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Evidence for the participation of calmodulin in stimulus–secretion coupling in the pancreatic β-cell

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1. The ability of a range of phenothiazines to inhibit activation of brain phosphodiesterase by purified calmodulin was studied. Trifluoperazine, prochlorperazine and 8-hydroxyprochlorperazine produced equipotent dose-dependent inhibition with half-maximum inhibition at 12 μM. When tested at 10 or 50 μM, 7-hydroxyprochlorperazine was a similarly potent inhibitor. However, trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine were ineffective at concentrations up to 50 μM, and produced only a modest inhibition at 100 μM. 2. The same phenothiazines were tested for their ability to inhibit activation of brain phosphodiesterase by boiled extracts of rat islets of Langerhans. At a concentration of 20 μM, 70–80% inhibition was observed with trifluoperazine, prochlorperazine, 7-hydroxyprochlorperazine or 8-hydroxyprochlorperazine, whereas trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine were less effective. 3. The effect of these phenothiazines on insulin release from pancreatic islets was studied in batch-type incubations. Insulin release stimulated by glucose (20 mM) was markedly inhibited by 10 μM-trifluoperazine or -prochlorperazine and further inhibited at a concentration of 20 μM. 8-Hydroxyprochlorperazine (20 μM) was also a potent inhibitor but 7-hydroxyprochlorperazine (20 μM) elicited only a modest inhibition of glucose-stimulated insulin release; no inhibition was observed with trifluoperazine-5-oxide or *N*-methyl-2-(trifluoromethyl)phenothiazine. 4. Trifluoperazine (20 μM) markedly inhibited insulin release stimulated by leucine or 4-methyl-2-oxopentanoate in the absence of glucose, and both trifluoperazine and prochlorperazine (20 μM) decreased insulin release stimulated by glibenclamide in the presence of 3.3 mM-glucose. 5. None of the phenothiazines affected basal insulin release in the presence of 2 mM-glucose. 6. Trifluoperazine (20 μM) did not inhibit islet glucose utilization nor the incorporation of [^{3}H]leucine into (pro)insulin or total islet protein. 7. Islet extracts catalysed the incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous protein substrates. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis resolved several phosphorylated bands, but incorporation was slight. However, calmodulin in the presence of Ca^{2+} greatly enhanced incorporation: the predominant phosphorylated band had an estimated mol.wt. of 55 000. This enhanced incorporation was abolished by trifluoperazine, but not by cyclic AMP-dependent protein kinase inhibitor protein. 8. These results suggest that islet phosphodiesterase-stimulating activity is similar to, although not necessarily identical with, calmodulin from skeletal muscle; that islet calmodulin may play an important role in Ca^{2+}-dependent stimulus–secretion coupling in the β-cell; and that calmodulin may exert part at least of its effect on secretion via phosphorylation of endogenous islet proteins.

An increase in the intracellular concentration of Ca^{2+} is implicated as a key event in the stimulation of insulin secretion. However, the way in which Ca^{2+} interacts with the exocytotic discharge system is unknown. Recently it has been suggested that the primary intracellular target for Ca^{2+} within the β-cell is the Ca^{2+}-dependent regulator protein calmodulin (Sugden & Ashcroft, 1979). Calmodulin is a heat-stable Ca^{2+}-binding protein first discovered as

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an activator of brain cyclic nucleotide phosphodiesterase (Cheung, 1970), but later found to confer sensitivity to Ca^{2+} to a number of other proteins (Cheung *et al.*, 1975; Wang *et al.*, 1975; Gopinath & Vincenzi, 1977; Cohen *et al.*, 1978). Indeed, the multiplicity of effects of calmodulin and its ubiquitous tissue distribution have led to the view that it may represent the major intracellular Ca^{2+}-receptor protein in non-muscle cells. The evidence for a role for calmodulin in Ca^{2+}-dependent insulin release is the demonstration of substantial amounts of calmodulin in extracts of rat islets of Langerhans, together with the observation that the phenothiazine tranquillizer trifluoperazine, a specific inhibitor of calmodulin (Levin & Weiss, 1977), inhibits glucose-stimulated insulin release (Sugden & Ashcroft, 1979). In the present study we have attempted to substantiate further this hypothesis by investigating the dose dependence of effects of trifluoperazine on calmodulin and on glucose-stimulated insulin release, and the relative ability of a range of other phenothiazines to affect these two parameters. We have also examined the effects of trifluoperazine on some other stimulators of insulin release. In addition we have investigated whether the inhibitory action of trifluoperazine is confined to the release process by assessing its effect on islet glucose utilization and glucose-stimulated insulin and protein biosynthesis.

