The inhibition of glucose-stimulated insulin secretion by primary amines

A role for transglutaminase in the secretory mechanism

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1. Rat pancreatic islets contain a Ca^{2+} -activated and thiol-dependent transglutaminase (EC 2.3.2.13) comparable in activity with that found in rat liver, lung and spleen. The Ca^{2+} -dependence of this enzyme is such that half-maximal velocity was obtained in the region of $40\ \mu\text{M}$. 2. Preincubation of rat islets with primary-amine substrates of transglutaminase (monodansylcadaverine, methylamine, ethylamine, propylamine and cystamine) led to an inhibition of glucose-stimulated insulin release by these amines. 3. Kinetic analysis of the competitive substrates methylamine, monodansylcadaverine, propylamine and ethylamine for their ability to inhibit islet transglutaminase activity indicated a potency that matched their ability to inhibit glucose-stimulated insulin release. 4. When these amines were tested for their effects on glucose-stimulated protein synthesis and glucose utilization, the most potent inhibitor of insulin release, monodansylcadaverine, had no effect on either process at $100\ \mu\text{M}$. The amines cystamine, ethylamine, methylamine and propylamine had variable effects on these metabolic processes. For ethylamine, methylamine and propylamine, concentrations were found which inhibited glucose-stimulated insulin release in a manner which was found to be independent of their effects on either glucose oxidation or protein synthesis. 5. Primary amines may therefore inhibit insulin release through their incorporation by islet transglutaminase into normal cross-linking sites. A role for protein cross-linking in the secretory mechanism is suggested.

Reactions catalysed by the Ca^{2+} -dependent enzyme transglutaminase (protein-glutamine: amine γ -glutamyltransferase, EC 2.3.2.13) lead to the post-translational modification of a protein either through the specific incorporation of amines into the γ -carboxamide groups of glutamine, or by the cross-linking of polypeptide chains by ϵ -(γ -glutamyl)lysine bridges (Mycek *et al.*, 1959; Lorand & Stenberg, 1976). Although transglutaminase enzymes occur widely in Nature (Folk & Finlayson, 1977), the function of the most widespread group of these enzymes, the 'tissue transglutaminase' (Chung, 1972), is still poorly understood. Studies with this transglutaminase are now suggesting that it may play an important role in the

mediation of events at the cell membrane. Observations supporting this idea include the demonstration of ϵ -(γ -glutamyl)lysine cross-links in cell-membrane proteins (Birckbichler *et al.*, 1973), the specificity of erythrocyte transglutaminase in the cross-linking of membrane proteins and associated structures (Siefring *et al.*, 1978; Bjerrum *et al.*, 1981), the cross-linking of fibronectin (Birckbichler & Patterson, 1978) and the cross-linking of β_2 -microglobulin (Fésüs *et al.*, 1981).

The physiological significance of these observations is as yet still unclear, but a functional role has been indicated for the enzyme in the membrane interactions involved in receptor-mediated endocytosis (Davies *et al.*, 1980). Evidence for this role was first suggested by the ability of competitive amine substrates of the enzyme to inhibit the receptor-mediated endocytosis of α_2 -macroglobulin and epidermal growth factor. This finding was later extended to the receptor-mediated endocytosis of low-density lipoprotein (Pastan &

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; KRB medium, Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932).

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Willingham, 1981), phage T3 (Cheng *et al.*, 1980) and *Shigella* toxin (Keusch, 1981).

Although some controversy exists over the site and mode of action of primary amines in this process (King *et al.*, 1980; Kaplan & Keogh, 1981), a functional role for transglutaminase in the receptor-recycling mechanism has now been indicated (Van Leuven *et al.*, 1980).

To test the involvement of transglutaminase in another cellular event involving specific membrane interactions, we have investigated a role for transglutaminase in the Ca^{2+} -mediated mechanism of stimulus-secretion coupling in the pancreatic β -cell (Wollheim & Sharp, 1982). Like receptor-mediated endocytosis, a rise in the cytosolic concentration of free Ca^{2+} plays a key role in this cellular event, but the mechanism whereby Ca^{2+} elicits the exocytotic release of insulin and its overall role in the secretory cycle is still unknown.

Evidence to suggest that transglutaminase may be involved in the membrane interactions occurring during glucose-stimulated insulin secretion has been indicated by our initial investigations, which have demonstrated the inhibition of glucose-stimulated insulin release from pancreatic islets by primary-amine substrates of transglutaminase (Griffin *et al.*, 1982; Bungay *et al.*, 1983). In the present paper we have extended this work and provided further evidence to suggest that the pancreatic-islet transglutaminase may play an important role in the secretory mechanism for insulin release.

