine determinations were first carried out on whole rat and mouse pancreas by h.p.l.c. In agreement with previous findings (Larsson *et al.*, 1982), very high polyamine concentrations were recorded, along with a high spermidine/spermine ratio (mouse 7:1, rat 3:1). Subsequent determinations on isolated rat and mouse pancreatic islets showed lower (rat) and slightly higher (mouse) concentrations of spermidine and much

higher spermine concentrations compared with total pancreatic concentrations (Table 1). Thus the ratio of spermidine to spermine (mouse 2:1, rat 0.6:1) was remarkably different from that seen in whole pancreas.

Incubation of isolated rat pancreatic islets with [3 H]ornithine in medium containing 11 mM-glucose for 24 h resulted in significant incorporation of label into putrescine and spermidine (separated by t.l.c.). This incorporation was decreased by 70% when the polyamine-biosynthesis inhibitor α -difluoromethylornithine was included in the medium. The degree of incorporation of 3 H label into putrescine and spermidine in the islets was dependent on the glucose concentration in the medium. When islets were incubated at a high glucose concentration (20 mM) for 24 or 48 h, the radioactivity in putrescine and spermidine fractions (separated by h.p.l.c.) was 3 times higher than when incubation was performed in low glucose (3.3 mM) (Table 2). As shown

Table 2. Effects of glucose on the radioactivity in putrescine and spermidine fractions in islets incubated with [³H]ornithine

Samples of approx. 100 islets were incubated for 24 or 48 h in 'supplemented RPMI' containing [³H]ornithine and 3.3 mM- or 20 mM-glucose. The polyamines were separated by h.p.l.c. and subjected to liquid-scintillation counting as described in the Materials and methods section. Significance of $b > a$ (for islets incubated with 10 μ Ci of [³H]ornithine/ml of medium): putrescine fractions, $P < 0.057$; spermidine fractions, $P < 0.028$ (Mann-Whitney U test). Abbreviation: N.D., not determined.

[³ H]Ornithine (μ Ci/ml of medium)	Time of incubation (h)	[Glucose] ...	Radioactivity (d.p.m./100 islets)					
			Putrescine fractions			Spermidine fractions		
			3.3 mM (a)	20 mM (b)	(b/a)	3.3 mM (a)	20 mM (b)	(b/a)
10	24		1450	4611	3.18	1100	3833	3.48
	48		1494	3211	2.15	1333	2694	2.02
10	24		N.D.	1417	—	N.D.	9494	—
	48		889	4056	4.56	2789	8611	3.09
2	24		556	639	1.15	400	1856	4.64
	48		144	506	3.51	527	1683	3.19

Table 3. Effects of glucose concentration on specific radioactivity of putrescine and spermidine in islets incubated with [³H]ornithine

The experiment was carried out as described in Table 2.

Time of incubation (h)	Concn. of glucose (mM)	Specific radioactivity (d.p.m./nmol)	
		Putrescine	Spermidine
24	3.3	6300	1500
	20	9500	5300
48	3.3	3500	2800
	20	7400	6000

in Table 3, part of this increase was due to an increased specific radioactivity of putrescine and spermidine. No radioactivity was observed in spermine fractions.

When islets were incubated with [³H]putrescine, substantial radioactivity was found in the spermidine fraction (separated by t.l.c. and h.p.l.c.) already after 1 h.

After 2 h of incubation the specific radioactivity of spermidine reached $10.8 (\pm 0.9) \times 10^5$ d.p.m./nmol. The incorporation of ³H label into spermidine and also into spermine was dependent on the glucose concentration in the medium. Thus, when the [³H]putrescine-pulsed islets were incubated for 2–4 h in non-radioactive medium with a high glucose concentration (20 mM), the radioactivity in the spermidine fraction was higher than in islets incubated at a low glucose concentration (3.3 mM). After 24 h of incubation in high glucose, the radioactivity in the spermidine fraction was twice that in islets incubated at low glucose (Table 4). At this time significant radioactivity occurred in the spermine fraction too, and islets incubated in 20 mM-glucose contained 4–11 times as much radioactivity in this fraction as did islets incubated in 3.3 mM-glucose (Table 4).