It has also been suggested (Ashcroft, 1980) that effects of Ca^{2+}–calmodulin on insulin secretion may involve protein phosphorylation–dephosphorylation. Previous studies have demonstrated that phosphorylation of endogenous islet protein by endogenous protein kinase is largely independent of cyclic AMP; and cyclic AMP-independent protein kinase activity in extracts of rat islets has been separated from the cyclic AMP-dependent protein kinases present by DEAE-cellulose chromatography (Sugden *et al.*, 1979). We have therefore investigated the effects of Ca^{2+}, calmodulin and trifluoperazine on the phosphorylation of endogenous islet proteins by endogenous protein kinase activity, using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to resolve the phosphorylated proteins.

Experimental

Materials

Bovine albumin (fraction V), 5′-nucleotidase and collagenase were from Sigma (London) Chemical Co., Poole, Dorset BM17 7NH, U.K. Trifluoperazine (Stelazine) and prochlorperazine maleate (Compazine) were gifts from Smith, Kline and French Laboratories. Dr. A. Manian, Pharmacology Research Branch, National Institute of Mental Health, MD, U.S.A., kindly provided the following phenothiazines: 7-hydroxyprochlorperazine, 8-hydroxyprochlorperazine, trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine. Sepharose 4B and QAE-Sephadex were from Pharmacia (G.B.), London W5 5SS, U.K. DE-52 DEAE-cellulose was from Whatman, H. Reeve Angel Scientific, London SE1 6BD, U.K. Guinea-pig anti-(porcine insulin) was obtained from Wellcome Reagents, Beckenham, Kent, U.K. Freeze-dried anti-insulin serum was from Miles–Yeda, Stoke Poges, Slough, Bucks., U.K. All radiochemicals were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Other reagents, of the purest available grade, were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Calmodulin prepared from skeletal-muscle phosphorylase kinase (Cohen *et al.*, 1978) was a gift from Dr. P. Cohen, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K. Calmodulin-deficient phosphodiesterase, prepared from bovine brain cortices, by the method of Klee & Krinks (1978), was a gift from Dr. P. Sugden of this Department. Cyclic AMP-dependent protein kinase inhibitor (Ashby & Walsh, 1972) from rat hearts and brains, partially purified as described by Sugden *et al.* (1979), was a gift from Dr. M. C. Sugden, Unit for Metabolic Research, Radcliffe Infirmary, Oxford, U.K. One unit of inhibitor activity is the amount that inhibited the transfer of 1 pmol of ^{32}P from [γ-^{32}P]ATP to histone per min at 30°C using purified catalytic subunit of cyclic AMP-dependent protein kinase.

Preparation of islets

Islets were prepared by a collagenase method (Coll-Garcia & Gill, 1969) from the pancreases of 300 g male allbino Wistar rats fed *ad libitum*.

Insulin release

Batches of five islets were incubated *in vitro* for 2 h at 37°C in Krebs bicarbonate medium (Krebs & Henseleit, 1932) containing albumin (2 mg/ml) and the additions stated in the text or Tables and Figures as previously described (Ashcroft *et al.*, 1973). Insulin release was measured by a charcoal radioimmunoassay as described by Ashcroft & Crossley (1975), except that Wellcome guinea-pig anti-insulin serum was used as antibody.

(Pro)insulin biosynthesis

Batches of 10 islets were incubated for 90 min at 37°C in 0.1 ml of bicarbonate medium containing albumin (2 mg/ml), L-[4,5-^{3}H]leucine (4 μCi; sp. radioactivity 50 Ci/mmol) and the additions stated. The incorporation of [^{3}H]leucine into insulin plus proinsulin [designated '(pro)insulin'] was measured by using insulin-binding affinity columns for (pro)-insulin, and into total islet protein by using trichloroacetic acid precipitation as previously described in detail (Ashcroft *et al.*, 1978).

Glucose utilization

Batches of 10 islets were incubated for 2h at 37°C in 20μl of bicarbonate medium containing albumin (2mg/ml) and [5-^{3}H]glucose (20mM; sp. radioactivity 1Ci/mol) in the absence or presence of trifluoperazine (25μM). Rates of glucose utilization were estimated as ^3H_2O formed as described previously (Ashcroft *et al.*, 1973).

Assay of calmodulin activity

Calmodulin was assayed by its ability to activate brain phosphodiesterase. The method has been described in detail (Sugden & Ashcroft, 1979). The reaction mixture, final volume 100μl, contained 50mM-Tris/HCl (pH 8.0)/3mM-$MgCl_2$/3′:5′-cyclic[^{3}H]AMP (0.1mM; sp. radioactivity 50Ci/mol) and 0.1mg of albumin/ml. Activator-dependent activity was measured in the presence of 0.05mM-$CaCl_2$ and sufficient calmodulin (100ng) or boiled islet extract (see below) to give approximately 5-fold activation. Trifluoperazine or other phenothiazines were present at concentrations stated in the Tables or Figures. Reaction was allowed to proceed for 15min at 37°C and was then terminated by boiling. 5′-[^{3}H]AMP formed was converted by 5′-nucleotidase and the [^{3}H]adenosine was separated from unreacted cyclic [^{3}H]AMP by column chromatography with columns (0.5cm × 1cm) of QAE-Sephadex A-25 in the formate form and quantified by liquid-scintillation spectrometry.

