Biochem J. (1986) 235, 269–278 (Printed in Great Britain)

A role for transglutaminase in glucose-stimulated insulin release from the pancreatic β-cell

Peter J. BUNGAY,* Robin A. OWEN,* Ian C. COUTTS† and Martin GRIFFIN*‡
*Department of Life Sciences and †Department of Physical Sciences, Trent Polytechnic, Clifton, Nottingham NG11 8NS, U.K.

1. Preincubation of rat islets of Langerhans with the potent inhibitors of islet transglutaminase activity, monodansylcadaverine (30–100 μM) and *N*-(5-aminopentyl)-2-naphthalenesulphonamide (100–200 μM), led to significant inhibition of glucose-stimulated insulin release from islets. In contrast, the respective *N'*-dimethylated derivatives of these two compounds, which did not inhibit islet transglutaminase activity, were much less effective as inhibitors of glucose-stimulated insulin release. 2. None of the compounds inhibited rat spleen protein kinase C activity at concentrations which gave rise to inhibition of glucose-stimulated insulin release. When tested for their effects on calmodulin-stimulated bovine heart phosphodiesterase activity, of the compounds that inhibited insulin release, only monodansylcadaverine did not act as an effective antagonist of calmodulin at concentrations (up to 50 μM) that gave rise to significant inhibition of glucose-stimulated insulin release. Furthermore, at 50 μM, monodansylcadaverine did not inhibit methylation of islet lipids. The inhibition of glucose-stimulated insulin release by monodansylcadaverine is therefore likely to be attributable to its interference with islet transglutaminase activity. 3. The sensitivity of islet transglutaminase to activation by Ca^{2+} was investigated by using a modified assay incorporating dephosphorylated *NN'*-dimethylcasein as a substrate protein. The K_m for Ca^{2+} obtained (approx. 3 μM) was an order of magnitude lower than previously reported for the islet enzyme [Bungay, Potter & Griffin (1984) Biochem. J. **219**, 819–827]. Mg^{2+} (2 mM) was found to have little effect on the sensitivity of the enzyme to Ca^{2+}. 4. Investigation of the endogenous substrate proteins of islet transglutaminase by using the Ca^{2+}-dependent incorporation of [^{14}C]methylamine into proteins of islet homogenates demonstrated that most of the incorporated radiolabel was present in cross-linked polymeric aggregates which did not traverse 3% (w/v) acrylamide gels. The radiolabelled polymeric aggregates were present in 71000 *g*-sedimented material of homogenates, and their formation was transglutaminase-mediated. 5. These findings provide new evidence for the involvement of islet transglutaminase in the membrane-mediated events necessary for glucose-stimulated insulin release.

INTRODUCTION

The process of stimulus–secretion coupling in the pancreatic β-cell is mediated by the Ca^{2+}-messenger system, whereby stimulation of insulin release by glucose and other secretagogues is thought to involve a rise in the cytosolic concentration of Ca^{2+} (Hedeskov, 1980; Wollheim & Sharp, 1981; Prentki & Wollheim, 1984; Rasmussen & Barrett, 1984). The way in which Ca^{2+} elicits the release of insulin is not understood, but is likely to be the result of the binding of the cation to intracellular receptor proteins, whose activities may then increase rates of hormone secretion. One of these possible Ca^{2+} receptors is the Ca^{2+}-dependent enzyme transglutaminase (EC 2.3.2.13), which catalyses an acyl-transfer reaction between peptide-bound glutamine residues and primary amine groups. The result of this activity is the post-translational modification of proteins, either through incorporation of amines or through protein cross-linking (for reviews see Folk & Finlayson, 1977; Lorand & Conrad, 1984). Evidence has suggested that the transglutaminase activity present in isolated islets of Langerhans may be important in the pancreatic β-cell during glucose-stimulated insulin release (Bungay *et al.*, 1982, 1984*a*, *b*; Gomis *et al.*, 1983). Reactions catalysed by transglutaminase have also been implicated in other cellular processes regulated by Ca^{2+}, notably receptor-mediated endocytosis (Davies *et al.*, 1980; Van Leuven *et al.*, 1980) and the activation of lymphocytes (Julian *et al.*, 1983) and macrophages (Fésüs *et al.*, 1981; Günzler *et al.*, 1982).

Of importance to the assessment of a functional role for the islet transglutaminase in the process of stimulus–secretion coupling is its sensitivity to activation by Ca^{2+}. Earlier reports from our laboratory (Bungay *et al.*, 1984*a*) and others (Gomis *et al.*, 1983) indicated that the islet transglutaminase operated at half-maximal velocity at concentrations of Ca^{2+} in the range 40–90 μM. Since concentrations of Ca^{2+} as high as these are unlikely to be found in the pancreatic β-cell during periods of stimulated insulin release it is of great importance to reassess the accuracy of these findings if a functional role for this enzyme in stimulus–secretion coupling in the pancreatic β-cell is to be established.

Other previous evidence for the involvement of transglutaminase in glucose-stimulated insulin release

Abbreviations used: KRB, Krebs–Ringer bicarbonate medium (Krebs & Hensleit, 1932); dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; MDCD, monodansylcadaverine; 2-NSA, *N*-(5-aminopentyl)-2-naphthalenesulphonamide.

‡ To whom all correspondence should be addressed.

was based on the observation that compounds which inhibited transglutaminase activity *in vitro* also inhibited glucose-stimulated insulin release from islets. However, the difficulty with using this type of approach was that some of these 'probe' molecules may inhibit insulin release by interference with targets other than islet transglutaminase (Bungay *et al.*, 1984*a*; Sener *et al.*, 1984). For instance, alkylamines (which are normally used in millimolar concentrations) may exert their effects by disruption of lysosomal and endosomal function. The more potent inhibitors of transglutaminase activity, the naphthalenesulphonamides (Lorand *et al.*, 1979), may be antagonists of the Ca^{2+}-binding protein calmodulin (Hidaka & Tanaka, 1983) and inhibitors of the phospholipid- and Ca^{2+}-sensitive protein kinase (protein kinase C) (Wise *et al.*, 1982*b*). These two proteins have been implicated as important regulators of stimulus–secretion coupling in the pancreatic β-cell (Gagliardino *et al.*, 1980; Lord & Ashcroft, 1984). It is therefore important to establish the specificity of such 'probe' molecules if they are to be used to determine the involvement of transglutaminase in glucose-stimulated insulin release.

The present study was therefore undertaken in order both to reassess previous evidence and to provide further evidence of the involvement of transglutaminase in glucose-stimulated insulin release.

EXPERIMENTAL

Materials

Collagenase used in the isolation of pancreatic islets of Langerhans was obtained from Serva Feinbiochimica, Heidelberg, West Germany. Monodansylcadaverine was purchased from Fluka A. G., Buchs, Switzerland. Materials for the synthesis of other naphthalenesulphonamides (dansyl chloride, 2-naphthalenesulphonyl chloride and 5-chlorovaleronitrile) were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. [1,4-^{14}C]Putrescine, [^{14}C]methylamine, L-[*methyl*-^{3}H]-methionine, [U-^{14}C]glucose, cyclic [^{3}H]AMP and [γ^{32}P]ATP were all purchased from Amersham International, Amersham, Bucks., U.K., and ^{125}I-labelled insulin was obtained from Novo, Bagsvaerd, Denmark. Calmodulin from bovine brain and bovine heart phosphodiesterase were obtained from Boehringer Mannheim, London W.5, U.K., and phosphatidylserine and 1,2-diolein were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Standard rat insulin and anti-insulin antibody were obtained from Novo.