Experimental

Materials

Collagenase used in the isolation of pancreatic islets was obtained from Serva Feinbiochemica, Heidelberg, W. Germany. Radiochemicals ($\text{D-}[U\text{-}^{14}\text{C}]\text{glucose}$, $\text{L-}[4,5\text{-}^3\text{H}]\text{leucine}$, $[1,4\text{-}^{14}\text{C}]\text{putrescine}$ and $[6,6'(\text{n})\text{-}^3\text{H}]\text{sucrose}$) were purchased from Amersham International, Amersham, Bucks., U.K., except for ^{125}I -labelled insulin, which was obtained from Novo, Bagsvaerd, Denmark. Methylamine hydrochloride, ethylamine, propylamine, cycloheximide and bovine serum albumin were obtained from Sigma Chemical Co., Poole, Dorset, U.K., Cystamine was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and monodansylcadaverine was from Fluka A.G., Buchs, Switzerland. The dimethylated analogue of monodansylcadaverine was kindly given by Professor Laszlo Lorand, Northwestern University, Evanston, IL, U.S.A. Standard rat insulin and insulin antibody were obtained from Novo, and human albumin was from Hoechst (U.K.), Hounslow, Middx., U.K.

Methods

Isolation of islets of Langerhans. Islets of Langerhans were isolated from the pancreas of 200–300g Sprague-Dawley rats by a collagenase digestion technique similar to that of Lacy & Kostianovski (1967). For the studies of insulin release, glucose oxidation and protein synthesis, islets were incubated at 37°C immediately after isolation in KRB medium containing 16.8mM-glucose for 5min, followed by 60min in the same medium containing 2.8mM-glucose in an atmosphere of O_2/CO_2 (19:1) before grouping for the experimental incubations.

Studies on insulin release. Batches of five islets were incubated at 37°C in KRB medium containing bovine serum albumin (5mg/ml) in an atmosphere of O_2/CO_2 (19:1). The incubations were for 45min in 0.5ml of KRB containing 2.8mM-glucose, followed by 60min in 1.0ml of KRB containing 16.8mM-glucose. The change to the higher glucose concentration was achieved by the addition of 0.5ml of KRB containing 30.5mM-glucose. For the effects of test compounds on basal rates of insulin release, 0.5ml of KRB containing 2.8mM-glucose was added instead. Experiments to determine the effects of test compounds on insulin release were performed with the concentrations of the test compounds shown in the Tables and Figures throughout the text; test compounds were present throughout the incubation period of 105min. The preincubation period of 45min was intended to allow test compounds to enter the islet cells before stimulation. After incubation, samples of medium were taken and stored at -20°C until radioimmunoassay was performed to determine insulin release (Heding, 1972). Before radioimmunoassay, serial dilutions ranging from 1:5 to 1:50 were carried out on the test samples. This allowed insulin measurements to be made on the linear part of the standard curve, and also tested for linearity between the respective dilutions made and the amount of insulin present. This acted as a preliminary screen for any interference by test compounds on the insulin assay.

Measurement of glucose oxidation by islets. Glucose oxidation was measured by determining the amount of $^{14}\text{CO}_2$ released from $[U\text{-}^{14}\text{C}]\text{glucose}$, a method essentially that of Malaisse *et al.* (1974); 10–20 islets were incubated in small plastic wells suspended in 10ml glass beakers, which were sealed with self-sealing rubber stoppers. Islets were preincubated in the presence or absence of test compounds for 45min in 50 μl of KRB incubation medium containing 2.8mM-glucose in an atmosphere of O_2/CO_2 (19:1) before the final addition of 50 μl of 30.5mM-glucose in KRB containing $[U\text{-}^{14}\text{C}]\text{glucose}$ (final sp. radioactivity 0.595Ci/mol). After incubation for 1h, 0.2ml of Hyamine hydroxide was added to the glass beaker to absorb

any released $^{14}\text{CO}_2$, and $50\mu\text{l}$ of 0.2M-HCl was injected into the islet incubation medium to release any further trapped $^{14}\text{CO}_2$. This amount of HCl proved to be adequate for lowering the pH of the medium to below 1.0 even in the incubations containing test compounds at their highest concentrations. After a further incubation period of 30 min after addition of the acid, the plastic wells were removed and the Hyamine hydroxide was diluted to 1.0ml with distilled water and then counted for radioactivity after addition of 10ml of Instagel (Packard). Counting efficiency was determined by internal standardization.

Measurement of total islet protein synthesis. Protein synthesis in intact islets was measured by the incorporation of $[^3\text{H}]$ leucine into trichloroacetic acid-precipitable protein. Batches of 10–20 islets were incubated at 37°C for 45 min in $50\mu\text{l}$ of KRB containing 2.8mM-glucose in the presence of test compound, followed by 60–90 min in $16.8\text{mM-glucose/KRB}$ medium containing $5\mu\text{Ci}$ of $[^3\text{H}]$ -leucine (55 or 60Ci/mmol) in a final volume of $100\mu\text{l}$. After incubation, $100\mu\text{l}$ of ice-cold 20% (w/v) trichloroacetic acid was added, the mixture was sonicated (two bursts of 5 s, $3\mu\text{m}$ peak-to-peak) and the resulting precipitate washed three times in 5% (w/v) ice-cold trichloroacetic acid containing 2mM-leucine , once in acetone/ethanol (1:1, v/v) and once in acetone. The dried precipitate was solubilized in 0.1ml of Soluene 350 (Packard) and counted for radioactivity in 1.0ml of Dimilume (Packard). Counting efficiency was determined by using the Quench Indicating Parameter of Packard.