Islet extracts were prepared for assay of calmodulin as described by Sugden & Ashcroft (1979).

Phosphorylation of islet homogenates

Islets (300–550) were disrupted by sonication (3 × 5s at position 3 on a Soniprobe, Dawe Instruments) in 100μl of reaction medium (see below). Phosphorylation of islet protein was carried out by a modification of the method of Schulman & Greengard (1978). The reaction mixture (final volume 50μl) contained 50mM-Mes (4-morpholineethanesulphonic acid) (pH 6.9)/0.1mM-dithiothreitol/1mM - $MgCl_2$/10mM - benzamidine/10mM - EGTA and 10μl of islet homogenate. Other additions are given in the legend to Fig. 1 and the text. Ca^{2+} when present was added as 10mM-$CaCl_2$ to give an estimated free Ca^{2+} concentration of 40μM. After pre-incubation for 2min the reaction was initiated by addition of [γ-^{32}P]ATP (3–7d.p.m./fmol; final concentration 20μM). After incubation for 2min at 30°C the reaction was terminated by addition of 25μl of 'stop solution' containing 50mM-Tris/HCl (pH 7.8)/9% (w/v) sodium dodecyl sulphate/15% (w/v) glycerol/0.01% (w/v) Bromophenol Blue/9% (w/v) 2-mercaptoethanol and heating for 5min on a boiling-water bath. Portions (30μl) were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography

Electrophoresis was performed by modification of the method of Laemmli (1970). The separation gel (7.0cm × 7.5cm × 0.1cm) contained 0.375M-Tris/HCl (pH 8.8)/12.5% (w/v) acrylamide/0.33% (w/v) *NN′*-methylenebisacrylamide/0.1% (w/v) sodium dodecyl sulphate/0.05% (v/v) *NNN′N′*-tetramethylethylenediamine/0.075% (w/v) ammonium persulphate. The stacking gel (0.5cm × 7.5cm × 0.1cm) contained 63mM-Tris/HCl (pH 6.7)/5% (w/v) acrylamide/0.13% (w/v) *NN′*-methylenebisacrylamide/0.1% (w/v) sodium dodecyl sulphate/0.09% (v/v) *NNN′N′*-tetramethylethylenediamine/0.075% ammonium persulphate. Electrophoresis was carried out at a constant voltage of 70V in 50mM-Tris/0.384M-glycine buffer containing 0.1% sodium dodecyl sulphate for approx. 4h, until the Bromophenol Blue was about 5mm above the bottom of the gel. The gels were stained overnight in 0.025% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid/25% (v/v) propan-2-ol, followed by 3–4h in 0.0025% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid/10% (v/v) propan-2-ol. The gels were destained in 10% (v/v) acetic acid. Gels were dried by heating under vacuum (Maizel, 1971) and the dried gels were subjected to autoradiography using Kodak Blue Brand Regulix X-ray film. The following were used for molecular-weight calibration of gels: human transferrin (mol.wt. 88000); bovine serum albumin (mol.wt. 68000); ovalbumin (mol.wt. 43000); pig heart lactate dehydrogenase (mol.wt. 36000); bovine pancreatic ribonuclease (mol.wt. 13700).

Miscellaneous methods

Phenothiazines other than trifluoperazine were prepared as a stock solution (10mM) in dimethyl sulphoxide and diluted with buffer to their final concentrations: equivalent concentrations of dimethyl sulphoxide were added to appropriate controls. Results are given as means ± S.E.M. for the number of observations in parentheses. The significance of differences was assessed by using Student's *t* test.

Results

Effects of phenothiazines on the activation of phosphodiesterase by purified calmodulin and by calmodulin activity in islet extracts

The effects of trifluoperazine and other phenothiazines on the Ca^{2+}-dependent activation of brain phosphodiesterase by purified calmodulin are given in Table 1. Under the assay conditions used,

Table 1. *Effects of phenothiazines on calmodulin-activated phosphodiesterase*

The effect of the phenothiazines given on brain phosphodiesterase activated by purified calmodulin (100ng) was measured as described in the text. The data are expressed as a percentage of phosphodiesterase activity in the absence of phenothiazine and are given as means ± S.E.M. for the numbers of observations in parentheses, or as means of closely agreeing duplicates. Control phosphodiesterase activity was approx. 12pmol/min in the absence and 60pmol/min in the presence of Ca^{2+} and calmodulin.

