Studies on the interaction of staphylococcal δ -haemolysin with isolated islets of Langerhans

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δ -Haemolysin, a small surface-active polypeptide purified from the culture media of *Staphylococcus aureus*, was observed to stimulate the release of insulin from isolated rat islets of Langerhans. This effect was dose-dependent and saturable, with the half-maximal response elicited by a δ -haemolysin concentration of 10 μ g/ml. Stimulation of insulin release by δ -haemolysin (10 μ g/ml) was not dependent on the presence of glucose in the incubation medium, but was augmented by increasing concentrations of the sugar. The release of insulin in response to δ -haemolysin could be inhibited by depletion of extracellular Ca^{2+} or by adrenaline (epinephrine) (10 μ M) and was readily reversible when δ -haemolysin was removed from the medium. In addition, the response was potentiated by incubation with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.2 mM). These observations suggest that δ -haemolysin induced a true activation of the β -cell secretory mechanism. Stimulation of islets of Langerhans with δ -haemolysin was found to be associated with a modest increase in intracellular cyclic AMP levels, although the adenylate cyclase activity of islet homogenates was not increased by δ -haemolysin. δ -Haemolysin was observed to induce a dose-dependent net accumulation of $^{45}\text{Ca}^{2+}$ by islet cells and to stimulate the efflux of $^{45}\text{Ca}^{2+}$ from preloaded islets. The efflux of $^{45}\text{Ca}^{2+}$ was modest in size and short-lived, but dramatically increased in medium depleted of $^{40}\text{Ca}^{2+}$. Incubation in the presence of verapamil augmented δ -haemolysin-induced $^{45}\text{Ca}^{2+}$ efflux and insulin secretion. δ -Haemolysin was found to be a potent $^{45}\text{Ca}^{2+}$ -translocating ionophore in an artificial system. This response was dose-dependent and could be augmented by verapamil. In addition, phosphatidylcholine (25 μ g/ml) was found to inhibit both δ -haemolysin induced $^{45}\text{Ca}^{2+}$ translocation and insulin release in a precisely parallel manner. These studies suggest that the ability of δ -haemolysin to stimulate insulin release may be due, in part, to the facilitation of Ca^{2+} entry into the β -cells of islets of Langerhans, mediated directly by an ionophoretic mechanism.

The mechanism by which the pancreatic β -cell is able to recognize and respond to a stimulus such as glucose remains to be defined in detail, although it has become evident that specific events occur both in the cell and at the level of the plasma membrane [see Hedeskov (1980) for review]. Most recent studies have focused on the intracellular events associated with stimulation. However, the possibility that the β -cell plasma membrane might directly contribute to the regulation of insulin secretion has been emphasized in studies utilizing a variety of enzyme preparations to alter the plasma membrane. These

studies have shown that small changes in membrane structure and composition achieved by digestion with enzymes such as trypsin (Krause *et al.*, 1973), neuraminidase (Hahn *et al.*, 1974), hyaluronidase (Macchi *et al.*, 1974) and phospholipase C (Sando *et al.*, 1977) could selectively alter the secretory response to specific stimuli. Recently, more direct changes at the level of the β -cell plasma membrane associated with stimulation have also been reported. These include changes in the extent of cation binding (Flatt *et al.*, 1981), calcium mobility (Taljedal, 1979) and membrane fluidity (Montague & Parkin, 1980).

In the present investigation an attempt has been made to study further the influence exerted by

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changes in the β -cell plasma membrane on the rate of insulin release. For this purpose a surface-active polypeptide isolated from *Staphylococcus aureus* (Heatley, 1971), δ -haemolysin, has been employed. This molecule is one of a family of toxins produced by *S. aureus* which are characterized by their ability to induce lysis in erythrocytes (Rogolsky, 1979). It has become increasingly evident, however, that treatment of certain cells with low concentrations of δ -haemolysin could induce more subtle responses which were not the result of cell lysis. In particular, the protein has been shown to inhibit water absorption, to stimulate cyclic AMP accumulation and to alter ion permeability in guinea-pig ileum (O'Brien & Kapral, 1976; O'Brien *et al.*, 1978). It has also been reported to stimulate arachidonic acid release and prostaglandin synthesis in a number of cell types in tissue culture (Umezawa *et al.*, 1980; Durkin & Shier, 1981). All of these effects were reported to occur under conditions when gross cell damage could not account for the response. Indeed, it has been proposed that the interaction of low concentrations of δ -haemolysin with the plasma membrane of certain cells may induce certain structural and functional perturbations in these membranes which then lead to activation of specific cellular processes (Umezawa *et al.*, 1980). For this reason, the toxin may represent a valuable tool with which to study aspects of cell activation.

