Effects of dehydrouretil on protein phosphorylation and insulin secretion in rat islets of Langerhans

Donna E. HARRISON,*† Mirko POJE,‡ Boris ROCIC§ and Stephen J. H. ASHCROFT*

*Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K., and ‡Laboratory of Organic Chemistry and §Institute for Diabetes, University of Zagreb, Zagreb, Yugoslavia

Dehydrouretil hydrate hydrochloride (DHU), a stable analogue of alloxan, inhibited the phosphorylation of an endogenous protein of M_r 53 000 catalysed by a Ca^{2+} -calmodulin-dependent protein kinase in extracts of islets of Langerhans. The concentration of DHU required for 50% inhibition was 0.09 mM. DHU did not inhibit islet cyclic AMP-dependent protein kinase and caused only slight inhibition of Ca^{2+} -phospholipid-dependent protein kinase. Inhibition of Ca^{2+} -calmodulin-dependent protein kinase was neither prevented nor reversed by dithiothreitol. DHU did not affect the ability of calmodulin to activate cyclic AMP phosphodiesterase. In intact islets, pre-exposure to DHU impaired the insulin-secretory response to glucose and blocked the potentiatory effect on insulin secretion of forskolin, an activator of adenylate cyclase, and of tetradecanoylphorbol acetate (TPA), an activator of Ca^{2+} -phospholipid-dependent protein kinase. The increase in islet cyclic AMP elicited by forskolin was not affected by DHU. The data are consistent with the hypothesis that protein phosphorylation catalysed by a Ca^{2+} -calmodulin-dependent protein kinase may play a central role in the regulation of insulin secretion.

INTRODUCTION

Diabetogenic compounds such as alloxan are selectively toxic to the pancreatic β -cell, causing rapid inhibition of insulin secretion and eventually β -cell destruction (reviewed by Cooperstein & Watkins, 1981). Alloxan itself, however, is very unstable in aqueous solution at pH 7 (half-time approx. 1 min). To investigate the mechanism of action of diabetogenic compounds, we have therefore used dehydrouretil hydrate hydrochloride (DHU), which is structurally related to alloxan but possesses the important advantage of stability in solution. We have shown that DHU is diabetogenic *in vivo* (Poje & Rocic, 1980; Dominis *et al.*, 1984); *in vitro* DHU inhibited insulin release stimulated by glucose, mannose or 4-methyl-2-oxopentanoate at concentrations that did not affect islet metabolism (Tait *et al.*, 1983). Inhibition was manifest after a short exposure (5 min) and persisted throughout subsequent incubation in the absence of DHU. As for alloxan, the presence of hexose during exposure of islets to DHU afforded substantial protection against the inhibitory effect. The mechanism of action of alloxan has not been firmly established. It has been shown that alloxan inhibits a Ca^{2+} -calmodulin-dependent protein kinase in extracts of rat islets (Colca *et al.*, 1983). This finding is of considerable interest in view of the growing evidence, reviewed by Harrison *et al.* (1984), implicating protein phosphorylation as a major control mechanism in the regulation of insulin release. However, protein kinases in addition to Ca^{2+} -calmodulin-dependent protein kinase are likely to be involved in the secretory process. Interpretation of the inhibitory effect of alloxan on Ca^{2+} -calmodulin-dependent protein kinase depends on whether or not other islet protein kinases are also sensitive to such drugs; the aim of the present study was to establish this point, which has not previously been

investigated. Since we find that DHU shares with alloxan the ability to inhibit phosphorylation catalysed by islet Ca^{2+} -calmodulin-dependent protein kinase, we examined the specificity of this effect by determining whether DHU also inhibited islet cyclic AMP-dependent or Ca^{2+} -phospholipid-dependent protein kinases. These kinases were resistant to inhibition by DHU; therefore, in studies with intact islets we examined whether agents that potentiate insulin release via activation of cyclic AMP-dependent or Ca^{2+} -phospholipid-dependent protein kinase (forskolin and TPA respectively) were able to by-pass the block in glucose-stimulated insulin secretion caused by DHU.