Phenothiazine	Concentration (μM)	Phosphodiesterase activity (% of control)
None	—	100
Trifluoperazine	5	86 ± 6 (5)
	10	55 ± 6 (9)
	20	25 ± 1 (6)
	40	23 ± 1 (6)
	50	18
Prochlorperazine	5	106 ± 4 (3)
	10	64 ± 4 (7)
	20	33 ± 1 (3)
	40	23 ± 1 (3)
	50	15
8-Hydroxyprochlorperazine	5	89 ± 2 (3)
	10	62 ± 3 (5)
	20	18 ± 1 (3)
	40	11 ± 1 (3)
	50	21
7-Hydroxyprochlorperazine	10	64
	50	18
Trifluoperazine-5-oxide	10	98 ± 3 (3)
	50	99
	100	60
N-Methyl-2-(trifluoromethyl)-phenothiazine	10	101 ± 3 (4)
	50	94
	100	68 ± 2 (5)

phosphodiesterase activity in the absence of calmodulin was approx. 12pmol/min and was increased 5-fold by calmodulin (100ng): activation required the presence of Ca^{2+} (Sugden & Ashcroft, 1979). With maximal rates of phosphodiesterase activity, not more than 10% of the 3′:5′-cyclic AMP originally present was hydrolysed. As previously described (Sugden & Ashcroft, 1979), trifluoperazine produced a dose-dependent inhibition of calmodulin-activated phosphodiesterase; we observed similar dose-dependent inhibition by prochlorperazine, and 8-hydroxyprochlorperazine with half-maximal inhibition at approx. 12μM. 7-Hydroxyprochlorperazine had a similar potency, but trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine were much less effective; no significant inhibition was observed with these two phenothiazines at 10 or 50μM and only a modest inhibition was seen at 100μM. The basal rate of phosphodiesterase seen in the absence of calmodulin was not affected by any of the phenothiazines (results not shown).

Table 2. *Effects of phenothiazines on phosphodiesterase activated by islet calmodulin*

The effect of the phenothiazines given (all at 20μM) on brain phosphodiesterase activated by boiled islet extract (islet calmodulin) was measured as described in the text. The amount of islet extract used, corresponding to 12 islets, increased the activity of the phosphodiesterase from approx. 12 to 60pmol/min. The results are expressed as percentages of phosphodiesterase activity in the absence of phenothiazine and are given as means ± S.E.M. for the numbers of observations given in parentheses.

Phenothiazine type	Phosphodiesterase activity (% of control)
None	100
Trifluoperazine	17 ± 1 (6)
Prochlorperazine	25 ± 2 (3)
8-Hydroxyprochlorperazine	15 ± 2 (3)
7-Hydroxyprochlorperazine	32 ± 2 (3)
Trifluoperazine-5-oxide	68 ± 1 (3)
N-Methyl-2-(trifluoromethyl)-phenothiazine	76 ± 1 (3)

It was necessary to determine whether the phosphodiesterase-stimulating activity in islet extracts, ascribed to islet calmodulin (Sugden & Ashcroft, 1979), showed similar responses to phenothiazines. The effects of these phenothiazines, at a concentration of 20μM, on islet calmodulin are shown in Table 2. By using the same assay conditions as had been established for showing activation by purified calmodulin, a similar degree of activation was achieved with an amount of boiled islet extract corresponding to 12 islets. Inhibition of activation was most marked with 20μM-trifluoperazine and 8-hydroxyprochlorperazine; prochlorperazine and 7-hydroxyprochlorperazine were slightly less effective, and trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)prochlorperazine produced only a modest inhibition.

Effects of phenothiazines on insulin release

Insulin release, measured in batch-type incubations of islets, was increased 28-fold on raising the glucose concentration from 2 to 20mM: the effects of trifluoperazine and other phenothiazines on glucose-stimulated insulin release are given in Table 3. Both trifluoperazine and prochlorperazine inhibited release of insulin when tested at a concentration of 10μM and inhibition was further increased at concentrations of 20μM. No effects of these agents were seen on basal insulin release with 2mM-glucose. Inhibition of glucose-stimulated in-

Table 3. *Effects of phenothiazines on glucose-stimulated insulin release*

Batches of five islets were incubated for 2h at 37°C in bicarbonate medium containing albumin (2mg/ml) and glucose (2 or 20mM) in the presence or absence of the phenothiazines stated. Insulin released into the medium was measured by radioimunoassay. Results are given as means ± S.E.M. for the numbers of batches of islets given in parentheses. The significance of the differences from the controls (no phenothiazine) was assessed by using Student's *t* test. Abbreviation used: NS, not significant. *, $P \leqslant 0.01$; **, $P \leqslant 0.001$.