Measurement of $[^3\text{H}]$ leucine uptake into islets. Uptake of leucine was measured by a method essentially that of Wollheim *et al.* (1978). Incubations were performed in $400\mu\text{l}$ polyethylene microcentrifuge tubes (Alpha Laboratories). Tubes contained $20\mu\text{l}$ of 6M-urea , which was overlaid with $200\mu\text{l}$ of a mixture containing dibutyl phthalate and dinonyl phthalate (10:3, v/v). KRB medium ($50\mu\text{l}$) containing batches of 10–20 islets was placed above the phthalate ester layer but separated from it by a small air space. Incubations were for 45 min in $50\mu\text{l}$ of KRB medium containing 2.8mM-glucose , followed by 60 min in $100\mu\text{l}$ of KRB medium containing 16.8mM-glucose and $5\mu\text{Ci}$ of $[^3\text{H}]$ leucine (55Ci/mol). The appropriate concentration of amine together with cycloheximide ($100\mu\text{M}$) was present throughout each period of incubation of $45 + 60\text{min}$. Incubations were terminated by sedimenting the islets for 3 s at $9390g_{\text{av}}$ in a Beckman microcentrifuge. The bottoms of the tubes containing the islets were cut off into suitable counting vials, left for 30 min to allow dissolution of the islets and then counted for radioactivity after addition of 10ml of Instagel. To

enable the true uptake of $[^3\text{H}]$ leucine into the islet cells to be calculated, the apparent uptake corresponding to islet extracellular space was also determined and subtracted accordingly. Islet extracellular space was measured with $[^3\text{H}]$ sucrose as an extracellular marker. Batches of 15 islets were incubated with $5\mu\text{Ci}$ of $[^3\text{H}]$ sucrose (9.8Ci/mmol) for 30 and 45 min in KRB medium ($50\mu\text{l}$) containing 2.8mM-glucose . The methodology used was essentially that described for measurement of $[^3\text{H}]$ -leucine uptake. The average sucrose space was calculated as $2.84 \pm 0.27\text{nl/islet}$ ($n = 11$). Counting efficiency was determined by using the Quench Indicating Parameter of Packard. Statistical significance was determined by Student's *t* test.

Homogenization of tissues. Homogenates of islets of Langerhans were prepared by sonication (two bursts of 5 s, $3\mu\text{m}$ peak-to-peak) in ice-cold $0.25\text{M-sucrose}/1\text{mM-Tris}/\text{HCl}/1\text{mM-EDTA}$, pH 7.4 (one to two islets/ μl). For cellular-distribution studies, homogenates were centrifuged at $71000g_{\text{av}}$ for 45 min to obtain particulate and particle-free-supernatant fractions. Homogenates of rat liver, lung and spleen were prepared in the same sucrose buffer with a Potter–Elvehjem homogenizer.

Transglutaminase assay. Transglutaminase activity was measured by the incorporation of $[^{14}\text{C}]$ -putrescine into *NN'*-dimethylcasein (Lorand *et al.*, 1972). The incubation mixture, at 37°C , consisted of $28\text{mM-Tris}/\text{HCl}$, pH 7.4, $3.85\text{mM-dithiothreitol}$, 2.5mM-CaCl_2 or 5mM-EDTA , *NN'*-dimethylcasein (5mg/ml) and $1.2\text{mM}-[^{14}\text{C}]$ putrescine (3.96Ci/mol) and $22.5\mu\text{l}$ of islet preparation in a final volume of $50\mu\text{l}$. Samples ($10\mu\text{l}$) were taken for the counting of trichloroacetic acid-insoluble radioactivity at appropriate time intervals, and rates of putrescine incorporation were determined after subtraction of EDTA controls. For enzyme assays investigating the effect of thiol-reactive inhibitors on the enzyme, dithiothreitol was omitted from the assay. Protein was determined by the method of Lowry *et al.* (1951).

Measurement of the effect of Ca^{2+} on transglutaminase activity. For this, islet supernatants were prepared as described above, except that 1mM-EGTA was substituted for EDTA in the homogenization buffer. Addition of $22.5\mu\text{l}$ of islet preparation to a final assay volume of $50\mu\text{l}$ then gave a final concentration of EGTA in the assay mixture of 0.45mM . Different amounts of CaCl_2 were then added to the assay medium in excess of the EGTA present, so that the free Ca^{2+} concentrations present ranged from $25\mu\text{M}$ to 2.0mM . For calculation of the free Ca^{2+} concentrations, an association constant at pH 7.4 of $10^{7.12}$ for $[\text{CaEGTA}]/[\text{Ca}][\text{EGTA}]$ was used (Schatzman, 1973).