MATERIALS AND METHODS

Materials

Collagenase was from Serva, Heidelberg, Germany. Bovine serum albumin, 5'-nucleotidase, Hepes, histone types II-A and V-S and cyclic AMP were from Sigma, Poole, Dorset, U.K. ^{125}I -insulin and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were from Amersham International, Amersham, Bucks., U.K. Cyclic AMP radioimmunoassay kits were from New England Nuclear, Dreiech, Germany. QAE (quaternary aminoethyl)-Sephadex was from Pharmacia (G.B.), Hounslow, Middx., U.K. Rat insulin standard was a gift from Dr. A. J. Moody, Novo Research Laboratories, Copenhagen, Denmark. Guinea-pig anti-insulin serum was from Wellcome Reagents, Beckenham, Kent, U.K. Bovine brain calmodulin was a gift from Dr. P. Esnouf, Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford. Calmodulin-deficient cyclic nucleotide phosphodiesterase was partially purified from bovine brains by the method of Klee & Krinks (1978).

Abbreviations used: DHU, dehydrouretil hydrate hydrochloride; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

† Present address: C.R.C. Medical Oncology Unit, Southampton General Hospital, Southampton SO9 4XY, U.K.

Preparation of islets of Langerhans

Islets were prepared by a collagenase digestion method (Coll-Garcia & Gill, 1969) from the pancreases of male Wistar rats fed *ad libitum* on standard laboratory diet. Islets were harvested by a wire loop under a dissecting microscope.

Protein kinase assays

For determination of the effects of DHU on islet protein kinases, extracts of islets were prepared by sonication (10 s, 50 W, Soniprobe 7532A) as described below and preincubated with various concentrations of DHU for 5 min at 30 °C before addition of buffer containing a mixture of the assay components. Each incubation contained extract from 15–20 islets. Preliminary experiments established that the time course

Fig. 1. Effect of DHU on Ca^{2+} -calmodulin-dependent protein kinase in the absence or presence of dithiothreitol

The extent of incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into an endogenous M_r -53000 protein catalysed by extracts of islets of Langerhans was measured as described in the Materials and methods section. Extracts were preincubated at 30 °C for 5 min with DHU (0–0.5 mM) before assay. Dithiothreitol (1 mM) was absent (Δ), or present after exposure to DHU ($\circ$), or present throughout ($\bullet$). The data are expressed as percentages of the phosphorylation observed in the presence of $10\text{ }\mu\text{M}$ - Ca^{2+} and $1.25\text{ }\mu\text{M}$ -calmodulin in extracts preincubated in the absence of DHU. Ca^{2+} plus calmodulin enhanced phosphorylation of the M_r -53000 protein 5-fold. The data shown are representative of three similar experiments.

of phosphorylation under the various conditions employed was linear for 2 min; a standard reaction time of 1 min was used.

Ca^{2+} -calmodulin-dependent protein kinase was assayed by its ability to phosphorylate its endogenous substrate of M_r 53000. Islet homogenates were prepared by sonicating 200–300 islets in 3 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose. The assay mixture (final volume 25 μl) contained imidazole, pH 7.0 (0.6 μmol), MgCl_2 (25 nmol), CaCl_2 (0.25 nmol), calmodulin (0.5 μg) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (825 pmol; 6–7 c.p.m./fmol). In some experiments the reaction mixture also contained dithiothreitol (25 nmol). Phosphorylation reactions were terminated by addition of 15 μl of SDS stop solution, containing SDS (3.75%, w/v), 20 mM-Tris/HCl, pH 6.6, Bromophenol Blue (0.02%, w/v) and glycerol (5%, w/v), and boiling for 5 min. After cooling, 2.5 μl of 2-mercaptoethanol was added to each tube. Proteins were separated by electrophoresis in 16% polyacrylamide gels as described previously (Harrison *et al.*, 1985). Phosphoproteins were detected by autoradiography using Kodak X-Omat AR-5 film, and the extent of incorporation of ^{32}P into the band of M_r 53000 was measured by densitometry.