Compound tested	Concentration	Insulin release (μunits/islet per h) Glucose (2mM)	Glucose (20mM)
None	—	13.5 ± 1.4 (19)	362.3 ± 23.0 (23)
Trifluoperazine	10μM	14.5 ± 4.5 (8) NS	110.5 ± 22.6 (6)**
	20μM	16.6 ± 4.6 (8) NS	52.3 ± 5.5 (15)**
Prochlorperazine	10μM	15.3 ± 4.2 (8) NS	111.7 ± 31.1 (8)**
	20μM	18.1 ± 3.3 (8) NS	21.5 ± 7.7 (7)**
8-Hydroxyprochlorperazine	20μM	—	50.4 ± 6.2 (8)**
7-Hydroxyprochlorperazine	20μM	—	231.2 ± 20.9 (9)*
Trifluoperazine-5-oxide	20μM	—	283.9 ± 42.7 (5) NS
N-Methyl-12-(trifluoromethyl)phenothiazine	20μM	—	340.6 ± 41.2 (5) NS

Table 4. *Effects of trifluoperazine and prochlorperazine in insulin release*

Batches of five islets were incubated for 2h at 37°C in bicarbonate medium containing albumin (2mg/ml) and the additions stated. Insulin released into the medium was measured by radioimmunoassay. Results are given as means ± S.E.M. for the numbers of batches of islets given in parentheses. The significance of the differences from the controls (no phenothiazine) was assessed by using Student's *t* test. *, $P \leqslant 0.05$; **, $P \leqslant 0.01$; ***, $P \leqslant 0.001$.

Compound tested	Concentration	Insulin release (μ-units/islet per h) Glucose (3.3mM)	3.3mM-Glucose + glibenclamide (10μg/ml)	15mM-4-Methyl-2-oxopentanoate	20mM-Leucine
None	—	22.3 ± 4.1 (5)	200.1 ± 39.3 (5)	338.6 ± 36.9 (9)	59.1 ± 6.8 (10)
Trifluoperazine	20μM	—	64.0 ± 17.9 (5)*	81.6 ± 21.4 (5)***	26.8 ± 3.9 (8)**
Prochlorperazine	20μM	—	50.1 ± 12.9 (5)**	—	—

sulin release was also observed with 20μM-8-hydroxyprochlorperazine. 7-Hydroxyprochlorperazine was much less effective and no inhibition was observed with either trifluoperazine-5-oxide or *N*-methyl-2-(trifluoromethyl)phenothiazine. None of the phenothiazines at the concentrations used affected the radioimmunoassay of insulin (results not shown).

The effects of phenothiazines on insulin release stimulated by other agents are given in Table 4. In the presence of 3.3mM-glucose, insulin release was stimulated 10-fold by glibenclamide (10μg/ml): marked inhibition by 20μM-trifluoperazine or -prochlorperazine was observed. Insulin release was stimulated by 4-methyl-2-oxopentanoate (15mM) to an extent similar to that seen with 20mM-glucose. 4-Methyl-2-oxopentanoate-induced release of insulin was inhibited markedly by 20μM-trifluoperazine. Leucine-induced insulin release was also strongly inhibited by 20μM-trifluoperazine.

Effects of trifluoperazine on glucose utilization and the synthesis of (pro)insulin and islet protein

It was important to try to exclude the possibility that trifluoperazine inhibited insulin release by a relatively non-specific mechanism. Therefore the effect of trifluoperazine on three other glucose-dependent parameters of islet function was tested. Results are given in Table 5. The rate of glucose utilization was increased 5-fold on raising the glucose concentration from 2 to 20mM: trifluoperazine (20μM) did not affect the rate of glucose utilization. The incorporation of [4,5-^{3}H]leucine into total trichloroacetate-precipitable material was increased 3-fold and into (pro)insulin 12-fold when the glucose concentration was raised from 2 to 20mM: the ratio of (pro)insulin to total protein synthesis increased from 0.035 to 0.162. None of these parameters was affected by 20μM-trifluoperazine.

Protein phosphorylation in islet homogenates

Previous studies (Sugden *et al.*, 1979) have shown that extracts of pancreatic islets catalyse the incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous islet protein. In the present study, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was used to resolve the phosphorylated

Table 5. *Effect of trifluoperazine on islet glucose utilization and incorporation of [^{3}H]leucine into (pro)insulin and protein*

Glucose utilization by islets was measured as the rate of ^3H_2O production from [5-^{3}H]glucose and rates of total protein and (pro)insulin biosynthesis was assessed by using [4,5-^{3}H]leucine incorporation as described in the Experimental section. Results are expressed as means ± S.E.M. for the number of batches of islets in parentheses.