We have recently presented evidence that δ -haemolysin interacts with the β -cells of the endocrine pancreas in a manner which leads to activation of the insulin-secretory mechanism of these cells (Morgan *et al.*, 1981). In the present study we have confirmed and extended these observations and present a systematic characterization of the response of the β -cell associated with the interaction of δ -haemolysin at the cell surface. Part of the present study has been reported in abstract form (Morgan & Montague, 1981).

Stimulation of insulin release in response to a variety of secretagogues is known to be dependent on the presence of extracellular Ca^{2+} (Milner & Hales, 1967; Curry *et al.*, 1968). A large body of evidence has accumulated which suggests that the reason for the sensitivity of insulin release to extracellular Ca^{2+} is that β -cell stimulation is associated with a net influx of the cation into the cell from the extracellular medium (for reviews, see Malaisse *et al.*, 1978; Hellman *et al.*, 1971). If this suggestion is correct, an agent which alters the permeability of the plasma membrane to Ca^{2+} may have the capacity to modulate the insulin-secretory activity of the β -cell. δ -Haemolysin is known to be surface-active (Kreger & Bernheimer, 1971) and it is possible, therefore, that interaction of δ -haemolysin with the β -cell plasma membrane might have induced Ca^{2+} -permeability changes in this mem-

brane which then contributed to the stimulatory activity of the agent. In the present study we have investigated the effect of δ -haemolysin on islet-cell Ca^{2+} handling and attempted to correlate this response with the ability of the agent to stimulate insulin release from the pancreatic β -cell.

Experimental

Materials

δ -Haemolysin purified from *S. aureus* strain 186X was obtained as a gift from Professor M. V. Shaw, Department of Biochemistry, University of Leicester. [^3H]Cyclic AMP, [$\text{U-}^{14}\text{C}$]glucose, $^{45}\text{Ca}^{2+}$ and [^3H]sucrose were obtained from Amersham International Ltd., Amersham, Bucks., U.K. Verapamil was a gift from Dr. W. J. Malaisse, Brussels University Medical School, Brussels, Belgium, and cyclic AMP-binding protein was a gift from Dr. S. R. Nahorski, Department of Pharmacology and Experimental Therapeutics, University of Leicester. Adrenaline (epinephrine) and phosphatidylcholine were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. and 3-isobutyl-1-methylxanthine from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. Collagenase and ionophore A23187 were purchased from Boehringer Co. Ltd., Lewes, Sussex, U.K. Di-n-butyl phthalate was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Dinonyl phthalate was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. All other reagents were of analytical grade or were the purest available commercially.

Methods

Isolation of islets of Langerhans. All experiments were performed with islets of Langerhans isolated by collagenase digestion (Montague & Taylor, 1968) of the pancreas of male Wistar rats (250–350 g) allowed free access to food and water. Islets were isolated in a bicarbonate-buffered medium, pH 7.4 (Gey & Gey, 1936), gassed with O_2/CO_2 (19:1) and containing glucose (4 mM), CaCl_2 (2 mM) and bovine serum albumin (0.5 mg/ml).

Insulin-secretion studies. In static incubation experiments, islets were preincubated for 30 min in gassed (O_2/CO_2 , 19:1) medium (37°C), then transferred in groups of four to vials containing 400 μl of medium, supplemented with test reagents as described for individual experiments. The tubes were gassed briefly, capped and incubated for 1 h (37°C). After incubation, portions of the medium were removed from each vial for determination of insulin content by radioimmunoassay (Cresto *et al.*, 1972). At the concentrations employed, test substances did not interfere with the radioimmunoassay.