Ca^{2+} -phospholipid-dependent protein kinase was assayed as described by Lord & Ashcroft (1984). Islets were sonicated in 50 μl of 20 mM-Tris/HCl (pH 7.4)/2 mM-EDTA/50 mM-mercaptoethanol. The sonicated extract was centrifuged at 20000 g for 20 min at 4 °C, and the supernatant was diluted in 20 mM-Tris/HCl, pH 7.4, for assay. The assay mixture (final volume 50 μl) contained Tris/HCl, pH 7.4 (1 μmol), magnesium acetate (0.25 μmol), histone type V-S (10 μg), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1 nmol; 2 c.p.m./fmol), CaCl_2 (2.5 nmol), phosphatidylserine (0.8 μg), diolein (0.06 μg) and dithiothreitol (50 nmol). When Ca^{2+} was omitted, the reaction medium also contained EGTA (50 nmol). The extent of incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into trichloroacetic acid-precipitable material was measured as previously described (Lord & Ashcroft, 1984).

Cyclic AMP-dependent protein kinase was assayed in islet homogenates as described by Sugden *et al.* (1979a). Homogenates were prepared by sonication of 200–400 islets in 50 mM-sodium phosphate, pH 7.0. The assay mixture (final volume 50 μl) contained: sodium phosphate, pH 7.0 (2.5 μmol), MgCl_2 (0.5 μmol), histone type II-A (0.2 mg), EGTA (5 nmol), dithiothreitol (25 nmol), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (25 nmol; 0.4 c.p.m./fmol). Samples were taken at appropriate times to determine the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into acid-precipitable material as described previously (Sugden *et al.*, 1979a).

Insulin release

Batches of five islets were preincubated on ice for 10 min in medium containing albumin (2 g/l) in the absence or presence of 1 mM-DHU. Islets were then incubated for 2 h at 37 °C with the further additions stated in the Tables or the text. When islet cyclic AMP content was determined, the medium used was a HEPES-buffered bicarbonate medium (Christie & Ashcroft, 1985) and the preincubation and incubation volumes were 50 and 100 μl respectively. For measurement of insulin release alone, bicarbonate medium equilibrated with O_2/CO_2 (19:1) (Christie & Ashcroft, 1984) was used and the respective volumes were 300 and 600 μl . Insulin

Fig. 2. Comparison of the effects of DHU on islet protein kinases

Ca²⁺-calmodulin-dependent protein kinase (○) was assayed by its ability to phosphorylate its endogenous M_r -53000 substrate in the presence of 10 μ M-Ca²⁺ and 1.25 μ M-calmodulin. Ca²⁺-phospholipid-dependent protein kinase (△) was assayed in the presence of 50 μ M-Ca²⁺, phosphatidylserine (16 μ g/ml) and diolein (1.2 μ g/ml) with histone type V-S as substrate. Cyclic AMP-dependent protein kinase (●) was assayed in the presence of 5 μ M-cyclic AMP and 0.1 mM-3-isobutyl-1-methylxanthine with histone type II-A as substrate. Extracts were preincubated with DHU at the concentrations indicated, and results are expressed as a percentage of the control in the absence of DHU. The data shown are representative of three similar experiments.

released into the medium was measured by radioimmunoassay (Ashcroft & Crossley, 1975).

Cyclic AMP content

After incubation and removal of medium for radioimmunoassay of insulin as above, cyclic AMP was immediately extracted from the islets by addition of 100 μ l of boiling 0.05 M-sodium acetate buffer, pH 6.2, followed by boiling for 5 min in a water bath. The boiled extract was cooled and sonicated for 5 s at position 1 of a Soniprobe (Dawe Instruments) and stored at -20 °C until assayed. The cyclic AMP content of the sonicated material was assayed with a commercial radioimmunoassay kit in accordance with the manufacturer's instructions, by using the procedure with acetylation except that half the recommended volumes were used.