Methods

Synthesis of test compounds. The *NN'*-dimethylcadaverine sulphonamides *N*-(5-dimethylaminopentyl)-2-naphthalenesulphonamide and *N*-(5-dimethylaminopentyl)-5-dimethylamino-1-naphthalenesulphonamide (free bases) were made by reaction of the appropriate napthalenesulphonyl chloride in pyridine with *NN'*-dimethylcadaverine, which was prepared from 5-chlorovaleronitrile as detailed in Lott & Krapcho (1957). For the *N*-(5-aminopentyl)-2-naphthalenesulphonamide (hydrobromide salt), 6-(2-naphthalenesulphonamide)-hexanamide was treated with bromine in methanol as described by Lorand *et al.*, (1979) and the resulting carbamate was hydrolysed with a saturated solution of HBr in acetic acid (Wani *et al.*, 1972). Purity of compounds was assessed by t.l.c., mass spectrometry, i.r. spectroscopy and n.m.r.

Isolation of islets of Langerhans. Islets of Langerhans were isolated from the pancreas of 200–300 *g* Sprague–Dawley rats of both sexes by a collagenase digestion technique similar to that of Lacy & Kostianovsky (1967). For studies with intact islets, islets were preincubated for 5 min in KRB containing 16.8 mM-glucose, followed by 60 min in KRB containing 2.8 mM-glucose.

Homogenization of islets. Before homogenization, islets were washed in ice-cold 0.25 M-sucrose/1 mM-Tris/HCl/1 mM-EDTA or -EGTA, pH 7.4, and suspended in 150–300 μl of the same medium to give a final islet concentration of approx. 2 per μl. Homogenization was then carried out either by ultrasonication with 2×5s bursts at 3 μm amplitude with an MSE sonicator or by use of three strokes of a 5 ml Potter–Elvehjem homogenizer. In the assays for islet transglutaminase activity, a particle-free supernatant was used, which was prepared by centrifugation of islet homogenates at 71000 $g_{av.}$ for 45 min.

Assay for transglutaminase activity. Transglutaminase activity was measured by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein by using the method of Lorand *et al.* (1972). The incubation mixture, at 37 °C, consisted of 30 mM-Tris/HCl, pH 7.4, 3.85 mM-dithiothreitol, 2.5 mM-$CaCl_2$ or 5 mM-EDTA, 1.2 mM-[^{14}C]putrescine (3.96 Ci/mol), *NN'*-dimethylcasein (5 mg/ml) and 20 μl of islet preparation in a final volume of 50 μl. Samples (10 μl) 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.

Measurement of the Ca^{2+}-sensitivity of transglutaminase. Islet supernatants were prepared as described above, except that 1 mM-EGTA was substituted for EDTA in the homogenization buffer. The islet supernatant extract was dialysed for at least 2 h at 4 °C against 50 mM-Tris/HCl (pH 7.4 at 37 °C)/1 mM-EGTA. Addition of 20 μl of islet preparation to the final assay volume of 50 μl then gave a final concentration of EGTA of 0.4 mM in 50 mM-Tris/HCl. Different amounts of $CaCl_2$ were then added to the assay medium such that the final desired concentration of free Ca^{2+} was obtained, ranging from 3.2 to 50.6 μM. For calculation of the free Ca^{2+} concentrations, an association constant at pH 7.4 of $10^{7.12}$ for [CaEGTA]/[Ca] [EGTA] was used (Schatzman, 1973). In these assays dephosphorylated *NN'*-dimethylcasein was used instead of native *NN'*-dimethylcasein. This protein was dephosphorylated by incubation with alkaline phosphatase as described by Cooke & Holbrook (1974).

Assay for protein kinase C activity. The potency of compounds as inhibitors of protein kinase C was determined by measuring protein kinase C activity in extracts obtained from rat spleen. With a Potter–Elvehjem homogenizer, rat spleen was homogenized in 3 vol. of 20 mM-Tris/HCl (pH 7.4)/2 mM-EGTA/50 mM-2-mercaptoethanol. The homogenate was centrifuged at 71000 *g* for 45 min at 4 °C and the supernatant

retained. This preparation was diluted 1:10 with 20 mM-Tris/HCl, pH 7.4, for use in the protein kinase C assays. The standard reaction mixture (100 μl) contained the following final concentrations of components: 20 mM-Tris/HCl, pH 7.4, 5 mM-magnesium acetate, 200 μg of histone H1/ml, 10 μM-[γ-^{32}P]ATP (0.6 Ci/mmol), 98 μM-$CaCl_2$, 48 μM-EGTA, 1.2 mM-2-mercaptoethanol, 16 μg of phosphatidylserine/ml, 2.4 μg of 1,2-diolein/ml and the appropriate concentration of test compound. Reactions were started by the addition of 24 μl of spleen extract (approx. 25 μg of protein). The incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 was measured by the modified procedure of Lord & Ashcroft (1984), whereby phosphorylated proteins were recovered by precipitation with 5% (w/v) trichloroacetic acid containing 0.25% (w/v) sodium tungstate. Protein kinase C activity was calculated by subtracting the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein and phosphatidylserine over a period of 2 min incubation at 37 °C. Addition of phospholipid and diolein stimulated enzyme activity by approx. 60%. Under these conditions, the incorporation of ^{32}P was linear with time, and the standard inhibitor of protein kinase C, trifluoperazine (Wise *et al.*, 1982*b*), was found to give 50% inhibition of enzyme activity at a concentration of 61 μM.

Assay for calmodulin-stimulated phosphodiesterase. The potency of compounds for their action as calmodulin antagonists was determined by using calmodulin-deficieint bovine heart phosphodiesterase, by the method of MacNeil *et al.* (1984). The reaction mixture at 37 °C contained, in a final volume of 400 μl: 40 mM-Tris/HCl, pH 7.0, 4 mM-2-mercaptoethanol, 5 mM-$MgCl_2$, cyclic [^{3}H]AMP (2×10^5 c.p.m./tube), 100 μM-cyclic AMP, 25 μM-$CaCl_2$, 2 munits of enzyme/tube and 12.5 ng of calmodulin. The amount of calmodulin in the assay was sufficient to produce approx. 80% of maximal activation of the phosphodiesterase enzyme. All assays were carried out at 37 °C for 15 min.

Incubation of islets for measurement of insulin release. This was done as described previously (Bungay *et al.*, 1984*a*). Insulin released into the incubation medium was determined by radioimmunoassay (Heding, 1972), with rat insulin as standard.

Measurement of glucose oxidation. Glucose oxidation by islets was measured by determination of the amount of $^{14}CO_2$ release from [U-^{14}C]glucose as described previously (Bungay *et al.*, 1984*a*).

Measurement of islet lipid methylation. Total islet lipid methylation was determined by measuring the incorporation of L-[*methyl*-^{3}H]methionine into chloroform/diethyl ether-extractable material, essentially as described by Best *et al.* (1984). Groups of 80–120 islets were preincubated for 45 min at 37 °C in 0.5 ml of KRB containing 2.8 mM-glucose with or without 50 μM-MDCD. After this preincubation, a further 0.5 ml of KRB containing 30.5 mM-glucose was added together with 10 μCi of L-[*methyl*-^{3}H]methionine (74 Ci/mol) to give final concentrations of 16.8 mM-glucose and 0.135 μM-methionine. After a further incubation period of 60 min, medium was removed from the islets, to which was added 1.0 ml of ice-cold 10% (w/v) trichloroacetic acid. The resulting precipitate was washed twice with 10% trichloroacetic acid and then transferred to a 15 ml glass centrifuge tube. To the precipitate was added 1.0 ml of distilled water, and the aqueous mixture was extracted with 1.0 ml of chloroform/methanol/11 M-HCl (200:100:1, by vol.). The lower organic layer was removed to a scintillation vial and the organic solvent evaporated with a stream of N_2. The residue was then counted for radioactivity. Counting efficiency was determined by using the Quench Indicating Parameter of Packard Instruments.