		Glucose utilization or [^{3}H]leucine incorporation		
Parameter	Units	Glucose (2mM)	Glucose (20mM)	Glucose (20mM) + 20μM-trifluoperazine
Glucose utilization	pmol/islet per h	16.5 ± 1.6 (10)	77.0 ± 4.3 (10)	81.5 ± 4.7 (10)
Total protein biosynthesis	d.p.m./10 islets per 90min	45793 ± 2178 (15)	115327 ± 7236 (15)	112639 ± 12203 (8)
(Pro)insulin biosynthesis	d.p.m./10 islets per 90min	1578 ± 164 (15)	18397 ± 1217 (15)	18194 ± 1943 (8)
(Pro)insulin/total protein ratio	—	0.035 ± 0.004 (15)	0.162 ± 0.009 (15)	0.165 ± 0.013 (8)

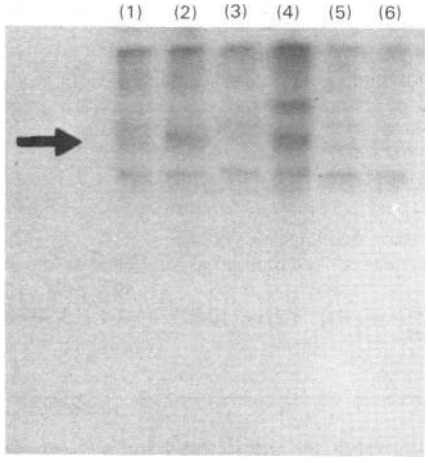

Fig. 1. *Autoradiogram showing calmodulin-dependent phosphorylation of islet proteins*

Islet extracts were incubated as described in the Experimental section with 20μM-[γ-^{32}P]ATP in Mes-buffered medium containing 0.10mM-dithiothreitol/1mM-$MgCl_2$/10mM-benzamidine/10mM-EGTA and the additions stated below. The phosphorylation reactions were terminated by addition of sodium dodecyl sulphate 'stop solution' and heating for 5min on a boiling-water bath. Electrophoresis was performed on a 12.5% sodium dodecylsulphate/polyacrylamide gel at constant voltage. After staining, the gel was dried under vacuum and subjected to autoradiography using Kodak Blue Band Regulix X-ray film. Additions to phosphorylation media were: lane 1, Ca^{2+} (as $CaCl_2$, to give an estimated free Ca^{2+} concentration of 40μM); lane 2, Ca^{2+} plus calmodulin (1μg); lane 3, Ca^{2+}, calmodulin and trifluoperazine (100μM); lane 4, Ca^{2+}, calmodulin and cyclic AMP-dependent protein kinase inhibitor (1000units/ml); lane 5, none; lane 6, calmodulin. The arrow marks the position of the 55000-mol.wt. phosphoprotein referred to in the text.

proteins. Control experiments established optimum conditions for demonstrating protein phosphorylation; the phosphorylation of several proteins was enhanced by the addition of Ca^{2+} (>1μM) and calmodulin (>100ng) and incorporation was maximal after 2min. The predominant Ca^{2+}-dependent phosphoprotein band had a mol.wt. of 55000 ± 600 (mean ± S.E.M.; $n = 7$) and Fig. 1 shows the effects of Ca^{2+}, calmodulin and trifluoperazine on this band (arrow). Similar results were obtained with four separate islet extracts. The faint incorporation observed in the absence of Ca^{2+} (lane 5) was slightly enhanced by the presence of 40μM free Ca^{2+} (lane 1), but not by calmodulin in the absence of Ca^{2+} (lane 6). Incorporation was, however, markedly increased by the addition of Ca^{2+} plus calmodulin (lane 2); the enhanced incorporation was abolished by trifluoperazine (lane 3), but not by 1000units of partially purified cyclic AMP-dependent protein kinase inhibitor/ml (lane 4).

Discussion

Specificity of effects of phenothiazines

In the present study and that of Sugden & Ashcroft (1979) the inhibitory action of trifluoperazine on insulin secretion has been ascribed to inhibition of calmodulin. The validity of this assumption requires comment. The specificity of binding of trifluoperazine to calmodulin has been studied by Levin & Weiss (1977) and by Weiss & Levin (1978). It was found that trifluoperazine bound to calmodulin prepared from several species and tissues; but, of a variety of purified proteins ranging from mol.wt. 12500 to mol.wt. 240000 and including several Ca^{2+}-binding proteins, only calmodulin and, to a small extent, troponin C displayed any significant Ca^{2+}-dependent binding of trifluoperazine. These findings attest to the specificity of trifluoperazine for calmodulin using purified