Assay of calmodulin

Calmodulin was assayed by its ability to stimulate calmodulin-depleted cyclic nucleotide phosphodiesterase by the method of Sugden *et al.* (1979b). Islet extracts were prepared by sonicating (10 s, 50 W) 150–200 islets in 10 mM-Tris/HCl, pH 8.0, and boiling for 3 min. After cooling, the extract was clarified by centrifugation (2 min at 10000 g) and diluted for assay. The assay mixture contained 50 mM-Tris/HCl (pH 8.0), 3 mM-MgCl₂, bovine albumin (0.1 mg/ml), 0.15 μ M-cyclic[³H]AMP (30 Ci/mmol) and 0.1 mM-cyclic AMP. Calmodulin-dependent activity was measured in the presence of 0.5 mM-CaCl₂ and either islet extract (equivalent to ten islets) or authentic calmodulin (up to 50 ng). The assay mixture was preincubated in the absence or presence of DHU (0.5–1 mM) for 5 min at 30 °C before addition of phosphodiesterase. After 15 min incubation, the reaction was stopped by boiling, and the [³H]AMP formed was converted into [³H]adenosine by 5'-nucleotidase. [³H]Adenosine was separated from cyclic [³H]AMP by using QAE-Sephadex and counted for radioactivity by liquid-scintillation spectrometry. Under the conditions used, the extent of reaction is proportional to time of incubation.

RESULTS

Effects of DHU on islet protein kinases

Homogenates of islets of Langerhans catalysed the incorporation of ³²P from [γ -³²P]ATP into endogenous islet protein. In the presence of calmodulin, Ca²⁺ stimulated phosphorylation of a protein of M_r 53000 as shown by SDS/polyacrylamide-gel electrophoresis. Characteristics of this activity have previously been described in detail (Harrison & Ashcroft, 1982). Fig. 1 shows that preincubation of islet homogenate with DHU produced a dose-dependent inhibition of the Ca²⁺-calmodulin-dependent phosphorylation of the M_r -53000 protein. The concentration of DHU required for 50% inhibition was 0.09 mM.

The inhibitory effect of DHU on phosphorylation catalysed by islet Ca²⁺-calmodulin-dependent protein kinase was not prevented by the simultaneous presence of the thiol reagent dithiothreitol, nor did the addition of dithiothreitol after exposure of the islet extracts to DHU reverse the inhibition by DHU (Fig. 1).

To determine the specificity of the inhibitory effect of DHU, the sensitivity to DHU of islet cyclic AMP-dependent and Ca²⁺-phospholipid-dependent protein kinases was examined (Fig. 2). In contrast with the marked inhibition of Ca²⁺-calmodulin-dependent protein kinase by DHU, islet cyclic AMP-dependent protein kinase, measured as the incorporation of ³²P from [γ -³²P]ATP into exogenous histone type V-S, was not significantly affected by DHU at concentrations up to 1 mM. Islet Ca²⁺-phospholipid-dependent protein kinase, measured as the incorporation of [³²P]P_i from [γ -³²P]ATP into exogenous histone type II-A, was only weakly inhibited by DHU; 0.5 mM-DHU gave 18% inhibition.

DHU did not significantly affect basal protein phosphorylation observed in the absence of activators.

Effects of DHU on forskolin-induced elevation of cyclic AMP and insulin secretion

The data are shown in Table 1. The rise in the rate of insulin secretion in response to an increase in glucose in

Table 1. Effects of DHU and forskolin on islet cyclic AMP content and insulin secretion

Batches of five islets were preincubated for 5 min at 4 °C in the absence or presence of 1 mM-DHU as indicated before addition of glucose and forskolin at the concentrations given. After incubation for 2 h at 37 °C, the medium was removed and a sample retained for determination of insulin by radioimmunoassay. Cyclic AMP was extracted from the islets as described in the Materials and methods section and measured by radioimmunoassay. Results are given as means $\pm$ S.E.M. for the numbers of observations in parentheses: ^a*P* < 0.001 versus line 1; ^b*P* < 0.001 versus line 2; ^c*P* < 0.001 versus line 3; ^dN.S. (*P* > 0.05) versus line 3.

Line	Preincubation with 1 mM-DHU	Incubation conditions		Insulin secretion (μ units/h per islet)	Islet cyclic AMP (fmol/islet)
		Glucose (mM)	Forskolin (μ M)		
1	—	2	0	3.9 $\pm$ 0.4 (14)	3.9 $\pm$ 0.2 (15)
2	—	10	0	138.2 $\pm$ 17.3 (14) ^a	4.5 $\pm$ 0.7 (15)
3	—	10	10	312.4 $\pm$ 37.7 (15) ^b	45.8 $\pm$ 5.0 (15) ^b
4	+	10	0	10.4 $\pm$ 1.9 (15) ^b	3.2 $\pm$ 0.5 (15)
5	+	10	10	33.8 $\pm$ 5.7 (15) ^c	33.5 $\pm$ 3.5 (15) ^d