Incorporation of [^{14}C]methylamine into islet homogenates. Homogenates of islets were prepared in 1 mM-Tris/HCl (pH 7.4)/0.25M-sucrose/1 mM-EGTA with a Potter–Elvehjem homogenizer as described above. The incubation mixture for incorporation of [^{14}C]methylamine consisted of 20 mM-Tris/HCl, pH 7.4, 3.85 mM-dithiothreitol, 1.8 mM-[^{14}C]methylamine (56 Ci/mol), 0.1 mM-phenylmethanesulphonyl fluoride and 2.5 mM-$CaCl_2$ or 0.625 mM-$CaCl_2$ or 5 mM-EGTA, in a final volume of 100 μl after the addition of 60 μl of islet homogenate. Under these conditions, the addition of 2.5 mM-$CaCl_2$ and 0.625 mM-$CaCl_2$ gave concentrations of free Ca^{2+} in the incubation of 1.9 mM and 26.7 μM respectively. The mixture was incubated at 37 °C for 15, 30 or 120 min, after which was added 100 μl of double-strength electrophoresis sample buffer so that the final concentrations of components in the homogenate sample were 62.5 mM-Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol. The sample for electrophoresis was boiled for 5 min and stored at 4 °C until required.

SDS/polyacrylamide-gel electrophoresis. Islet homogenate proteins were separated by discontinuous SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The solubilized sample (approx. 50 μg of protein in 45–75 μl) was applied to the gels, which consisted of a stacking gel of 3% (w/v) acrylamide, 0.125 M-Tris/HCl, pH 6.8, and 0.1% SDS, and a resolving gel of 10% acrylamide, 0.375 M-Tris/HCl, pH 8.8, and 0.1% SDS. Samples were run for 4–5 h together with the M_r standard proteins β-galactosidase (116000), bovine serum albumin (66000), ovalbumin (45000), trypsinogen (24000), β-lactoglobulin (18400) and lysosyme (14300), which were solubilized in the same buffer. After electrophoresis, the gels were fixed in a solution of 10% trichloroacetic acid and 3.4% (w/v) sulphosalicylic acid and then stained with a solution of 1.25 g Coomasie Blue (PAGE 83; BDH):/l in 18% (v/v) methanol/5% (v/v) acetic acid. After destaining in 18% methanol/5% acetic acid containing 2 mM unlabelled methylamine, gels were photographed and resolving gels sectioned with a razor blade every 2 mm. The whole stacking gel above the respective protein lanes was taken as one section. The sections were thoroughly dried in scintillation vials, dissolved in H_2O_2 (30%, w/v)/conc. NH_3 (sp. gr. 0.88) (19:1, v/v) and counted for radioactivity in 10 ml of Instagel (Packard).

Addition of test compounds to enzyme assays and incubation media. Compounds were initially dissolved in 100 μl of dimethyl sulphoxide, except for MDCD, which was initially dissolved in 100 μl of dilute HCl. From a stock 10 mM solution in 10% (v/v) dimethyl sulphoxide,

the compounds were added to the assay or incubation medium after serial dilution to give the desired final concentration. Control incubations contained the same final concentration of dimethyl sulphoxide as the incubations containing test compounds. For hydrobromide salts of test compounds, NaBr was added to control incubations so that the same final concentration of Br^- was present in control and test incubations.

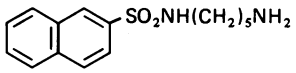
RESULTS

Effects of naphthalenesulphonamides on insulin release

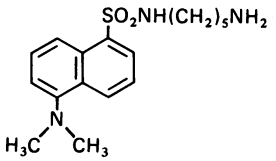
In order to investigate the involvement of islet transglutaminase in the mechanism of insulin release from the pancreatic β-cell, isolated islets of Langerhans were incubated with two known potent inhibitors (competitive substrates) (Lorand *et al.*, 1979) of the enzyme activity, monodansylcadaverine (MDCD) and *N*-(5-aminopentyl)-2-naphthalenesulphonamide (2-NSA). In addition, the *N*-dimethylated derivatives of these compounds, *N*-dimethylmonodansylcadaverine (dimethyl-MDCD) and *N*-(5-dimethylaminopentyl)-2-naphthalenesulphonamide (dimethyl-2-NSA), were tested for their effects on insulin release, since these act as control compounds in that they lack the primary amine group which is crucial to inhibition of transglutaminase activity (Fig. 1). The effects on insulin release of these compounds and their *N*-dimethylated derivatives are shown in Table 1. These chemically related compounds displayed varied effects on glucose-stimulated insulin release from islets. The most potent inhibitor was MDCD, which was effective over the concentration range 30–100 μM. 2-NSA was also an effective inhibitor of glucose-stimulated insulin release, but not as potent as MDCD, concentrations of 100–200 μM being required for effective inhibition.

In contrast with these primary amines, much higher concentrations of the *N*-dimethylated derivatives of these naphthalenesulphonamides were required for any effects on insulin release. Dimethyl-2-NSA did not inhibit glucose-stimulated insulin release at either 100 or 200 μM, but was able to cause 76% inhibition at 400 μM. Dimethyl-MDCD did not inhibit glucose-stimulated insulin release at either 200 or 400 μM. Therefore inhibition of glucose-stimulated insulin release by the range of compounds used appeared to depend on the possession of a primary amine moiety, since the tertiary-amine analogues were not as potent inhibitors as their primary-amine counterparts.

(*a*) Inhibitors

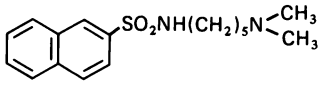


N-(5-Aminopentyl)-2-naphthalenesulphonamide (2-NSA)

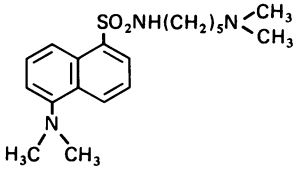


Monodansylcadaverine (MDCD)

(*b*) Control compounds for inhibitors



N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide (dimethyl-2-NSA)



N-Dimethylmonodansylcadaverine (dimethyl-MDCD)

Fig. 1. Inhibitors of transglutaminase activity (*a*) and control compounds for the inhibitors (*b*)

Effects of naphthalenesulphonamides on glucose-stimulated glucose oxidation

In view of the importance of glucose metabolism to stimulus–secretion coupling in the pancreatic β-cell, we compared the compounds used above for their effects on glucose oxidation by islets. The release of $^{14}CO_2$ from [U-^{14}C]glucose was measured over the same time-course as that used in the studies on insulin release. The results of these experiments are shown in Table 2. Of the compounds used that were shown to inhibit insulin release, 2-NSA significantly inhibited glucose oxidation when incubated with islets at a concentration of 200 μM, but not at a concentration of 100 μM. Dimethyl-2-NSA (400 μM) was also tested and caused no significant inhibition of glucose-stimulated glucose oxidation. In our previous experiments with MDCD, we demonstrated that it was non-toxic to islets at concentrations up to 100 μM (Bungay *et al.*, 1984*a*). Therefore, inhibition of glucose-stimulated insulin release by concentrations of MDCD below 100 μM, of 2-NSA below 100 μM and of dimethyl-2-NSA below 400 μM was independent of any effects of these compounds on islet glucose oxidation.