Perfusion experiments were performed in an apparatus described in detail by Green *et al.* (1980).

For these studies, groups of 100 islets were transferred to perfusion chambers (Millipore-Swinnex 13) immediately after isolation. Islets were perfused at a constant flow rate (1 ml/min) with gassed medium (O_2/CO_2 , 19:1; 37°C) for 45 min before the introduction of test substances. Samples of perfusate were subsequently collected over successive 2-min periods for assay of insulin content by radioimmunoassay.

Lactate dehydrogenase assay. To assess the extent of cell damage in isolated islets, the release of lactate dehydrogenase was measured. For these experiments, groups of 120–150 isolated islets of Langerhans were incubated in 130 μ l of gassed medium at 37°C for 1 h. The medium was supplemented as described for individual experiments. After incubation the islets were pelleted by a short spin in a Beckman Microfuge (9000 g for 5 s) and the supernatant medium decanted and stored at 4°C before assay of lactate dehydrogenase activity. The islet pellet was resuspended in 250 μ l of ice-cold phosphate buffer (50 mM; pH 7.5) containing pyruvate (0.6 mM), and treated ultrasonically for 15 s. The sonicated extract was spun in a Microfuge (9000 g for 30 s) and the supernatant removed for assay of lactate dehydrogenase activity.

For assay of lactate dehydrogenase activity, all reagents and samples were equilibrated to 25°C. A 50 μ l portion of NADH solution (3 mM) was added to 3 ml of a solution of pyruvate (0.6 mM in 50 mM-phosphate buffer, pH 7.5) and the ΔA_{340} was monitored in a spectrophotometer (Gilford) for 5 min, in order to determine the basal rate of NADH oxidation. After this time, 100 μ l of sample was added and the decrease in A_{340} continuously recorded over a further period of 5 min.

Measurement of islet [3H]sucrose space. The [3H]sucrose space of isolated islets of Langerhans was measured by a modification of the method of Wollheim *et al.* (1977). A 200 μ l portion of a mixture of di-n-butyl phthalate and dinonyl phthalate (10:3, v/v) was dispensed into 400 μ l polythene tubes and islets were transferred in groups of ten to each tube in 50 μ l of gassed (O_2/CO_2 , 19:1) medium. The medium containing the islets was carefully placed against the walls of the tube to leave an air space between the buffer and the oil mixture. Incubation was commenced by addition of 50 μ l of incubation medium containing the test substances, followed immediately by a further 50 μ l of medium supplemented with [3H]sucrose (40 μ Ci/ml). Tubes were incubated for 1 h (37°C) and islets separated from the bulk medium by rapid centrifugation (9000 g for 5 s) through the phthalate layer. The tubes were immersed in liquid N_2 and the bottom 5 mm cut from each, above the islet pellet. The pellet was then shaken in 500 μ l of 0.2 M-HClO₄ to disrupt the cells. A portion (4.5 ml) of scintillant (Instagel;

Packard) was added and the radioactivity determined in a liquid-scintillation spectrometer. Blanks containing no islets were included in each experiment and results from them did not differ significantly from background values. The extracellular volume was estimated as the [3H]sucrose space, assuming a specific activity equivalent to that of the bulk medium.

Determination of islet cyclic AMP content. In experiments performed to determine the amount of cyclic AMP present in islet cells, groups of 100 isolated islets of Langerhans were dispensed into tubes containing 1 ml of gassed medium (37°C) supplemented as described for individual experiments. The tubes were capped and incubated at 37°C for 30 min. After incubation, the islets were sedimented by a short spin in a Microfuge (9000 g for 5 s) and the supernatant medium discarded. The islet pellet was frozen at the temperature of liquid N_2 , then resuspended in 400 μ l of 80% (v/v) ethanol. This was treated ultrasonically at low amplitude for 10 s to disrupt the cells and a portion removed for determination of protein content by the method of Lowry *et al.* (1951); bovine serum albumin was used as the standard. All samples were dried under nitrogen and stored at -20°C before assay for either protein or cyclic AMP content.