Results

Characterization of islet transglutaminase

When islet homogenates were assayed for transglutaminase activity by [14 C]putrescine incorporation into *NN'*-dimethylcasein, a Ca^{2+} -dependent enzyme of activity comparable with that found in homogenates of rat liver, lung and spleen could be demonstrated (Table 1). The islet transglutaminase displayed no observable activity when Mg^{2+} (2.5 mM) was substituted for Ca^{2+} in the enzyme assay, indicating the Ca^{2+} -specificity of this enzyme.

Fractionation of the islet homogenate ($n = 4$) into a particulate and a cytosolic fraction by centrifugation at $71\,000g_{\text{av}}$ for 45 min indicated that most of the enzyme activity ($94 \pm 3\%$) was present in the cytosolic fraction. Subsequent studies on the enzyme were therefore performed with this supernatant fraction.

Further characterization of the islet enzyme as a typical transglutaminase was performed by investigation of the thiol-dependence and Ca^{2+} -activation of the enzyme. Measurement of enzyme activity in the presence of different concentrations of the thiol-reactive compounds *p*-chloromercuribenzoic acid or iodoacetamide ($1\text{--}50\ \mu\text{M}$) indicated that the enzyme was very susceptible to these two compounds, with concentrations of $2\ \mu\text{M}$ -iodoacetamide and $15\ \mu\text{M}$ -*p*-chloromercuribenzoic acid giving approx. 80% loss of enzyme activity. Further, when enzyme activity was measured in the presence of different concentrations ($0.025\text{--}2.0\text{ mM}$) of the amine cystamine, an inhibitor of the enzyme which is thought to undergo a disulphide exchange with the active thiol group of transglutaminase enzymes (Siefring *et al.*, 1978), a progressive decrease in enzyme activity was observed with increasing cystamine concentrations, with a 50% loss of enzyme activity occurring at 0.6 mM -cystamine.

Table 1. Activity of transglutaminase in homogenates of various rat tissues

Homogenates of rat liver, lung, spleen and islets of Langerhans were prepared in 0.25 M -sucrose/ 1 mM -Tris/HCl/ 1 mM -EDTA, pH 7.4, and assayed for transglutaminase activity as described in the Experimental section. The numbers of determinations are shown in parentheses. Unit of enzyme activity is 1 nmol of putrescine incorporated/h.

Tissue	Transglutaminase activity (units/mg of protein)
Liver	33.2 ± 3.5 (3)
Lung	31.5 ± 4.2 (4)
Spleen	30.0 ± 0.6 (3)
Pancreatic islets	26.2 ± 5.7 (6)

Fig. 1. Ca^{2+} activation of pancreatic-islet transglutaminase. Transglutaminase activity was measured by incorporation of [14 C]putrescine into *NN'*-dimethylcasein at 5 (●), 10 (○) and 30 min (▲) with variable concentrations of free Ca^{2+} from 25 to $2000\ \mu\text{M}$ as described in the Experimental section.

The demonstration of Ca^{2+} as an activator of islet transglutaminase was performed by titrating enzyme activity with various concentrations of free Ca^{2+} in the range $25\ \mu\text{M}\text{--}2\text{ mM}$. As demonstrated in Fig. 1, the islet enzyme was readily activated by small additions of Ca^{2+} in the concentration range $25\text{--}100\ \mu\text{M}$. If it is assumed that the *NN'*-dimethylcasein present in the assay has negligible effect on the amount of free Ca^{2+} present, then treatment of the data by the Lineweaver–Burk method gave the enzyme an apparent half-saturation value for Ca^{2+} of $39\ \mu\text{M}$.

Inhibition of islet transglutaminase activity by primary amines

To determine the potency of potential inhibitors of islet transglutaminase activity, which could later be compared with the inhibitors' effects on glucose-stimulated insulin release, various primary-amine substrates of the enzyme were tested for their effects on [14 C]putrescine incorporation into *NN'*-dimethylcasein catalysed by islet transglutaminase. The apparent inhibition constants (K_i) for each of the compounds monodansylcadaverine, methylamine, ethylamine and propylamine were calculated by the method of Dixon (Dixon & Webb, 1964) from data prepared from Lineweaver–Burk plots. The apparent K_m for putrescine for the islet transglutaminase was calculated as $0.59 \pm 0.10\text{ mM}$ ($n = 8$) from these data. Example plots for the results obtained for monodansylcadaverine and the alkylamine methylamine are illustrated in Fig. 2. The plots obtained for mono-

dansylcadaverine, methylamine, ethylamine and propylamine with three different concentrations of each inhibitor were all found to intersect at $1/V_{\max}$ on the $1/v$ axis of the Lineweaver–Burk plot, a result in keeping with the findings of others (Lorand *et al.*, 1979) for the competitive nature of these amines when measured under these conditions. Dixon plots of $1/v$ against $[I]$ for calculation of K_i values were linear in each case.