Effects of compounds on activities of transglutaminase, calmodulin-stimulated phosphodiesterase and protein kinase C

Since the β-cell contains a number of possible targets for the action of probe compounds such as the naphthalenesulphonamides used in the present work, it was crucial to determine the specificity of the compounds for intracellular targets whose inhibition could lead to inhibition of glucose-stimulated insulin release. The compounds were therefore screened for their possible action as inhibitors of islet transglutaminase and rat spleen phospholipid- and Ca^{2+}-dependent protein kinase (protein kinase C). In addition, the inhibition of the activation of bovine heart phosphodiesterase by calmodulin was used to measure the potency of the compounds as calmodulin antagonists (MacNeil *et al.*, 1984).

By comparison with control rates of enzyme activity obtained in the absence of the test compounds, the percentage inhibition of enzyme activity was calculated (Figs. 2 and 3). From these data, the concentration of compound that gave 50% inhibition of enzyme activity (IC_{50}) for each enzyme system was determined (Table 3).

Table 1. Effects of naphthalenesulphonamides on glucose-stimulated insulin release

Batches of five islets were incubated in KRB medium in the presence or absence of the concentrations of compounds stated as described in the Experimental section. Compounds were preincubated with islets for 45 min in KRB containing 2.8 mM-glucose, followed by 60 min in KRB containing 16.8 mM-glucose. The insulin content of the medium was determined by radioimmunoassay. Stimulation of insulin release over the 60 min period was determined by subtracting basal rates of insulin release from islets maintained in KRB containing 2.8 mM-glucose incubated simultaneously over the same total time period of 45+60 min. The inhibition of glucose-stimulated insulin release for each compound tested was then expressed as a percentage (±S.E.M.) of the rate 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., no significant difference. Typical rates of insulin release were 6.7±0.9 ($n = 33$) μunits/h per islet for islets incubated in 2.8 mM-glucose and 94.0±6.4 μunits/h per islet ($n = 48$) for islets incubated in 16.8 mM-glucose.

Test compound	Conc. (μM)	Inhibition of glucose-stimulated insulin release (%)
Monodansylcadaverine	30	33±6 (5) $P \leqslant 0.05$
	50	65±11 (6) $P \leqslant 0.002$
	100	85±8 (5) $P \leqslant 0.001$
N-Dimethylmonodansylcadaverine	200	24±9 (6) N.S.
	400	9±7 (6) N.S.
N-(5-Aminopentyl)-2-naphthalenesulphonamide	100	55±9 (6) $P \leqslant 0.001$
	200	86±4 (6) $P \leqslant 0.01$
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	100	0 (7)
	200	0 (6)
	400	76±9 (7) $P \leqslant 0.01$

Table 2. Effects of naphthalenesulphonamides on islet glucose oxidation

Glucose oxidation was determined for groups of 10–20 islets by measuring $^{14}CO_2$ release from [U-^{14}C]glucose as described in the Experimental section. Incubations were carried out for 1 h after the addition of a stimulatory concentration (16.8 mM) of glucose. For each concentration of test compound, control rates were determined for untreated islets from the same isolated batch. The inhibition of glucose oxidation by test compounds was expressed as a mean percentage (±S.E.M.) relative to control incubations, with the numbers of observations shown in parentheses. The significance of difference from controls was determined by Student's *t* test. Typical rates of glucose-stimulated glucose oxidation were 344±35 pmol/h per 10 islets ($n = 17$).

Test compound	Concn. (μM)	Inhibition of glucose-stimulated glucose oxidation (%)
N-(5-Aminopentyl)-2-naphthalenesulphonamide	100	0 (4) N.S.
	200	40±7 (5) $P < 0.005$
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	400	18±3 (3) N.S.

Since the naphthalenesulphonamides used in these experiments are known to act as competitive substrates of transglutaminase (Lorand *et al.*, 1979), inhibition constants (K_i) were calculated (Table 3) using the data shown in Fig. 3.

The effects of the compounds on protein kinase C activity were determined over the concentration range 0.1–1.0 mM. Under the conditions of assay, inhibition of enzyme activity required concentrations of the compounds of at least 1.0 mM (Table 3). The compounds that inhibited protein kinase C activity (2-NSA and dimethyl-2-NSA) were tested for their effects on basal enzyme activity (measured in the absence of diolein and phospholipid) and were found not to inhibit this activity, indicating that their effects were directed against the phospholipid-stimulated protein kinase C activity.

When tested for their effects on the activation of phosphodiesterase by calmodulin, the naphthalenesulphonamides inhibited this enzyme more potently and to varying degrees. The most potent inhibitor was 2-NSA ($IC_{50} = 105$ μM), with dimethyl-2-NSA ($IC_{50} = 277$ μM) being approx. $\frac{1}{3}$ as potent as 2-NSA and MDCD ($IC_{50} = 387$ μM) being approx. $\frac{1}{4}$ as potent as 2-NSA (Fig. 2; Table 3). Dimethyl-MDCD was found to be the least effective inhibitor of calmodulin-stimulated phosphodiesterase activity ($IC_{50} > 500$ μM) when tested at concentrations up to 500 μM. The effects of these compounds on basal phosphodiesterase activity measured in the absence of calmodulin were also determined. Basal activity was not significantly affected by concentrations of MDCD up to 500 μM, of 2-NSA up to 200 μM and of dimethyl-MDCD and dimethyl-2-NSA up to 250 μM. The two dimethylated analogues significantly stimulated basal phosphodiesterase activity between 2- and 4-fold at a concentration of 500 μM. These results therefore indicate that the compounds inhibited calmodulin-stimulated phosphodiesterase activity as a result of calmodulin antagonism.

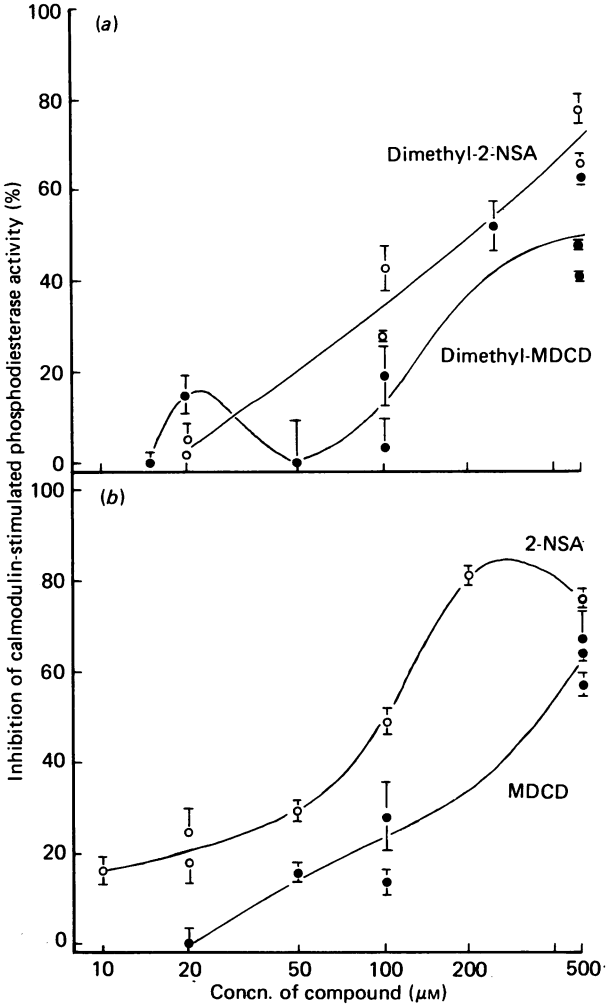


Fig. 2. Inhibition of calmodulin-stimulated phosphodiesterase activity by naphthalenesulphonamides