Table 2. Effects of DHU and TPA on insulin secretion

Batches of five islets were preincubated for 5 min at 4 °C in the absence or presence of 1 mM-DHU as indicated before addition of glucose and TPA at the stated concentrations. After incubation for 2 h at 37 °C, insulin released into the medium was determined by radioimmunoassay. Results are given as means $\pm$ S.E.M. for ten batches of islets: ^a*P* < 0.001 versus line 1; ^b*P* < 0.001 versus line 2; ^c*P* < 0.001 versus line 3.

Line	Preincubation with 1 mM-DHU	Incubation conditions		Insulin secretion (μ units/h per islet)
		Glucose (mM)	TPA (nM)	
1	—	2	0	9.9 $\pm$ 1.6
2	—	10	0	193.9 $\pm$ 7.9 ^a
3	—	10	50	299.6 $\pm$ 19.0 ^b
4	+	10	0	57.1 $\pm$ 8.8 ^b
5	+	10	50	54.4 $\pm$ 7.5 ^c

the medium from 2 to 10 mM was markedly enhanced by 10 μ M-forskolin. When islets were exposed to 1 mM-DHU for 10 min before challenge with stimulus, both the effect of glucose alone and that of glucose plus forskolin were drastically impaired. The effect of forskolin on insulin release was accompanied by a marked elevation of islet cyclic AMP content. Pre-exposure to DHU did not significantly affect islet cyclic AMP content.

Effects of DHU on TPA-stimulated insulin secretion

Results are given in Table 2. TPA (50 nM) markedly potentiated insulin secretion elicited by 10 mM-glucose. Pre-exposure to 1 mM-DHU impaired the secretory response to glucose and abolished the potentiatory effect of TPA.

Effect of DHU on islet calmodulin

Boiled extracts of islets were assessed for their calmodulin content by their ability to activate, in a Ca²⁺-dependent manner, brain cyclic AMP phosphodiesterase. As shown in Table 3, DHU had no effect on phosphodiesterase activation either by islet extracts or by authentic calmodulin.

DISCUSSION

Administration of alloxan to rats leads to β -cell necrosis and production of diabetes. Studies *in vitro* have

shown that alloxan also has a rapid inhibitory effect on insulin secretion that precedes evidence of morphological change (Tomita *et al.*, 1974; McDaniel *et al.*, 1975; Weaver *et al.*, 1978; Tait *et al.*, 1983). The mechanisms of these effects have not been elucidated, although several studies have suggested that toxic free-radical production may result from the interaction of alloxan with the β -cell (Heikkila *et al.*, 1976; Grankvist *et al.*, 1979; Fisher & Hamberger, 1980; Asayama *et al.*, 1984). It is important to note that the rapid action *in vitro* on insulin secretion may not necessarily involve the same mechanisms as the chronic β -cell destruction. Indeed, although at high concentrations alloxan elicits impairment of metabolic function (Idahl *et al.*, 1977; Tait *et al.*, 1983), at concentrations below 1 mM the drug causes rather specific inhibition of secretion (Tait *et al.*, 1983). Studies *in vitro* on the uptake of alloxan by isolated islets have indicated that the specificity of the drug for the β -cell may result from the relatively high rate of uptake of alloxan by the β -cell (Gorus *et al.*, 1982) and also from the relatively low content of peroxidase in the tissue (Malaisse *et al.*, 1982). A possible intracellular target for the acute effect of alloxan has emerged from the finding that an islet Ca²⁺-calmodulin-dependent protein kinase is inhibited by the drug (Colca *et al.*, 1983). This effect was seen in extracts of islets; it was also observed that intact islets exposed to alloxan had a decreased activity of Ca²⁺-calmodulin-dependent protein kinase activity on subse-

Table 3. Effect of DHU on the activation of cyclic nucleotide phosphodiesterase by calmodulin

The activity of purified brain cyclic AMP-dependent phosphodiesterase depleted of calmodulin was assayed in the presence of authentic calmodulin or boiled islet extract equivalent to ten islets in the presence of 0.5 mM- Ca^{2+} or 1 mM-EGTA. The assay mixture was preincubated in the absence or presence of DHU as indicated for 5 min at 30 °C before addition of phosphodiesterase. Results are given as means $\pm$ S.E.M. for three separate preparations of islet extract or as means of closely agreeing duplicates.