proteins. However, in an intact cell system, the possible interaction of trifluoperazine, which is hydrophobic, with hydrophobic environments such as the cell membrane should also be considered. For example, in voltage-clamp studies of frog sciatic nerve, Hille (1966) found that prochlorperazine affected Na^+ conductance in a manner similar to that seen with a local anaesthetic. Although it is difficult to eliminate conclusively the possibility of calmodulin-independent actions of trifluoperazine, the following points argue against such an explanation for our results. First, the failure of trifluoperazine to affect islet metabolic fluxes and rates of insulin biosynthesis excludes a gross non-specific impairment of islet function by the drug. Secondly, the present study has avoided reliance on a single drug and has investigated a range of phenothiazines whose relative abilities to inhibit insulin release were well correlated with their effects on calmodulin. Thirdly, in unpublished experiments, we have found that *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W7), an inhibitor of calmodulin chemically unrelated to the phenothiazines (Hidaka *et al.*, 1979), also inhibits glucose-induced insulin release (compound W7 was kindly supplied to us by Professor H. Hidaka, Mie University, Tsu, Japan). Finally, it should be noted that although Hille (1966) interpreted his results as a direct interaction of prochlorperazine with a component of the Na^+ channel, other mechanisms cannot be excluded.

Calmodulin and exocytosis

The key role for Ca^{2+} in triggering exocytosis and the possibility that calmodulin may constitute a major intracellular receptor for Ca^{2+} have prompted investigation of a possible involvement of calmodulin in stimulus–secretion coupling. It has been demonstrated that trifluoperazine inhibits exocytosis in sea-urchin eggs (Baker & Whitaker, 1979), and in adrenal medulla cells rendered leaky by electric discharge, trifluoperazine inhibited the release of catecholamines (Baker & Knight, 1979). In pancreatic islets we previously demonstrated the presence of high concentrations of calmodulin, and found that glucose-stimulated insulin secretion was inhibited by concentrations of trifluoperazine shown to inhibit Ca^{2+}-dependent activation of phosphodiesterase by calmodulin (Sugden & Ashcroft, 1979). The suggestion that calmodulin may play a role in insulin secretion receives considerable support from the present study.

Activation of brain phosphodiesterase provided a convenient assay for calmodulin and initial experiments investigated the relative effects of several phenothiazines on the activation of phosphodiesterase by purified calmodulin. It was found that trifluoperazine, prochlorperazine, 7-hydroxyprochlorperazine and 8-hydroxyprochlorperazine had similar potency as inhibitors, whereas trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)-phenothiazine were relatively ineffective. The same phenothiazines were then tested for their ability to inhibit the activation of phosphodiesterase by boiled islet extracts, first to substantiate the view that the phosphodiesterase-stimulating activity in islets is attributable to calmodulin, and secondly to permit direct comparison with effects of phenothiazines on insulin release. In qualitative terms, the phenothiazines had similar relative effects on purified calmodulin and on islet phosphodiesterase-stimulating activity. Thus trifluoperazine, prochlorperazine, 7-hydroxyprochlorperazine and 8-hydroxyprochlorperazine were all more potent inhibitors than trifluoperazine-5-oxide or *N*-methyl-2-(trifluoromethyl)phenothiazine. Quantitatively, the degree of inhibition produced by the four most active phenothiazines was closely similar for both systems: however, the islet calmodulin appeared somewhat more sensitive than purified calmodulin to inhibition by trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine. When the relative ability of these phenothiazines to inhibit islet calmodulin was compared with their inhibitory effects on glucose-stimulated insulin release, the order of potency of the phenothiazines was similar for these two parameters. Moreover the degree of inhibition (75–95%) was also similar for the two phenomena for the three most potent phenothiazines at 20μM. The lack of effect of 20μM-trifluoperazine-5-oxide and *N*-methyl-2-(trifluoromethyl)phenothiazine on insulin release compares well with the ineffectiveness of these agents at this concentration on purified calmodulin, but correlates only qualitatively with their effects on islet calmodulin. The magnitude of the inhibitory effect of 20μM-7-hydroxyprochlorperazine was also greater on calmodulin (68%) than on glucose-stimulated insulin release (38%). These quantitative differences could be due to a diffference in sensitivity to calmodulin of phosphodiesterase and the insulin secretory process.