Of the amines tested, monodansylcadaverine was the most potent inhibitor of islet transglutaminase (K_i 0.0073 mM), followed by ethylamine (K_i 1.75 mM), methylamine (K_i 2.00 mM) and propylamine (K_i 3.8 mM).

Effects of primary-amine inhibitors of transglutaminase on glucose-stimulated insulin release from intact islets

To test the hypothesis that the pancreatic-islet transglutaminase may be involved in the mechanism of insulin release from the islet β -cell, primary-amine inhibitors of islet transglutaminase activity were tested for their effects on glucose-stimulated insulin release. Insulin release measured in batch-type incubations of islets was normally increased 6–10-fold when the glucose concentration in the incubation medium was raised from 2.8 to 16.8 mM, the variation in the capacity of islet populations to respond to glucose being a function of different islet populations used from different isolations. To minimize this inconsistency when examining the effects of different inhibitors on insulin release, control rates of basal and stimulated insulin release were always determined for each batch of islets that was tested.

The effects of primary amines on insulin release are shown in Table 2. With reference to the work of Davies *et al.* (1980), the amines were chosen not only for their ability to inhibit transglutaminase but also on the basis of their likelihood to gain entry to the islet β -cell.

Of the amines tested, all appeared to inhibit glucose-stimulated insulin release from islets incubated at 16.8 mM-glucose in a dose-dependent manner. Incubation of cystamine, ethylamine or methylamine with rat insulin standards during the radioimmunoassay indicated that these amines had no effect on the insulin assay when tested at the maximum concentrations used in the inhibition studies. This rules out the possibility that the observed inhibition of insulin release by these amines might be due to interference with insulin measurement. When the amines were tested for their effects on the basal release of insulin in the presence of 2.8 mM-glucose for a total time period of 105 min (45 + 60 min), monodansylcadaverine (0.1 mM), cystamine (1 mM) and propylamine (10 mM) had no significant effect. Methylamine,

Fig. 2. Lineweaver–Burk plots for the inhibition of pancreatic-islet transglutaminase by monodansylcadaverine and methylamine

Islet supernatant prepared by the protocol described in the Experimental section was assayed for transglutaminase activity by [14 C]putrescine incorporation into *NN'*-dimethylcasein by using different concentrations of putrescine (1.2, 0.5, 0.3 and 0.2 mM). (a) Transglutaminase activity in the presence of various concentrations of monodansylcadaverine: Δ , 5 μ M; $\circ$, 10 μ M; $\blacktriangle$, 20 μ M. (b) Transglutaminase activity in the presence of various concentrations of methylamine; Δ , 2 mM; $\circ$, 5 mM; $\blacktriangle$, 10 mM. The symbol $\bullet$ represents the control system without inhibitor. Unit of enzyme activity is 1 nmol of putrescine incorporated/h.

however showed a small but significant stimulation of insulin release when tested under these conditions (60% increase over control basal rates; $n = 4$; $P \leq 0.001$), a result that we are unable to explain at present. The most potent inhibitor of transglutaminase activity, monodansylcadaverine, also proved to be the most potent inhibitor of glucose-stimulated insulin release, and was effec-

Table 2. *Effects of test compounds on glucose-stimulated insulin release*

Batches of five islets were incubated in KRB medium, in the presence or absence of the concentrations of primary amines stated, according to the protocol described in the Experimental section. Insulin released into the medium was determined by radioimmunoassay. Stimulation of insulin release in control and test incubations over a 60 min period was determined by subtracting basal rates of insulin release in control incubations performed simultaneously over the same total time period of 45 + 60 min. The inhibition of glucose-stimulated release for each amine tested is then expressed as the mean percentage ($\pm$ S.E.M.) of that in control incubations, for the numbers of determinations shown in parentheses. The significance of difference from controls was determined by Student's *t* test; N.S. denotes no significant difference from controls. Typical rates of insulin release (μ units/h per islet) from isolated islets were 8.9 ± 0.8 ($n = 56$) at 2.8 mM-glucose and 95.4 ± 6.2 ($n = 49$) at 16.8 mM-glucose.