Cyclic AMP assay. Cyclic AMP was measured by using the protein-binding method described by Brown *et al.* (1971). Samples were resuspended in 250 μ l of Tris/HCl buffer (50 mM), pH 7.4, containing EDTA (4 mM). Duplicate 100 μ l portions of each sample were then incubated at 4°C with 0.5 pmol of [3H]cyclic AMP and 200 μ l of a solution of cyclic AMP-binding protein in 50 mM-Tris/HCl buffer. After incubation overnight (4°C), 500 μ l of a suspension of charcoal (2 mg/ml) in a solution of bovine serum albumin (2 mg/ml) was added and the tubes centrifuged immediately (1500 g; 15 min; 4°C). Portions (500 μ l) of the supernatant were transferred to scintillation vials and 4.5 ml of scintillant [toluene/PPO (2,5-diphenyloxazole)/Triton X-100, 700 ml/4 g/300 ml] added. The radioactivity in each sample was determined in a Packard liquid-scintillation spectrometer. The cyclic AMP content of each sample was determined by reference to a series of standards assayed concurrently with the test samples.

Assay of islet-cell adenylate cyclase activity. The adenylate cyclase activity of islet homogenates was determined by using the method of Ismail & Montague (1977). Cyclic AMP formation was determined by a protein-binding assay (Brown *et al.*, 1971).

Islet-cell glucose oxidation. The rate of islet-cell glucose metabolism was measured by a modification of the method of Malaisse *et al.* (1974). Isolated islets of Langerhans were placed in groups of 15 into

small plastic wells (14 mm × 8 mm) to each of which was added 200 μ l of incubation medium containing [^{14}C]glucose (5 $\mu\text{Ci/ml}$). The wells were suspended inside scintillation vials in an atmosphere of O_2/CO_2 (19:1) and the vials sealed with self-sealing rubber stoppers. The vials containing the islets were then placed in an incubator (37°C) and incubated for 2 h.

After incubation, 200 μ l of Hyamine (Packard) was injected through the rubber stopper into each scintillation vial to absorb any released $^{14}\text{CO}_2$. A 150 μ l portion of 2M-HCl was then injected into each well containing the islets to release any $^{14}\text{CO}_2$ which remained in the medium. The vials were shaken gently at room temperature for 60 min, after which the wells containing the islets were removed, and 10 ml of scintillation fluid (Fisofluor 3; Fisons) added to each vial. The extent of $^{14}\text{CO}_2$ evolution was determined by liquid-scintillation spectrometry. Blanks containing no islets were included in each experiment and the values subtracted from all test samples.

Islet-cell $^{45}\text{Ca}^{2+}$ accumulation. The accumulation of $^{45}\text{Ca}^{2+}$ by the cells of isolated islets of Langerhans was determined by a modification of the method of Wollheim *et al.* (1977). A mixture of di-n-butyl phthalate and dinonyl phthalate (10:3; v/v), 200 μ l in all, was dispensed into small polythene tubes. Islets of Langerhans, isolated in medium containing 0.5 mM- $^{40}\text{CaCl}_2$, were transferred in groups of ten to each tube in 50 μ l of gassed (O_2/CO_2 , 19:1) medium containing glucose (4 mM) and bovine serum albumin (0.5 mg/ml). The medium containing the islets was carefully placed against the walls of the tubes to leave an air space between the buffer and the oil mixture. The incubation was commenced by addition of 50 μ l of incubation medium containing the test substances, followed immediately by a further 50 μ l of medium supplemented with $^{45}\text{Ca}^{2+}$ (20 $\mu\text{Ci/ml}$) and [^3H]sucrose (40 $\mu\text{Ci/ml}$). The tubes were incubated at 37°C for the times indicated in individual experiments, and the islets separated from the radioactive medium by rapid centrifugation (9000g for 5 s) through the phthalate layer. The tubes were immersed in liquid N_2 and the bottom 5 mm cut from each, above the islet pellet. The pellet was then shaken in 500 μ l of 0.2M- HClO_4 to disrupt the cells. A 4.5 ml portion of scintillant (Instagel) was added and the radioactivity determined in a liquid-scintillation spectrometer. Blanks containing no islets were included in each experiment and their radioactivities did not differ significantly from background values.