Calmodulin-stimulated phosphodiesterase activity was determined as described in the Experimental section in both the presence and the absence of the concentrations of compounds shown in the Figure. By reference to control rates of [^{3}H]adenosine production, results (obtained from three separate experiments) were expressed as a mean percentage (±S.E.M.) inhibition of enzyme activity, with each point the mean of three determinations. (*a*) ○, Dimethyl-2-NSA;●, dimethyl-MDCD; (*b*) ●, MDCD; ○, 2-NSA. Typical control rates (±S.E.M.) representing 80% stimulation of maximal activity were 48.0±1.6 (n = 12) nmol of cyclic AMP hydrolysed/h.

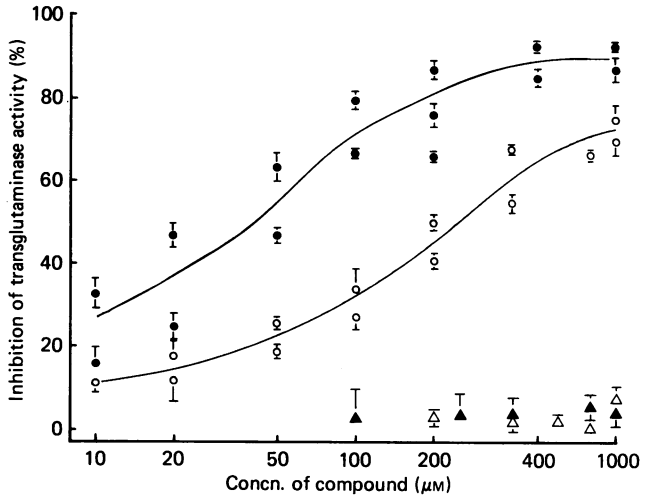


Fig. 3. Inhibition of islet transglutaminase activity by naphthalenesulphonamides

Transglutaminase activity in islet extracts was measured by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein as described in the Experimental section in the presence and absence of the concentrations of MDCD (●), 2-NSA (○), dimethyl-MDCD (▲) and dimethyl-2-NSA (△) shown in the Figure. Rates of putrescine incorporation were calculated and, by reference to control values obtained in the same experiment, results were expressed as a mean (±S.E.M.) percentage inhibition of enzyme activity. All points are the mean of three determinations, and those for 2-NSA and MDCD represent data taken from three separate experiments. Typical control rates (±S.E.M.) for putrescine incorporation into *NN'*-dimethylcasein were 0.90±0.11 nmol of putrescine/h (n = 22).

When the same compounds were tested for their effects on islet transglutaminase activity, MDCD was found to be a potent inhibitor (IC_{50} = 43.3 μM; K_i = 9.8 μM) compared with 2-NSA (IC_{50} = 252 μM; K_i = 62.6 μM), a result comparable with those obtained by Lorand *et al.*, (1979), when these compounds were tested for their effects on guinea-pig liver transglutaminase. The two dimethylated analogues of these compounds did not significantly inhibit islet transglutaminase activity below concentrations of 1 mM (Fig. 3).

Reference to the data on insulin release (Table 1) reveals that the inhibition of transglutaminase activity by MDCD and 2-NSA and their respective dimethylated analogues correlated well with the potency with which they inhibited insulin release. For 2-NSA, the potency of the effect shown by this compound in its ability to inhibit the activation of phosphodiesterase by calmodulin suggests an overlap in the inhibitory action of the compound. However, such an overlap was not evident for MDCD, since the IC_{50} for calmodulin inhibition and the IC_{50} and K_i for transglutaminase inhibition were an order of magnitude apart. Furthermore, concentrations of MDCD could be defined (30–50 μM) that gave rise to significant inhibition of glucose-stimulated insulin release, but had little or no effect on the activation of phosphodiesterase by calmodulin.

Effect of MDCD on total islet methylation

It has been reported that MDCD incubated with neutrophils and fibroblasts caused an inhibition of phospholipid methylation, representing a non-specific action of the compound (Mato *et al.*, 1983). Phospholipid methylation has been shown to occur in islets, and its inhibition may lead to inhibition of glucose-stimulated insulin release (Best *et al.*, 1984). Therefore, as a further test for the specificity of MDCD as a probe for investigating transglutaminase involvement in glucose-stimulated insulin release, we investigated the effect of MDCD on total islet lipid methylation. Groups of 50–100 islets were preincubated for 45 min in the absence or presence of MDCD (50 μM), and the incorporation of methyl groups from [*methyl*-^{3}H]methionine into acidic

Table 3. Inhibition of transglutaminase, protein kinase C and calmodulin-stimulated phosphodiesterase activity by naphthalenesulphonamides

IC_{50} data represent the mean values calculated from three separate experiments by using the data shown in Figs. 2 and 3. The ranges of values obtained from these experiments are shown in parentheses. K_i values for inhibition of transglutaminase activity were calculated from the equation

$$K_i = \frac{[I]_{0.5}}{1+(s/K_m)}$$

by using a K_m of 0.59 mM for putrescine incorporation into *NN'*-dimethylcasein (Bungay *et al.*, 1984*a*). The $[I]_{0.5}$ values (concentration for half-maximal inhibition) used were the mean values calculated from the slopes of Eadie–Hofstee plots of fractional inhibition against fractional inhibition/inhibitor concentration, and obtained from three experiments by using the data shown in Fig. 3. $[I]_{0.5}$ (±S.E.M.) for MDCD = 29.9±11.1 μM and for 2-NSA = 190±20.6 μM. Numbers in square brackets show the percentage inhibition at the concentration shown.

Compound	IC_{50} (μM) or K_i* (μM)			
	Transglutaminase		Phosphodiesterase activation by calmodulin	Protein kinase C
Monodansylcadaverine	43.3 (34–56)	9.8*	387 (380–400)	> 1000 [0%]
N-Dimethylmonodansylcadaverine	> 1000 [6%]		> 500	> 1000 [2.1%]
N-(5-Aminopentyl)-2-naphthalenesulphonamide	252 (200–300)	62.5*	105 (90–110)	> 2500 [25%]
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	> 5000 [7%]		275 (210–370)	> 1000 [21%]

chloroform/methanol extracts of islets was determined after a further incubation for 60 min under glucose-stimulated conditions (16.8 mM-glucose), also in the absence or presence of MDCD. Comparison of the incorporation of radiolabel in control incubations of 29508±4118 d.p.m./h per 100 islets ($n = 5$) with the value obtained for islets incubated in the presence of 50 μM-MDCD, 34315±3321 d.p.m./h per 100 islets ($n = 5$) indicated that no significant inhibition of islet lipid methylation was caused by the compound (Student's *t* test). Therefore inhibition of glucose-stimulated insulin release by MDCD is unlikely to be the result of inhibition of islet phospholipid methylation.