In [³H]putrescine-labelled islets significant radioactivity also appeared in t.l.c. spots and h.p.l.c. fractions occurring in the positions of γ -aminobutyric acid. The amount of radioactivity in these fractions was constantly about 3–5% of that in the putrescine fractions and steadily decreased to zero during the incubation period, independent of the glucose concentration in the medium.

Table 4. Effects of glucose concentration on radioactivity in spermidine and spermine fractions of islets preincubated with [³H]putrescine and chased for 24 h

Samples of 100 islets were pulse-labelled for 2 h in 'supplemented RPMI' containing [³H]putrescine and 11 mM-glucose, and subsequently chased for 24 h in 'supplemented RPMI' containing 3.3 mM- or 20 mM-glucose. The polyamines were separated by h.p.l.c. and subjected to liquid-scintillation counting as described in the Materials and methods section. Data are presented for two experiments.

[Glucose] ...	Radioactivity (d.p.m./100 islets)					
	Spermidine fractions			Spermine fractions		
	3.3 mM (a)	20 mM (b)	b/a	3.3 mM (a)	20 mM (b)	b/a
	67400	114900	1.70	1900	6640	3.49
	85100	196100	2.31	1220	13900	11.4

DISCUSSION

Our data show that, in the rat and mouse, spermine and spermidine respectively occur in high concentrations in islets. This agrees with findings by Bungay *et al.* (1984). We have localized the polyamines to insulin-producing cells and have found that polyamine biosynthesis *in vitro* is stimulated by glucose. Glucose is the major stimulus for insulin release and also stimulates islet-cell replication (Andersson, 1975; Swenne, 1982). Unlike in most other mammalian cells, protein synthesis in islet cells is stimulated by glucose within physiological concentrations of 2–20 mM (Giddings *et al.*, 1985; Andersson *et al.*, 1976). Polyamines are known to be important for both protein biosynthesis and cell division. Moreover, they are substrates for transglutaminases, which are known to be present in insulin cells and have been postulated to be important for release of the hormone (Folk *et al.*, 1980; Gomis *et al.*, 1983). Thus the stimulation of polyamine biosynthesis by glucose may be related to any of these three mechanisms. Interestingly, our data show that glucose stimulates polyamine biosynthesis not only from [³H]ornithine but also from [³H]putrescine. Thus it is possible that glucose affects the activity or amount of both ornithine decarboxylase and polyamine-biosynthetic enzymes distal to this step (e.g. *S*-adenosylmethionine decarboxylase, spermidine synthase or spermine synthase). Whether this action is mediated by glucose directly or indirectly is unknown. Moreover, it cannot be excluded that polyamines or their derivatives may be released from islet cells as intercellular messengers or cybernins. This theory is supported by our findings in hydrolysed media after 24–72 h of incubation of nanomolar concentrations of spermidine and spermine. In such media the spermidine/spermine ratio was 3–6 times higher than in the corresponding islets. However, it has so far not been possible to establish any correlation between the glucose and polyamine concentrations in the media (results not shown). We have also found that about 5% of [³H]putrescine is converted into the neurotransmitter γ -aminobutyrate. A similar biosynthetic pathway has previously been described in neural tissue (Seiler, 1980). Moreover, insulin-producing cells have been shown to contain γ -aminobutyrate and the enzyme glutamate decarboxylase, which is known to be responsible for γ -aminobutyrate synthesis. It is thus possible that within the insulin-producing cells both glutamate decarboxylase and the putrescine pathway for γ -aminobutyrate formation exist. Our cytochemical results clearly show that most of the polyamines are restricted to the insulin-producing cell cytoplasm, where they occur in secretory granules. Thus the weight of evidence points to a role for the polyamines in transglutaminase-mediated insulin release and/or as intercellular messengers.

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