Test compound	Concn. (mM)	Inhibition of glucose-stimulated insulin release (%)
Monodansylcadaverine	0.01	31 ± 8 (4) $P \leq 0.05$
	0.02	41 ± 1 (4) $P \leq 0.01$
	0.05	90 ± 12 (6) $P \leq 0.01$
	0.10	91 ± 8 (4) $P \leq 0.001$
Cystamine	0.10	47 ± 17 (6) $P \leq 0.25$
	0.50	79 ± 10 (6) $P \leq 0.05$
	1.0	98 ± 4 (6) $P \leq 0.002$
Methylamine	1.0	51 ± 5 (6) $P \leq 0.001$
	5.0	78 ± 8 (4) $P \leq 0.01$
	15.0	99 ± 6 (4) $P \leq 0.001$
Propylamine	0.5	61 ± 6 (5) $P \leq 0.01$
	1.0	47 ± 11 (4) $P \leq 0.05$
	5.0	95 ± 2 (5) $P \leq 0.001$
Ethylamine	1.0	50 ± 9 (7) $P \leq 0.005$
	5.0	81 ± 8 (6) $P \leq 0.001$
Cycloheximide	0.01	19 ± 7 (5) N.S.
N'-Dimethyldansylcadaverine	0.1	24 ± 9 (3) $P \leq 0.1$

tive over the concentration range 10–100 μ M. The dimethyl analogue of monodansylcadaverine, N'-dimethyldansylcadaverine, showed only $24 \pm 9\%$ ($n = 3$; $P \leq 0.1$) inhibition of glucose-stimulated insulin release at 100 μ M. Of the other amines tested, cystamine proved to be an effective inhibitor of insulin release over the concentration range 0.1–1.0 mM, whereas methylamine, propylamine and ethylamine were of a similar potency to each other, being effective in the concentration range 1.0–5.0 mM. Reference to the K_i values calculated for monodansylcadaverine, methylamine, ethylamine and propylamine for their effects on islet transglutaminase activity indicates that these concentration ranges are in the same order of magnitude with regard to their effects on insulin release.

Effects of primary amines on islet glucose oxidation and synthesis of total islet protein

To exclude the possibility that primary amines may inhibit insulin release by non-specific mechanisms, the effects of amines on two other glucose-stimulated parameters, namely glucose oxidation and protein synthesis, were investigated. In our experiments, increasing the glucose concentration from 2.8 mM to 16.8 mM stimulated glucose oxidation 7–10-fold and protein synthesis 3–4-fold. To minimize errors between experiments, control rates of glucose oxidation and protein synthesis were always determined for untreated islets in each investigation.

Table 3 shows that the amines displayed a variation in their effects on $^{14}\text{CO}_2$ release from [^{14}C]glucose and on [^3H]leucine incorporation into total islet protein when tested at the concentrations used in the experiments on insulin release. At 100 μ M, monodansylcadaverine did not significantly inhibit either glucose oxidation or protein synthesis in stimulated islets. Cystamine, however, was a potent inhibitor of glucose oxidation, causing almost total inhibition of the stimulated rate at 1.0 mM and 24% inhibition at 0.1 mM. Methylamine, ethylamine and propylamine also inhibited glucose oxidation in glucose-stimulated islets, but with a far lower potency than cystamine. Only at concentrations below 5 mM for methylamine and ethylamine and 1 mM for propylamine did these amines show no significant effect on glucose utilization in stimulated islets.

When these primary amines were tested for their effects on glucose-stimulated incorporation of [^3H]leucine into total islet protein, only at 1 mM did methylamine and propylamine show no significant inhibitory effect on this process. At the same concentration, however, ethylamine still gave 38% inhibition of [^3H]leucine incorporation. When glucose-stimulated uptake of [^3H]leucine into islets was measured in the presence and absence of methylamine and ethylamine, but in the presence of 0.1 mM-cycloheximide to equalize any 'metabolic pull' from protein synthesis, inhibition of [^3H]leucine uptake was observed when the amines were present at 5 mM. Comparison with the rate of uptake of [^3H]leucine measured for controls (0.317 ± 0.059 pmol/h per 10 islets, $n = 5$) gave percentage inhibition values of 48 ± 9 ($n = 5$, $P \leq 0.05$) for 5 mM-ethylamine and 35 ± 17 ($n = 5$, $P \leq 0.25$) for 5 mM-methylamine. When the concentrations of these amines in the incubation medium were decreased to 1 mM-ethylamine and 2 mM-methylamine, no significant effect on [^3H]leucine uptake into islets was detectable. It may be concluded therefore that the observed inhibition of glucose-stimulated [^3H]leucine incorporation by these amines when tested at their

Table 3. *Effects of test compounds on islet glucose oxidation and protein synthesis*