Assay conditions		Phosphodiesterase activity (nmol of cyclic AMP hydrolysed) in the presence of:			
		Islet extract	Calmodulin (ng):		
DHU (mM)	Ca^{2+}		0	10	50
0	—	0.14 $\pm$ 0.01			
0	+	1.13 $\pm$ 0.05	0.15	0.57	1.33
0.5	+	1.16 $\pm$ 0.05			
1.0	—	0.16 $\pm$ 0.03			
1.0	+	1.14 $\pm$ 0.08	0.18	0.52	1.36

quent homogenization and assay; and islets prepared from rats injected with alloxan also showed decreased Ca^{2+} -calmodulin-dependent protein kinase activity (Norling *et al.*, 1984). Ca^{2+} -calmodulin-dependent protein kinase in brain extracts was also inhibited by alloxan, but the amount of activity extractable from brain was not diminished by administration of the drug *in vivo* (Norling *et al.*, 1984), consistent with previous views on the mechanism of tissue specificity.

In aqueous solution alloxan rapidly decomposes to the inactive alloxanic acid. This instability has prompted a search for new diabetogenic compounds related to alloxan but stable in solution. We have synthesized a range of such compounds and have demonstrated the potent diabetogenic activity of DHU, of dehydroisouramil hydrate hydrochloride and 5-hydroxypseudouric acid (Dominis *et al.*, 1984). These compounds are of interest not only as useful tools for exploring the mechanism of action of diabetogenic compounds but also because they are potential metabolites of uric acid. We have suggested (Dominis *et al.*, 1984; Tait *et al.*, 1983) that endogenous production of such compounds destructive towards the β -cell could be involved in the pathogenesis of Type 1 diabetes.

Like alloxan, DHU also has rapid acute effects on the β -cell. Since the present study was directed towards investigating a possible mechanism for inhibition of insulin secretion by DHU, it is important to note that several lines of evidence attest to the fact that such inhibition does not arise through non-specific cell damage caused by the drug. Thus Tait *et al.* (1983) showed that: (i) marked inhibition of insulin release by DHU occurred without impairment of islet energy metabolism; (ii) the presence of glucose during exposure to DHU afforded protection against the inhibitory effects of DHU; (iii) although exposure of islets to 1 mM-DHU in the absence of glucose caused some inhibition of insulin biosynthesis, the effect was modest compared with that on secretion. The present study shows in addition that DHU has no effect on either basal or stimulated islet contents of cyclic AMP.

Previous studies *in vitro* with DHU have shown that it rapidly and irreversibly inhibits insulin release stimulated by glucose, mannose or 4-methyl-2-oxopentanoate (Tait *et al.*, 1983). These stimuli are all initiators

of insulin secretion, i.e. they are able to elicit secretion in the absence of other agents. However, certain other agents are potentiators of secretion, i.e. their effect on secretion is negligible when present alone, but they are capable of augmenting the secretory response to glucose or other initiator. Two major classes of potentiators are recognized: those agents that increase β -cell cyclic AMP, e.g. glucagon, and are presumed to act via cyclic AMP-dependent protein kinase (for review see Malaisse & Malaisse-Lagae, 1984), and those agents that elicit enhanced turnover of inositol phospholipids, e.g. acetylcholine (for review see Best *et al.*, 1984). The latter event is believed to lead to two distinct intracellular signals, namely inositol trisphosphate, which may serve to mobilize intracellular Ca^{2+} (Prentki *et al.*, 1984), and diacylglycerol, which may activate Ca^{2+} -phospholipid-dependent protein kinase (Tanigawa *et al.*, 1982). We have suggested (Harrison *et al.*, 1984) that initiation of secretion, which appears to require primarily an increase in intracellular Ca^{2+} , involves protein phosphorylation catalysed by Ca^{2+} -calmodulin-dependent protein kinase. We have further suggested that the phosphorylation of protein catalysed by cyclic AMP-dependent and Ca^{2+} -phospholipid-dependent protein kinases serves an amplificatory role in the action of the potentiators of secretion. The present study provides further support for these views.