Standards of the radioactive medium and samples containing $^{45}\text{Ca}^{2+}$ only, for estimation of spill-over of $^{45}\text{Ca}^{2+}$ radioactivity counts into the ^3H channel, were included for spectrometry. $^{45}\text{Ca}^{2+}$ uptake was calculated from the $^{45}\text{Ca}^{2+}$ space in excess of the

extracellular volume, estimated as the [^3H]sucrose space.

$^{45}\text{Ca}^{2+}$ efflux from preloaded islets of Langerhans. Studies on the kinetics of $^{45}\text{Ca}^{2+}$ efflux from preloaded islets of Langerhans were performed by using a method modified from that described by Frankel *et al.* (1978). Groups of 150 isolated islets of Langerhans were incubated for 30 min (37°C) in 500 μ l of gassed (O_2/CO_2 , 19:1) medium containing $^{45}\text{Ca}^{2+}$ (200 $\mu\text{Ci/ml}$), glucose (20 mM) and bovine serum albumin (0.5 mg/ml). This medium contained no added $^{40}\text{Ca}^{2+}$, and the total $^{45}\text{Ca}^{2+}$ concentration was approx. 0.2 mM.

After preincubation, the islets were washed with $3 \times 500 \mu\text{l}$ of gassed medium containing 5 mM-glucose and 2 mM- $^{40}\text{CaCl}_2$, but no $^{45}\text{Ca}^{2+}$. They were then transferred to a perfusion chamber and perfused at a flow rate of 1 ml/min with medium containing 4 mM-glucose, bovine serum albumin (0.5 mg/ml) and, unless otherwise stated, 2 mM- $^{40}\text{CaCl}_2$. Islets were perfused for 35 min before any alteration in the composition of the influent medium. The effluent medium was collected over successive 1-min periods throughout the experiment. Portions of each sample (400 μ l) were transferred to scintillation vials and 4.5 ml of scintillant (Fisofluor 2; Fisons) added, for determination of $^{45}\text{Ca}^{2+}$ content by liquid-scintillation spectrometry. The extent of $^{45}\text{Ca}^{2+}$ efflux was measured as the rate of change of effluent radioactivity with time.

Ionophoretic translocation of $^{45}\text{Ca}^{2+}$ in an artificial system. The ionophoretic properties of δ -haemolysin were investigated by using an artificial Ca^{2+} -translocation system (Pfeiffer & Lardy, 1976). A 200 μ l portion of the medium normally employed for islet incubation (Gey & Gey, 1936) and containing 2 mM- $^{40}\text{CaCl}_2$ and $^{45}\text{Ca}^{2+}$ (5 $\mu\text{Ci/ml}$) was dispensed into a polystyrene tube and 200 μ l of a mixture of toluene/butan-1-ol (7:3, v/v), containing the substances under study, was added. The contents were vortex-mixed for 30 s to allow $^{45}\text{Ca}^{2+}$ translocation to approach equilibrium. After mixing, the tubes were given a short spin in a Microfuge (9000g; 5 s) and 75 μ l of the upper organic phase was removed. Scintillant (2 ml; Fisofluor 3) was added and the $^{45}\text{Ca}^{2+}$ content determined by liquid-scintillation spectrometry.

Statistical analysis of results

Levels of significance of differences between mean values were assessed by Student's *t* test. All results are expressed as mean values $\pm$ S.E.M., with the numbers of observations in parentheses.

Results

Stimulation of insulin release by δ -haemolysin

Table 1 shows the dose-response for δ -

Table 1. Dose-response for δ -haemolysin-induced insulin release

Groups of four isolated islets were incubated for 1 h in medium containing 4 mM-glucose supplemented with increasing concentrations of δ -haemolysin (as shown). After incubation, portions were removed for determination of insulin content by radioimmunoassay. Results are expressed as mean rates of insulin release $\pm$ S.E.M.