Since glucose-stimulated insulin release is believed to be closely linked with β-cell glucose metabolism (for review see Ashcroft, 1980) it was necessary to assess possible effects of trifluoperazine on glucose metabolism. In agreement with a previous observation on glucose oxidation (Sugden & Ashcroft, 1979), we found no evidence for impairment of islet glucose utilization by trifluoperazine. This finding is consistent with the lack of effect of trifluoperazine on glucose-stimulated insulin synthesis, since the latter also appears to depend on the integrity of islet glucose metabolism (Ashcroft *et al.*, 1978). The lack of effect of trifluoperazine on glucose-stimulated insulin biosynthesis also gives support to the view

that the action of trifluoperazine on calmodulin involves Ca^{2+}, since glucose-stimulated biosynthesis, unlike release of insulin, is not dependent on Ca^{2+} (Pipeleers *et al.*, 1973). If phenothiazines are acting, via calmodulin, on exocytosis, than their inhibitory action should not be confined to insulin release stimulated by glucose. This prediction was verified by showing that insulin release in response to three other stimulants, glibenclamide, 4-methyl-2-oxopentanoate and leucine, was impaired by trifluoperazine to a similar degree to that of glucose-stimulated insulin release.

The following conclusions seem warranted. Rat islets of Langerhans contain calmodulin. Inhibition of calmodulin by phenothiazines is associated with impaired insulin release in response to glucose and other stimulants, but is without effect on glucose-stimulated biosynthesis of insulin or islet glucose metabolism. Hence calmodulin is a plausible mediator of the action of Ca^{2+} in stimulus–secretion coupling in the pancreatic β-cell.

Calmodulin and islet protein phosphorylation

The multiplicity of effects of calmodulin suggests, as discussed elsewhere (Sugden & Ashcroft, 1979; Ashcroft 1980), several possible sites of action in insulin secretion. These include effects on Ca^{2+}-dependent ATPases (Gopinath & Vincenzi, 1977), on microtubule function (Marcum *et al.*, 1978) and on the cyclic AMP system itself: effects of calmodulin on islet adenylate cyclase have indeed been reported (Valverde *et al.*, 1979) and we have observed Ca^{2+}-dependent activation of islet phosphodiesterase by calmodulin (M. C. Sugden, unpublished work). However, in the present study we wish to draw attention to the possibility that calmodulin may exert all, or part, of its postulated effect on insulin release via protein phosphorylation. First, in other systems, several of the effects of calmodulin are exerted in such a manner e.g. regulation of phosphorylase kinase (Cohen *et al.*, 1978), activation of myosin light-chain kinase (Wang *et al.*, 1975). Secondly, there is persuasive evidence that such Ca^{2+}-dependent protein phosphorylation may be involved in secretion: it has been shown that Ca^{2+}-dependent protein phosphorylation accompanies ionophore A23187-induced release of histamine from mast cells (Sieghart *et al.*, 1978) and neurotransmitter release from synaptic vesicles (DeLorenzo *et al.*, 1979). In pancreatic islets, protein kinase activity has been found to be heterogeneous (Sugden *et al.*, 1979): when histone is added as an exogenous substrate it is possible to demonstrate the existence of two isoenzymes of cyclic AMP-dependent protein kinase. The latter activities are stimulated by cyclic AMP and inhibited by the heat-stable cyclic AMP-dependent protein kinase inhibitor. However, the existence of cyclic AMP-independent protein kinase unaffected by the inhibitor has also been shown, and has been separated by DEAE-cellulose chromatography from the cyclic AMP-dependent protein kinases (Sugden *et al.*, 1979). In the absence of exogenous substrate, the cyclic AMP-independent protein kinase appears to be responsible for the major part of the incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous islet protein (Sugden *et al.*, 1979). In the present study these observations have been expanded by using sodium dodecyl sulphate/polyacrylamide-gel chromatography to resolve the proteins phosphorylated in islets by endogenous protein kinase activity. Several discrete bands are obtained. The major new finding is that incorporation of ^{32}P into islet protein is markedly enhanced by exogenous calmodulin in the presence of Ca^{2+}: moreover the enhanced incorporation is abolished by trifluoperazine, but unaffected by cyclic AMP-dependent protein kinase inhibitor. Thus islets contain calmodulin-dependent protein kinase activity and endogenous substrates for such activity. While this manuscript was in preparation, an essentially similar conclusion was drawn by Schubart *et al.* (1980) from the results of studies on hamster insulinoma cells. These workers demonstrated that cytosol from single-cell suspensions of insulinoma catalysed the incorporation of ^{32}P from [γ-^{32}P]ATP into a number of endogenous proteins. ^{32}P-labelling of several proteins was enhanced by Ca^{2+}. Phosphorylation of the predominant Ca^{2+}-dependent phosphoprotein (mol.wt. 98000) and of another protein (molecular weight not stated) was further enhanced by calmodulin and was inhibited by trifluoperazine. Trifluoperazine also inhibited insulin release from insulinoma cells stimulated by 40mM-K^+ or by ouabain.

Further studies are required to define the nature of calmodulin-activated protein kinase and substrates for such activity in pancreatic islets and to examine their relationship to the exocytotic discharge of insulin. In particular, it will be necessary to demonstrate protein phosphorylation occurring in the intact stimulated islet.

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