Ca^{2+}-sensitivity of islet transglutaminase

Important to the evidence for the involvement of islet transglutaminase in insulin release is its sensitivity to activation by Ca^{2+}. Results from our laboratory (Hand *et al.*, 1985) have indicated that the use of dephosphorylated *NN'*-dimethylcasein in the enzyme assay is essential in order to obtain a true reflection of the activation of transglutaminase by Ca^{2+}, since significant amounts of Ca^{2+} can be bound by protein-bound phosphate groups. Therefore, in order to reassess the responsiveness of islet transglutaminase to Ca^{2+}, extracts of islets were incubated with dephosphorylated *NN'*-dimethylcasein in the presence of concentrations of free Ca^{2+} in the range 3.2–50.6 μM. Under these conditions, islet transglutaminase was found to be sensitive to concentrations of Ca^{2+} below 10 μM, and had a K_m for Ca^{2+} of 3.2 μM (Fig. 4). This value is a factor of 10 or so lower than values obtained previously (Bungay *et al.*, 1984*a*; Gomis *et al.*, 1983). This discrepancy probably reflects the use of a phosphorylated protein substrate in the enzyme assay before the present study.

Since Mg^{2+} is reported to be present in the cell at concentrations at least an order of magnitude higher than Ca^{2+} (Veloso *et al.*, 1973), it was feasible that this cation could affect the Ca^{2+}-sensitivity of islet transglutaminase. The use of dephosphorylated *NN'*-dimethylcasein allowed us to determine the effect of Mg^{2+} on the Ca^{2+}-sensitivity of the enzyme in the absence of any interference by Mg^{2+} through displacement of Ca^{2+} from protein-bound phosphate groups (Hand *et al.*, 1985). Measurement of the Ca^{2+}-sensitivity of islet transglutaminase in the presence of 2 mM-$MgCl_2$ revealed a K_m for Ca^{2+} of 2.2 μM (results not shown). Therefore Mg^{2+} appeared to have little effect on the Ca^{2+}-sensitivity of islet transglutaminase.

Identification of protein substrates for transglutaminase in islet homogenates

Experiments were carried out to determine which proteins in islets may be modified by transglutaminase action in the β-cell. Homogenates of islets were prepared and incubated with [^{14}C]methylamine in order to label proteins containing γ-glutamyl residues available for transglutaminase-catalysed reactions. Firstly, homogenates prepared by disruption of islets with a Potter–Elvehjem homogenizer were incubated with [^{14}C]methylamine in the presence of either 1.9 mM-Ca^{2+} or 5 mM-EGTA for 120 min. Homogenates labelled in this way with [^{14}C]methylamine were fractionated by centrifugation at 71000 g_{av} for 45 min, and the trichloroacetic acid-insoluble radioactivity in the pellet and supernatant was determined. Comparison of the Ca^{2+}-dependent labelling ($n = 3$) of the pellet (9371±373 c.p.m.) and supernatant (732±290 c.p.m.) fractions demonstrated that 93% of the label was present in the pellet fraction, indicating that the proteins labelled by the action of islet transglutaminase were probably membrane-associated.

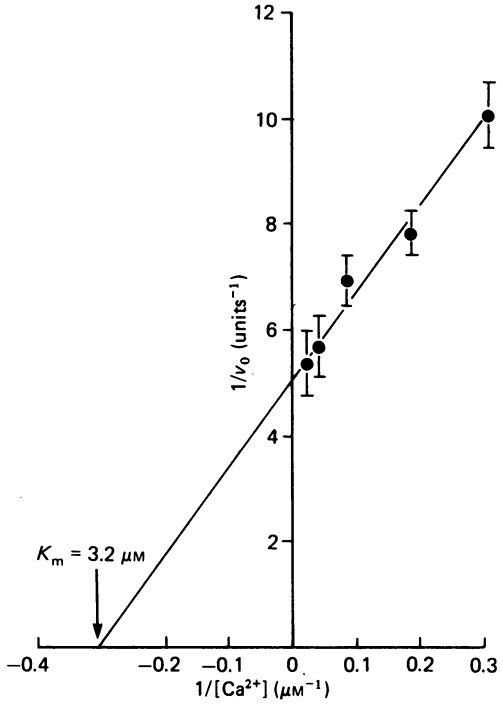


Fig. 4. Activation of islet transglutaminase by Ca^{2+}

Transglutaminase activity in islet extracts was measured by [^{14}C]putrescine incorporation into dephosphorylated *NN'*-dimethylcasein in the presence of 3.22, 5.45, 12.14, 26.1 and 50.6 μM free Ca^{2+} as described in the Experimental section. After calculation of rates of putrescine incorporation, data were treated to give a double-reciprocal plot. One unit of enzyme activity was equal to 1 nmol of putrescine incorporated/h. Points are means (±S.E.M.) for three determinations.

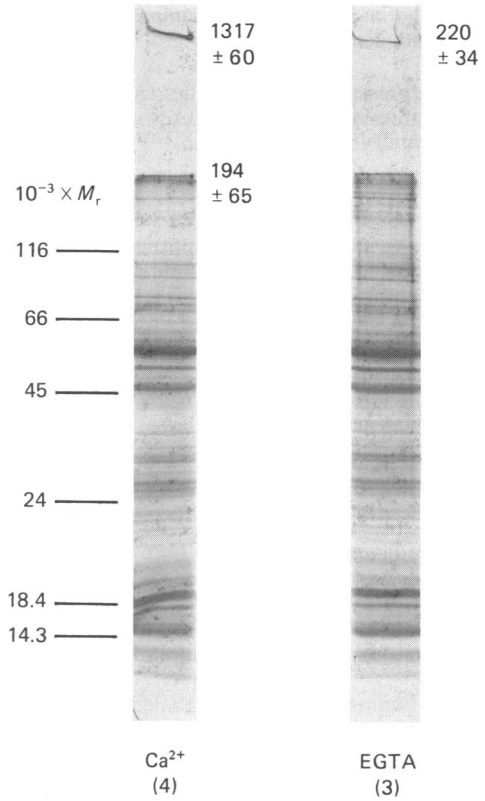


Fig. 5. Labelling of islet homogenate proteins with [^{14}C]methylamine

Islet homogenates were incubated with [^{14}C]methylamine as described in the Experimental section. The samples were dissolved in electrophoresis sample buffer and electrophoresed by the method of Laemmli (1970). Stained gels were sectioned every 2 mm, apart from the stacking gel, which was treated as one unit, and the amount of radioactivity in the sections was determined. The photographs show examples of protein staining obtained from homogenates. The radioactivity (c.p.m.) incorporated was standardized per 50 μl incubation sample and is shown at the points indicated in the electrophoretograms. Amounts of radioactivity equal to or below control values are not shown. The bars indicate the mobility of standard M_r protein markers, which were electrophoresed under identical conditions. The number of experiments is shown in parentheses under each gel.

Further analysis of the labelled islet proteins was carried out by SDS/polyacrylamide-gel electrophoresis. Separation of islet proteins from homogenates incubated in the presence of [^{14}C]methylamine and 1.9 mM-Ca^{2+} for 120 min revealed that most of the radiolabel incorporated remained in the stacking gel, with a much smaller proportion remaining at the top of the resolving gel (Fig. 5). This labelling was inhibited by EGTA (5 mM) or by iodoacetamide (1.0 mM) in the incubation mixture. Furthermore, with homogenates incubated with 1.9 mM–Ca^{2+}, stained material of a very high M_r, presumably of polymeric nature, could be discerned at the top of the stacking gel. This material could not be discerned with homogenates incubated with either EGTA (5 mM) or iodoacetamide (results not shown). The patterns of bands observed after staining of the gels were otherwise similar regardless of the incubation conditions used. These results indicate that both the incorporation of [^{14}C]methylamine and the formation of the high-M_r material were a result of islet transglutaminase activity.