δ -Haemolysin concn. (μ g/ml)	Insulin release (ng/h per islet)
0	1.8 ± 0.1 (14)
5	3.2 ± 0.1 (7)
10	5.6 ± 0.2 (11)
25	8.0 ± 0.4 (7)
50	10.0 ± 0.6 (7)
100	10.1 ± 0.3 (7)

haemolysin-stimulated insulin release. δ -Haemolysin was a potent stimulant of insulin release from isolated islets of Langerhans over the concentration range 0–100 μ g/ml, and the extent of release was dependent on the concentration of δ -haemolysin employed. The rate of insulin release in response to δ -haemolysin was saturable at concentrations in excess of 50 μ g/ml, with the maximum rate of secretion representing a 5–6-fold elevation relative to the basal rate. Close to half-maximal effects were elicited by 10 μ g of δ -haemolysin/ml, and this concentration was selected for routine use in subsequent experiments.

Effect of the glucose concentration of the incubation medium on δ -haemolysin-stimulated insulin release

Table 2 shows the effect of the glucose concentration of the incubation medium on δ -haemolysin-stimulated insulin release. Increasing the glucose concentration in the absence of δ -haemolysin resulted in an increase in the rate of insulin release to a value some 4-fold above basal in the presence of 20 mM-glucose. δ -Haemolysin accentuated the rate of insulin release at each glucose concentration studied and was effective in the absence of glucose.

Reversibility of δ -haemolysin-stimulated insulin release

An elevated rate of insulin release could be maintained for at least 60 min from islets perfused with medium containing δ -haemolysin (Fig. 1). Removal of the agent from the perfusion medium after a 20-min period of stimulation was followed by an immediate and continuous decrease in the rate of insulin secretion over the subsequent period of perfusion. The rate of release, however, still remained slightly elevated relative to the basal rate 40 min after removal of δ -haemolysin (Fig. 1); this

was in contrast with the rate of decline in insulin secretion seen on removal of a glucose stimulus (Fig. 2). Decrease of the glucose concentration from 20 mM to 4 mM resulted in a rapid decrease in the rate of insulin secretion, which returned close to basal levels within 20 min.

Response of islets of Langerhans to sequential stimulation

Perfused islets of Langerhans previously stimulated with glucose readily responded to a subsequent δ -haemolysin stimulus by a renewed increase in the rate of insulin secretion (Fig. 2). Similarly, islets initially stimulated with δ -haemolysin rapidly responded to a subsequent glucose stimulus (20 mM), achieving a final rate of release some 3-fold above control values (Fig. 2). This rate was not significantly different from that induced by the initial period of stimulation with δ -haemolysin.

Effect of agents that modulate glucose-induced insulin secretion on δ -haemolysin-stimulated insulin release

The presence of extracellular Ca^{2+} is an absolute requirement for glucose-stimulated insulin release, and removal of this ion from the incubation medium inhibited the stimulation of release induced by

Fig. 1. Reversibility of δ -haemolysin-induced insulin release

Groups of 100 isolated islets were perfused in medium containing 4 mM-glucose, then stimulated by introduction of δ -haemolysin (10 μ g/ml). In one channel the stimulus was maintained throughout the remainder of perfusion (●), whereas in the second channel δ -haemolysin was removed from the influent medium after a 20-min period (○). The insulin content of successive samples of the perfusate was determined by radioimmunoassay. Results are expressed as mean rates of insulin release $\pm$ S.E.M. ($n=3$) relative to the basal rate. Significances: ★ $P<0.05$ relative to control; ★★ $P<0.02$ relative to control. Arrows indicate when the medium composition was changed.

Table 2. *Effect of various incubation conditions on δ -haemolysin-induced insulin release*

Groups of four isolated islets were incubated in medium containing glucose and δ -haemolysin as shown in the presence and absence of the additions shown. After incubation for 1 h, portions were removed for estimation of insulin content by radioimmunoassay. Results are expressed as mean rates of insulin release $\pm$ s.e.m. Abbreviation used = IBMX, 3-isobutyl-1-methylxanthine. Levels of significance: * $P < 0.025$ relative to appropriate glucose control; ** $P < 0.025$ relative to appropriate δ -haemolysin control.