Glucose oxidation by islets was determined by $^{14}\text{CO}_2$ release from $[\text{U-}^{14}\text{C}]$ glucose as described in the Experimental section. Incubations were carried out for 1 h after the addition of stimulatory concentrations of glucose, except for those incubations containing monodansylcadaverine, which were carried out for both 1 h and 2 h(*). For each concentration of amine tested, control values were determined for untreated islets from the same isolation to accommodate any variability seen between different islet preparations in their ability to utilize glucose. Total islet protein synthesis was assessed by ^3H leucine incorporation into trichloroacetic acid-precipitable material. Control batches of islets were used in each experiment from the same preparation, for the same reasons given for the experiments on glucose oxidation. The inhibition of glucose oxidation and protein synthesis by test compounds is expressed as a mean percentage ($\pm$ S.E.M.) relative to control incubations; the numbers of determinations are shown in parentheses. The significance of the difference from controls was determined by Student's *t* test; N.S. denotes no significant difference from controls, N.T. denotes not tested. Typical values for glucose-stimulated total protein synthesis were 306471 ± 21392 d.p.m./h per 10 islets ($n = 35$) and for glucose utilization 306 ± 21 pmol/h per 10 islets ($n = 31$).

Test compound	Concn. (mM)	Inhibition (%) of glucose-stimulated	
		Glucose utilization	Total protein synthesis
Monodansylcadaverine	0.1	0 (4) N.S.	0 (3) N.S.
	0.1	* 19.8 ± 17 (3) N.S.	—
Cystamine	0.1	23.8 ± 11 (5) $P \leq 0.25$	N.T.
	1.0	90.3 ± 9 (4) $P \leq 0.002$	N.T.
Methylamine	1.0	N.T.	10.2 ± 3.2 (4) N.S.
	2.0	N.T.	25.5 ± 5.5 (5) $P \leq 0.1$
	5.0	5.1 ± 7.1 (4) N.S.	79.3 ± 4 (5) $P \leq 0.02$
	15.0	55.4 ± 4 (5) $P \leq 0.001$	N.T.
Propylamine	1.0	19 ± 19 (4) N.S.	19.5 ± 18 (3) N.S.
	5.0	48 ± 8 (3) $P \leq 0.01$	N.T.
Ethylamine	1.0	N.T.	38.2 ± 7.1 (5) $P \leq 0.01$
	5.0	8.7 ± 6.9 (4) N.S.	49 ± 12.6 (4) $P \leq 0.05$
Cycloheximide	0.01	N.T.	73.3 ± 3.5 (4) $P \leq 0.001$
	0.1	N.T.	88.8 ± 0.5 (4) $P \leq 0.001$

higher concentrations may in part be due to inhibitory effects on ^3H leucine uptake. At lower concentrations of these amines, however, our results suggest that inhibition of ^3H leucine uptake does not contribute to inhibition of ^3H leucine incorporation, which may therefore represent true inhibition of protein synthesis. To determine what effect the inhibition of glucose-stimulated protein synthesis would have on insulin release over the time course of our experiments, cycloheximide was incubated with islets by using an experimental protocol identical with that used with primary amines. At a final concentration of $10\text{ }\mu\text{M}$, cycloheximide inhibited glucose-stimulated protein synthesis by 73% of the stimulated rate, a value comparable with non-stimulated rates of protein synthesis. At this same concentration cycloheximide did not significantly inhibit glucose-stimulated insulin release (Table 2). It therefore appears that, under the conditions of assay, the nature of inhibition of glucose-stimulated insulin release by methylamine, ethylamine and propylamine at concentrations that do not significantly affect glucose-stimulated glucose utilization is independent of the effects these primary amines may have on protein synthesis.

Discussion

These studies were undertaken to demonstrate the presence of a typical transglutaminase enzyme in islets of Langerhans and to test the hypothesis that this enzyme may be involved in the Ca^{2+} -dependent mechanism of insulin release from the islet β -cell.

Our results indicate that pancreatic islets do contain a typical thiol-dependent transglutaminase enzyme which requires Ca^{2+} for its activation. Initial studies on the Ca^{2+} -dependence of the enzyme have demonstrated that half-maximal velocity of the enzyme occurs in the region of $40\text{ }\mu\text{M-Ca}^{2+}$.

If it is assumed that the Ca^{2+} concentration in the cytosol of the resting β -cell is in the region of $0.1\text{ }\mu\text{M}$ (Wollheim & Sharp, 1982) and that other localized stores are not available to the enzyme, then the suggestion is that the islet transglutaminase may be latent under these conditions and that its activation may occur during periods of β -cell stimulation, when an increase in the cytosolic Ca^{2+} concentration takes place (Wollheim & Sharp, 1982).

In order to test the hypothesis for trans-

glutaminase involvement in the Ca^{2+} -mediated stimulatory mechanism of insulin release, known primary-amine substrates of the enzyme (inhibitors of transglutaminase-catalysed protein cross-linking) were tested for their effects on insulin release from glucose-stimulated pancreatic islets.

Of the primary amines tested, monodansylcadaverine was by far the most potent inhibitor of glucose-stimulated insulin release and was approx. 50 times more potent than methylamine, ethylamine or propylamine, which were of similar potency to each other. These relative potencies were found to correlate well with the relative potency of the amines when tested for their effects on islet transglutaminase activity.