As previously shown for alloxan (Colca *et al.*, 1983), DHU, at concentrations which markedly and specifically inhibit glucose-stimulated insulin secretion (Tait *et al.*, 1983), inhibits phosphorylation of an endogenous protein of M_r 53000 catalysed by islet Ca^{2+} -calmodulin-dependent protein kinase. Moreover, the inhibitory effect of DHU on insulin release is not confined to inhibition of initiation of secretion. We show here that the effects on insulin release of two potentiators are also blocked by DHU. Forskolin acts via activation of adenylate cyclase and has been shown to cause enhanced protein phosphorylation catalysed by cyclic AMP-dependent protein kinase in intact islets of Langerhans (Christie & Ashcroft, 1985). TPA has been shown to activate β -cell Ca^{2+} -phospholipid-dependent protein kinase (Lord & Ashcroft, 1984), and its potentiatory effect on insulin secretion is accompanied by enhanced phosphorylation catalysed by this kinase (Harrison *et al.*, 1984). One

possible explanation for the effect of DHU on potentiation of secretion is that DHU may inhibit all three protein kinases. However, our data suggest that this is not so. At concentrations of DHU that markedly inhibit Ca^{2+} -calmodulin-dependent protein phosphorylation, the activity of islet cyclic AMP-dependent and Ca^{2+} -phospholipid-dependent protein kinases was little affected. Since the action of forskolin involves increase in cyclic AMP, it was also possible that DHU may act on forskolin-potentiated secretion by interfering with the ability of forskolin to activate adenylate cyclase. Again, however, this possibility can be discounted, since DHU did not lower islet cyclic AMP concentrations augmented by forskolin.

It is important to note that DHU did not affect the phosphorylation of endogenous substrates observed in the absence of Ca^{2+} and cyclic AMP. Since the latter has been attributed to casein kinase activity (Sugden *et al.*, 1979a), this observation further supports the view that DHU is rather specific for Ca^{2+} -calmodulin-dependent protein kinase.

These data are therefore consistent with the hypothesis that Ca^{2+} -calmodulin-dependent protein kinase plays a central role in the insulin-secretory process and that activation of cyclic AMP-dependent or Ca^{2+} -phospholipid-dependent protein kinase leads only to amplification of insulin release when this initiation pathway has been activated.

The Ca^{2+} -calmodulin-dependent protein kinase that we have studied phosphorylates an endogenous protein of M_r 53000 as shown by SDS/polyacrylamide-gel electrophoresis. Properties of this kinase have previously been documented in detail (Harrison & Ashcroft, 1982), and the activity has been reported in various insulin-secretory tissues (Landt *et al.*, 1982; Brocklehurst & Hutton, 1983; Schubart & Fields, 1984), including human islets of Langerhans (Harrison *et al.*, 1985). The effect of DHU on the measured activity of this kinase may be on the kinase or on its substrate; this point cannot yet be resolved, since separation of kinase and substrate has not been achieved and other substrates for the kinase are unknown. However, the effect of DHU is unlikely to be on calmodulin, since DHU did not affect the ability of calmodulin to bind and activate cyclic AMP phosphodiesterase. Our data also suggest that the effect of DHU on Ca^{2+} -calmodulin-dependent protein kinase does not arise from reaction with thiol groups, since dithiothreitol neither prevented nor reversed the inhibitory action of DHU. The mode of action remains to be established, but DHU is likely to be a valuable tool for investigating further the role of islet protein kinases in the insulin secretory mechanism.

These studies were supported by grants from the British Diabetic Association and the Medical Research Council.