Incubation conditions					Insulin release (ng/h per islet)
[Glucose] (mM)	δ -Haemolysin (10 μ g/ml)	EGTA (0.5 mM)	IBMX (0.2 mM)	Adrenaline (epinephrine) (10 μ M)	
0	—	—	—	—	1.4 $\pm$ 0.1 (7)
0	+	—	—	—	3.2 $\pm$ 0.2 (7)*
4	—	—	—	—	1.6 $\pm$ 0.2 (21)
4	+	—	—	—	3.9 $\pm$ 0.3 (7)*
4	—	+	—	—	1.4 $\pm$ 0.2 (7)
4	—	—	+	—	1.6 $\pm$ 0.1 (7)
4	—	—	—	+	1.4 $\pm$ 0.2 (7)
4	+	+	—	—	2.4 $\pm$ 0.3 (7)**
4	+	—	+	—	7.0 $\pm$ 0.5 (7)**
4	+	—	—	+	2.1 $\pm$ 0.1 (8)**
8	—	—	—	—	2.5 $\pm$ 0.1 (26)
8	+	—	—	—	5.2 $\pm$ 0.5 (22)*
8	—	+	—	—	1.9 $\pm$ 0.1 (25)*
8	—	—	+	—	5.2 $\pm$ 0.3 (20)*
8	—	—	—	+	1.8 $\pm$ 0.1 (10)*
8	+	+	—	—	3.9 $\pm$ 0.2 (21)**
8	+	—	+	—	9.4 $\pm$ 0.7 (11)**
8	+	—	—	+	3.0 $\pm$ 0.3 (10)**
20	—	—	—	—	4.1 $\pm$ 0.4 (7)
20	+	—	—	—	6.2 $\pm$ 0.5 (7)*

glucose (8 and 20mM) (Table 2), to a value not significantly different from control values.

Removal of extracellular Ca^{2+} also antagonized the ability of δ -haemolysin to stimulate the release of insulin from isolated islets. In the presence of either 4mM or 8mM-glucose, omission of extracellular Ca^{2+} resulted in marked inhibition of the secretory response to δ -haemolysin (Table 2). This diminution in the extent of insulin secretion was not, however, total, since release was not decreased to basal values. Even in Ca^{2+} -depleted media, δ -haemolysin was found to be capable of significant stimulation of hormone release from isolated islets (Table 2).

The results presented in Table 2 demonstrate that insulin secretion in response to glucose and δ -haemolysin was also sensitive to the hormone adrenaline (epinephrine) (10 μ M). This agent was observed to inhibit significantly the release of insulin induced by 8mM-glucose and by δ -haemolysin (10 μ g/ml).

3-Isobutyl-1-methylxanthine is a potent phosphodiesterase inhibitor that induces the accumulation of cyclic AMP in islet cells (Table 3). In the presence of a sub-maximal glucose stimulus, this agent was found to enhance the insulin-secretory response of isolated islets of Langerhans (Table 2) by some 115%. Under similar conditions, δ -haemolysin

(10 μ g/ml) caused a slightly larger increase in the extent of insulin release than did 3-isobutyl-1-methylxanthine, namely 150%. However, islets treated simultaneously with 3-isobutyl-1-methylxanthine and δ -haemolysin showed a potentiated response, achieving a rate of secretion nearly 4-fold greater than that seen under control conditions. This rate was also significantly higher than that observed in the presence of either agent alone ($P < 0.001$).

Assessment of the extent of cell damage on incubation of islets of Langerhans with δ -haemolysin

Lactate dehydrogenase release. The extent of release of the intracellular marker enzyme lactate dehydrogenase from islet cells after incubation under control conditions amounted to some 4% of the total activity and was not significantly increased by incubation with δ -haemolysin (10 μ g/ml).

[^3H]Sucrose space. [^3H]Sucrose has been widely employed as a non-penetrating marker (Wollheim *et al.*, 1977) for estimation of the extracellular volume of islet cells. After 60 min incubation under control (4mM-glucose) conditions the extracellular space was estimated as 6.4nl/islet. This value was not significantly changed after incubation for a similar time period with either 20mM-glucose or δ -haemolysin (10 μ g/ml).