When amines were tested for their effect on two other metabolic processes intimately associated with the secretory response of the β -cell, namely glucose oxidation and protein synthesis, the most potent inhibitor of insulin release, monodansylcadaverine, had no significant effect on either of these processes when tested at a concentration of $100\text{ }\mu\text{M}$. Methylamine, ethylamine and propylamine did, however, show variable effects on these two metabolic processes, a finding that should be borne in mind by other investigators when using these alkylamines in studies related to receptor-mediated endocytosis (King *et al.*, 1980). Similarly, cystamine, the thiol-directed active-site inhibitor of transglutaminase (Siefring *et al.*, 1978), which, of the compounds tested, proved to be the second most potent inhibitor of glucose-stimulated insulin release, was also a potent inhibitor of stimulated islet glucose oxidation when tested at concentrations of 0.1 and 1.0 mM. Considering the importance of increased glucose utilization in glucose-stimulated insulin release, it is difficult to separate this non-specific effect of cystamine from any effect this compound may have on islet transglutaminase activity in the intact islet.

For methylamine, ethylamine and propylamine, however, concentrations of these alkylamines were found (5 mM for methylamine and ethylamine and 1 mM for propylamine) that gave significant inhibition of glucose-stimulated insulin release but did not affect glucose oxidation. At 1 mM, methylamine and propylamine were also found not to affect glucose-stimulated incorporation of [^3H]leucine into islet protein, although ethylamine when tested at the same concentration still gave a significant inhibition of this process, which could not be attributed to any effect of the amine on [^3H]leucine uptake. However, comparable experiments performed with the protein-synthesis inhibitor cycloheximide indicated that, in the time course of the experiments carried out, inhibition of glucose-stimulated protein synthesis is unlikely to affect insulin release. This result compares favourably

with those obtained by Morris & Korner (1970).

Our results therefore suggest that monodansylcadaverine, methylamine, ethylamine and propylamine affect events catalysed by islet transglutaminase which are involved in the glucose-stimulated mechanism of insulin release. This is further substantiated by the observation that the dimethyl analogue of monodansylcadaverine, which is not a substrate of transglutaminase, possessed only a small percentage (24%) of the inhibitory power of the primary-amine analogue, indicating that the effect of monodansylcadaverine is mainly a consequence of the primary-amine group.

Primary amines when incubated with cells can, however, affect other processes, which may feasibly lead to inhibition of the secretory process, and it is important that these should be considered. For example, it has been shown that the weak bases imidazole (10 mM) and NH_4^+ (20 mM) can inhibit glucose-stimulated insulin release by up to 70%. This is thought to be due to an increase in the intracellular pH of the β -cell, which in turn may affect the coupling between glucose utilization and insulin secretion (Smith & Pace, 1983). Primary amines, being weak bases, may therefore inhibit glucose-stimulated insulin release by increasing the internal pH of the β -cell. However, three points argue against this. Firstly, the amine concentrations used in our experiments, which were found not to inhibit either glucose oxidation or protein synthesis, were relatively low ($20\text{ }\mu\text{M}$ –1 mM) compared with those used in the studies on pH alteration, and thus may not affect intracellular pH. Secondly, although monodansylcadaverine and methylamine are of similar basicity, monodansylcadaverine is some 50 times more potent as an inhibitor of insulin release, suggesting that specificity for the transglutaminase enzyme is the differentiating factor. Thirdly, the basic dimethylated analogue of monodansylcadaverine has only a small effect on insulin release.

Our findings therefore suggest that the membrane interactions involved in the exocytotic mechanism of insulin release are dependent on reactions that may be inhibited by primary amines. The evidence suggests that the nature of this inhibition is consistent with a role for transglutaminase in the secretory mechanism. Such a role for the enzyme may lie in the translocation and/or exocytosis of the secretory granule. In other systems studied, a role for transglutaminase has been suggested in the mechanism of receptor recycling (Van Leuven *et al.*, 1980). Membrane recycling is now thought to be a necessary feature of continued exocytotic activity (Farquhar, 1981), and such a process will occur in the β -cell during glucose-stimulated insulin release (Orci *et al.*,

1978; Herzog, 1981).

During membrane recycling, it is at present not known how chemical specificity is achieved or maintained by interacting membranes of different compartments (Palade, 1982), although stabilization by membrane infrastructures has received much attention (Pearse & Bretscher, 1981). The cross-linking of membrane proteins or associated structures of the endocytosed membrane by transglutaminase would provide an alternative or additional mechanism of membrane stabilization, thus limiting the intermixing of membrane components on fusion and making possible their subsequent non-random removal from membrane components such as the Golgi body in the recycling process. A role for membrane-protein stabilization by the ϵ -(γ -glutamyl)lysine cross-link in this mechanism is therefore an attractive proposal, and its importance in glucose-stimulated insulin release requires further investigation.

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