In an attempt to identify the individual proteins labelled by [^{14}C]methylamine in the absence of their cross-linking and incorporation into high-M_r material, various modifications were made to the incubation conditions. Firstly, the free Ca^{2+} concentration in the incubation mixture was decreased to 26.7 μM, which resulted in a decrease both in the incorporation of [^{14}C]methylamine and in the visibility of the high-M_r material in the stacking gel. However, all the radiolabel incorporated was found to be present in the stacking gel. Secondly, a time-course experiment was undertaken whereby incubations in the presence of 1.9 mM-Ca^{2+} were carried out for 15 min or 30 min and compared with incubations carried out for 120 min. Although decreases in the amount of labelling and polymer were apparent in the 15 min and 30 min samples, the label was detectable mainly in the stacking gel and to a much lesser extent in the top of the resolving gel (results not shown).

DISCUSSION

The potency with which naphthalenesulphonamides inhibited glucose-stimulated insulin release appeared to depend on the possession by these molecules of a primary amine group. Thus MDCD was effective in the inhibition

of insulin release (33–86% inhibition) in the concentration range 30–100 μM, whereas its dimethylated analogue failed to inhibit glucose-stimulated insulin release at concentrations as high as 400 μM. Comparison of the compounds possessing a primary amine group indicated that MDCD was a more effective inhibitor of glucose-stimulated insulin release than 2-NSA. At first glance, these findings correlate with the effects of these compounds on islet transglutaminase activity. However, further studies indicated that, with 2-NSA, more than one cellular process may be sensitive to this compound. Although islet glucose oxidation was not inhibited by 100 μM-NSA, the effects of the compound on the biological activity of calmodulin measured *in vitro* indicated that 2-NSA may not be specific in its action within islet cells. The sensitivity of calmodulin to antagonism by 2-NSA suggests that calmodulin may be the prime (though not the sole) target for this compound in the β-cell. Similarly, inhibition of insulin release by dimethyl-2-NSA at a concentration of 400 μM may be attributable to calmodulin antagonism. In this case, however, it is possible that dimethyl-2-NSA specifically inhibits calmodulin in the β-cell, since glucose oxidation, protein kinase C activity and transglutaminase activity were all unaffected by 400 μM-dimethyl-2-NSA.

In contrast with 2-NSA, MDCD gave significant inhibition of glucose-stimulated insulin release (33–65%) at low concentrations of the drug (30–50 μM) such that a specific mode of action for MDCD as an inhibitor of transglutaminase may be inferred. Thus we have previously shown that at 100 μM MDCD did not interfere with islet glucose oxidation (Bungay *et al.*, 1984*a*), and in addition the present findings suggest that MDCD (50 μM) did not significantly affect islet lipid methylation, nor did it affect the activity of protein kinase C measured *in vitro*. In common with its analogue, 2-NSA, MDCD was found to act as an antagonist of calmodulin, as measured in a standard assay using the calmodulin-sensitive phosphodiesterase. However, the potency of this effect was almost an order of magnitude lower than that measured for the effect of MDCD on islet transglutaminase. Thus concentrations of MDCD could be defined (30–50 μM) which gave rise to significant inhibition of glucose-stimulated insulin release, but which had little ($< 20\%$) or no effect on calmodulin-stimulated phosphodiesterase activity. The specificity of MDCD for islet transglutaminase thus demonstrated is therefore compatible with a role for this enzyme in the mechanism of glucose-stimulated insulin release. Furthermore, our results demonstrate that, within the naphthalenesulphonamide family of compounds, structural variations in the parent molecule can lead to substantial differences in their potency as inhibitors of various cellular targets which may lead to inhibition of glucose-stimulated insulin release.

A large body of evidence supports the view that a rise in the cytosolic concentration of Ca^{2+} is responsible for eliciting the secretory response of the β-cell to glucose (Prentki & Wollheim, 1984). The magnitude of this rise in intracellular [Ca^{2+}] has been estimated to be in the order of 0.1 μM, so that the concentration of free Ca^{2+} in the stimulated β-cell may reach approx. 0.2 μM (Wollheim & Pozzan, 1984; Deleers *et al.*, 1985). Therefore, for islet transglutaminase to act as a Ca^{2+}-receptor protein during β-cell activation, this enzyme must be sensitive to small changes in the free [Ca^{2+}] in the β-cell. Previous studies on the islet transglutaminase have suggested that half-maximal enzyme activity may occur between 40 and 90 μM-Ca^{2+} (Bungay *et al.*, 1984*a*; Gomis *et al.*, 1983). However, these estimates were based on assays conducted with native *NN'*-dimethylcasein as the protein substrate, under which conditions the actual free [Ca^{2+}] may be overestimated as a result of the binding of the cation to protein-bound phosphate groups (Hand *et al.*, 1985). Similarly, interference of this kind also provides difficulties in assessing the effects of other cellular cations (such as Mg^{2+}) on the sensitivity of transglutaminase to Ca^{2+}. In the present paper we measured the sensitivity of the islet transglutaminase to Ca^{2+}, using a dephosphorylated *NN'*-dimethylcasein substrate. Under these more authentic conditions, the islet transglutaminase was found to be at least an order of magnitude more sensitive to Ca^{2+} than had been previously demonstrated, with a K_m for Ca^{2+} in the region of 2–3 μM. Furthermore, the presence of Mg^{2+} (2 mM) appeared to have little effect on the sensitivity of the enzyme to Ca^{2+}. Therefore Mg^{2+}, which is present in cells at millimolar concentrations, is unlikely to affect the sensitivity of islet transglutaminase to activation by Ca^{2+}. The sensitivity of islet transglutaminase to Ca^{2+} is comparable with that measured for the phospholipid- and Ca^{2+}-dependent protein kinases (protein kinase C) obtained from islets, which possess a K_m for Ca^{2+} of 3.9 μM (Lord & Ashcroft 1984), and from bovine heart, for which a K_m for Ca^{2+} of 5 μM was measured (Wise *et al.*, 1982*a*). Our results therefore suggest that islet transglutaminase may be sufficiently sensitive to activation by Ca^{2+} for this enzyme to directly act as a receptor for Ca^{2+} in the stimulated β-cell.

It is therefore proposed that, during stimulus–secretion coupling in the pancreatic β-cell, islet transglutaminase may be activated by a rise in the free [Ca^{2+}] and that the result of the activation of this enzyme may be the post-translational modification of specific β-cell proteins which participate in the secretory mechanism. We have previously shown that primary amines such as methylamine and putrescine may be incorporated into islet proteins both *in vitro* and in the intact islet (Bungay *et al.*, 1984*b*). In the present work, we investigated these proteins further, using islet homogenates incubated with [^{14}C]methylamine. Crude subcellular fractionation of these homogenates into a particle-free supernatant and a 71000 *g*-sedimentable particulate (membranous) fraction indicated that most (93%) of the Ca^{2+}-dependent incorporation of radiolabel occurred in the membrane fraction. This finding is in keeping with other reports for the specificity of the tissue transglutaminase in the cross-linking of membrane or membrane-associated proteins (Bjerrum *et al.*, 1981; Pober & Strominger, 1981; Birckbichler *et al.*, 1972).