REFERENCES

- Asayama, K., Byfeler, F., English, D., Pilkis, S. J. & Burr, L. M. (1984) *Diabetes* **33**, 1008-1011
- Ashcroft, S. J. H. & Crossley, J. R. (1975) *Diabetologia* **11**, 279-284
- Best, L., Dunlop, M. & Malaisse, W. J. (1984) *Experientia* **40**, 1085-1090
- Brocklehurst, K. W. & Hutton, J. C. (1983) *Biochem. J.* **210**, 533-539
- Christie, M. R. & Ashcroft, S. J. H. (1984) *Biochem. J.* **218**, 87-99
- Christie, M. R. & Ashcroft, S. J. H. (1985) *Biochem. J.* **227**, 727-736
- Colca, J. R., Kotagal, N., Brooks, C.-L., Lacy, P. E., Landt, M. & McDaniel, M. L. (1983) *J. Biol. Chem.* **258**, 7260-7263
- Coll-Garcia, E. & Gill, J. R. (1969) *Diabetologia* **5**, 61-66
- Cooperstein, S. J. & Watkins, D. (1981) in *The Islets of Langerhans: Biochemistry, Physiology and Pathology* (Cooperstein, S. J. & Watkins, D., eds.), pp. 387-425, Academic Press, New York
- Dominis, M., Rocic, S., Ashcroft, S. J. H., Rocic, B. & Poje, M. (1984) *Diabetologia* **27**, 403-406
- Fisher, L. J. & Hamberger, S. A. (1980) *Diabetes* **29**, 213-216
- Gorus, F. K., Malaisse, W. J. & Pipeleers, D. G. (1982) *Biochem. J.* **208**, 513-515
- Grankvist, K., Marklund, S., Sehlin, J. & Taljedal, I.-B. (1979) *Biochem. J.* **182**, 17-25
- Harrison, D. E. & Ashcroft, S. J. H. (1982) *Biochim. Biophys. Acta* **714**, 313-319
- Harrison, D. E., Ashcroft, S. J. H., Christie, M. R. & Lord, J. M. (1984) *Experientia* **40**, 1075-1084
- Harrison, D. E., Christie, M. R. & Gray, D. W. R. (1985) *Diabetologia* **28**, 99-103
- Heikkila, R. E., Winston, B. & Cohen, G. (1976) *Biochem. Pharmacol.* **25**, 1085-1092
- Idahl, L. A., Lernmark, A., Sehlin, J. & Taljedal, I.-B. (1977) *Biochem. J.* **162**, 9-18
- Klee, C. B. & Krinks, M. H. (1978) *Biochemistry* **17**, 120-126
- Landt, M., McDaniel, M. L., Bry, C. G., Kotagal, N., Colca, J. R., Lacy, P. E. & McDonald, J. M. (1982) *Arch. Biochem. Biophys.* **213**, 148-154
- Lord, J. M. & Ashcroft, S. J. H. (1984) *Biochem. J.* **219**, 547-551
- Malaisse, W. J. & Malaisse-Lagae, F. (1984) *Experientia* **40**, 1068-1074
- Malaisse, W. J., Malaisse-Lagae, F., Sener, A. & Pipeleers, D. G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 927-930
- McDaniel, M., Roch, C., Fink, J., Fyfe, G. & Lacy, P. E. (1975) *Biochem. Biophys. Res. Commun.* **66**, 1089-1096
- Norling, L. L., Colca, J. R., Brooks, C. L., Kloepper, R. F., McDaniel, M. L. & Landt, M. (1984) *Biochim. Biophys. Acta* **801**, 197-205
- Poje, M. & Rocic, B. (1980) *Experientia* **36**, 78-79
- Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Nature (London)* **309**, 562-564
- Schubart, U. K. & Fields, K. L. (1984) *J. Cell Biol.* **98**, 1001-1009
- Sugden, M. C., Ashcroft, S. J. H. & Sugden, P. H. (1979a) *Biochem. J.* **180**, 219-229
- Sugden, M. C., Christie, M. R. & Ashcroft, S. J. H. (1979b) *FEBS Lett.* **105**, 95-100
- Tait, S. P. C., Poje, M., Rocic, B. & Ashcroft, S. J. H. (1983) *Diabetologia* **25**, 360-364
- Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y. & Nishizuka, Y. (1982) *FEBS Lett.* **138**, 183-186
- Tomita, T., Lacy, P. E., Matschinsky, F. M. & McDaniel, M. L. (1974) *Diabetes* **23**, 517-524
- Weaver, D. C., McDaniel, M. L., Naker, S. P., Barry, C. D. & Lacy, P. E. (1978) *Diabetes* **27**, 1205-1214