Attempts to resolve the islet proteins labelled by transglutaminase by using SDS/polyacrylamide-gel electrophoresis indicated that most of the incorporation of [^{14}C]methylamine occurred in proteins which did not traverse, but remained in, the stacking gel (3% acrylamide) during electrophoresis. These protein(s) therefore possessed a very high M_r, probably in excess of 10^6. In some incubations, a much smaller proportion of radiolabel was detectable at the top of the running gel (10% acrylamide), suggesting an M_r for these proteins in excess of 250000, as was the case for incubations carried out with 1.9 mM-Ca^{2+} for 120 min. These experiments also indicated that, the greater the Ca^{2+} concentration or

the longer the incubation period, the greater was the amount of polymeric material and extent of radiolabelling of this material. Since this material was not detectable in homogenates of islets which were incubated in the absence of Ca^{2+}, it seems unlikely that large amounts of this material are present in the β-cell. However, these studies demonstrate the ability of the islet transglutaminase to cross-link islet proteins which appear to be membrane-associated. Furthermore, the absence of any incorporation of methylamine into individual islet proteins involved in the formation of the polymer may suggest that the islet transglutaminase possesses a high specificity for the cross-linking of these proteins. Alternatively, these substrate proteins may already be associated in a non-covalent matrix which may be cross-linked covalently by the action of transglutaminase. Such a mechanism is comparable with that found in the human erythrocyte, where both integral membrane proteins and the associated membrane infrastructures may become cross-linked into a high-M_r polymer under the action of erythrocyte transglutaminase (Bjerrum *et al.*, 1981). Although it seems unlikely that such a drastic modification to the β-cell membrane and associated proteins would play in glucose-stimulated insulin release, these observations *in vitro* may represent the extreme of what may be a carefully regulated process *in vivo*.

In conclusion, our results detailing (1) the specificity of MDCD as an inhibitor of transglutaminase in the β-cell, (2) the sensitivity of islet transglutaminase to activation by Ca^{2+} and (3) the substrate specificity of islet transglutaminase are consistent with a role for this enzyme in glucose-stimulated insulin release. Furthermore they add further evidence to our original proposal (Bungay *et al.*, 1984*a*) that the role of the islet transglutaminase in insulin secretion may be in the cross-linking of membrane proteins and/or membrane infrastructures, a process which may serve to selectively stabilize areas of membrane during membrane recycling.

This work was funded by the Wellcome Trust and by the Sir Halley Stewart Trust, to whom we are grateful. We are also grateful to Dr. S. MacNeil for helpful discussions and for assistance with the screening of test compounds for calmodulin antagonism.

REFERENCES

Best, L., Lebrun, P., Saceda, M., Garcia-Morales, P., Hubinont, C., Juvent, M., Herchuelz, A., Malaisse-Lagae, F., Valverde, I. & Malaisse, W. J. (1984) Biochem. Pharmacol. **33**, 2033–2039

Birckbichler, P. J., Dowben, R. M., Matacic, S. S. & Loewy, A. G. (1972) Biochim. Biophys. Acta. **291**, 149–155

Bjerrum, O. J., Hawkins, M., Swanson, P., Griffin, M. & Lorand, L. (1981) J. Supramol. Struct. **16**, 289–301

Bungay, P. J., Potter, J. M. & Griffin, M. (1982) Diabetologia **23**, 159

Bungay, P. J., Potter, J. M. & Griffin, M. (1984*a*) Biochem. J. **219**, 819–827

Bungay, P. J., Potter, J. M. & Griffin, M. (1984*b*) Biosci. Rep. **4**, 869–877

Cooke, R. D. & Holbrook, J. J. (1974) Biochem. J. **141**, 71–78

Davies, P. J. A., Davies, D. R., Levitzki, A., Maxfield, R. R., Milhaud, P., Willingham, M. C. & Pastan, I. (1980) Nature (London) **283**, 162–167

Deleers, M., Mahy, M. & Malaisse, W. J. (1985) Biochem. Int. **10**, 97–103

Fésüs, L., Sándor, M., Horváth, L. I., Bagyinka, C., Erdei, A. & Gergely, J. (1981) Mol. Immunol. **18**, 633–638

Folk, J. E. & Finlayson, J. S. (1977) Adv. Protein. Chem. **31**, 1–133

Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino, E. E. & Ashcroft, S. J. H. (1980) Biochem. J. **192**, 919–927

Gomis, R., Sener, A., Malaisse-Lagae, F. & Malaisse, W. J. (1983) Biochim. Biophys. Acta **760**, 384–388

Günzler, V., Schopf, R. E., Hanauske-Abel, H. M. & Schulte-Wissermann, H. (1982) FEBS Lett. **150**, 390–396

Hand, D., Bungay, P. J., Elliott, B. M. & Griffin, M. (1985) Biosci. Rep. **5**, 1079–1086

Hedeskov, C. J. (1980) Physiol. Rev. **60**, 442–509

Heding, L. (1972) Diabetologia **8**, 260–266

Hidaka, H. & Tanaka, T. (1983) Methods Enzymol. **102**, 185–194

Julian, C., Speck, N. A. & Pierce, S. K. (1983) J. Immunol. **130**, 91–96

Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–66

Lacy, P. E. & Kostianovsky, M. (1967) Diabetes **16**, 35–39

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lorand, L. & Conrad, S. M. (1984) Mol. Cell Biochem. **58**, 9–35

Lorand, L., Campbell-Wilkes, L. K. & Cooperstein, L. (1972) Anal. Biochem. **50**, 623–631

Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jönsson, N. A., Mikiver, L. & Moses, P. (1979) Biochemistry **18**, 1756–1765

Lord, J. M. & Ashcroft, S. J. H. (1984) Biochem. J. **219**, 547–551

Lott, W. A. & Krapcho, J. (1957) U.S. Patent 2,813,904

MacNeil, S., Walker, S. W., Senior, H. J., Bleehen, S. S. & Tomlinson, S. (1984) J. Invest. Dermatol. **83**, 15–19

Mato, J. M., Pencev, D., Vasanthakumar, G., Schiffmann, E. & Pastan, I. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 1929–1932

Pober, J. A. & Strominger, J. L. (1981) Nature (London) **289**, 819–821

Prentki, M. & Wollheim, C. B. (1984) Experientia **40**, 1052–1060

Rasmussen, H. & Barrett, P. O. (1984) Physiol. Rev. **64**, 938–984

Schatzman, J. H. (1973) J. Physiol. (London) **235**, 551–569

Sener, A., Gomis, R., Lebrun, P., Herchuelz, A., Malaisse-Lagae, F. & Malaissee, W. J. (1984) Mol. Cell. Endocrinol. **36**, 175–180

Van Leuven, F., Cassiman, J. J. & Van Den Berghe, H. (1980) Cell **20**, 37–43

Veloso, D., Guynn, R. W., Oskarsson, M. & Veech, R. L. (1973) J. Biol. Chem. **248**, 4811–4819

Wani, M. C., Campbell, H. F., Brine, G. A., Kepler, J. A. & Wall, M. E. (1972) J. Am. Chem. Soc. **94**, 3631–3632

Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982*a*) J. Biol. Chem. **257**, 8481–8488

Wise, B. C., Glass, D. B., Jen Chou, C. H., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. S., Kibler, R. F. & Kuo, J. F. (1982*b*) J. Biol. Chem. **257**, 8489–8495

Wollheim, C. B. & Sharp, G. W. G. (1981) Physiol. Rev. **61**, 914–973

Wollheim, C. B. & Pozzan, T. (1984) J. Biol. Chem. **259**, 2262–2267

Received 21 August 1985/20 November 1985; accepted 5 December 1985