Effect of δ-haemolysin on islet-cell cyclic AMP levels

The results presented in Table 3 show that incubation of isolated islets of Langerhans for 30 min in the presence of δ-haemolysin (10 µg/ml) resulted in a significant (2-fold) increase in the intracellular cyclic AMP concentration, relative to control islets.

Inclusion of the phosphodiesterase inhibitor 3-

isobutyl-1-methylxanthine in the incubation medium of control islets increased the cyclic AMP content of the cells to a value some 13-fold higher than that observed in the absence of this agent. This value was unaffected by a decrease in the extracellular calcium concentration.

When incubated in media containing 3-isobutyl-1-methylxanthine, islets simultaneously exposed to δ-haemolysin again displayed a marked increase in the extent of cyclic AMP accumulation relative to controls. In this case, cyclic AMP levels were increased a further 3-fold by δ-haemolysin. However, the extent of this stimulation was dependent on the presence of extracellular Ca²⁺.

Effect of δ-haemolysin on islet-cell adenylate cyclase activity

δ-Haemolysin in the concentration range 0.5–25 µg/ml had no effect on the adenylate cyclase activity of islet-cell homogenates.

Effect of δ-haemolysin on islet-cell glucose metabolism

Islets of Langerhans incubated in the presence of 4 mM-glucose were found to oxidize 26.9 ± 3.6 pmol of glucose/2 h per islet, and this was increased to a value of 62.5 ± 10.7 pmol/2 h per islet in the presence of 20 mM-glucose (*P* < 0.025). In contrast, islets of Langerhans incubated in the presence of 4 mM-glucose plus δ-haemolysin (10 µg/ml) failed to show any increase in the rate of glucose utilization, even though, under these conditions, the rate of insulin secretion is increased to that observed with 20 mM-glucose.

Effect of δ-haemolysin on islet-cell ⁴⁵Ca²⁺ accumulation

The extent of accumulation of ⁴⁵Ca²⁺ by the cells

Fig. 2. Response of perfused islets of Langerhans to sequential stimulation

Groups of 100 isolated islets were perfused in medium containing 4 mM-glucose before stimulation for a 20-min period with either 20 mM-glucose (○) or 10 µg of δ-haemolysin/ml (●). After a recovery period, a second stimulus was then introduced, being either 10 µg of δ-haemolysin/ml (○) or 20 mM-glucose (●). The insulin content of successive samples of the perfusate was determined by radioimmunoassay. Results are expressed as mean rates of insulin release ± S.E.M. (*n* = 3) relative to the basal rate. Significance: ★*p* < 0.02 relative to basal rate. Arrows indicate when the medium composition was changed.

Table 3. Effect of δ-haemolysin on islet-cell cyclic AMP levels

Groups of 100 isolated islets were incubated in medium containing 8 mM-glucose together with δ-haemolysin and 3-isobutyl-1-methylxanthine (IBMX) as shown, in the presence and absence of Ca²⁺. Ca²⁺-depleted medium was supplemented with EGTA (1 mM). After incubation for 30 min the islets were frozen and disrupted ultrasonically. The cyclic AMP content of the tissue extract was determined by using a competitive binding assay. Results are expressed as mean values ± S.E.M. for islet cyclic AMP content. Significances: **P* < 0.02 relative to absence of δ-haemolysin; ***P* < 0.001 relative to absence of δ-haemolysin; ****P* < 0.02 relative to presence of Ca²⁺.

Additions to incubation medium			Islet cyclic AMP content (fmol/µg of protein)
δ-Haemolysin (10 µg/ml)	IBMX (0.2 mM)	EGTA (1 mM)	
—	—	—	1.2 ± 0.2 (4)
+	—	—	2.5 ± 0.4 (4)*
—	+	—	15.0 ± 3.2 (12)
—	+	+	13.7 ± 3.8 (6)
+	+	—	49.8 ± 8.3 (10)**
+	+	+	20.3 ± 5.9 (7)***

of isolated islets of Langerhans is shown in Table 4. Islets incubated in the presence of both 4 mM- and 20 mM-glucose accumulated $^{45}\text{Ca}^{2+}$ after 60 min of incubation. However, the total extent of accumulation at the end of this time was some 79% higher when islets were incubated in 20 mM-glucose compared with that seen with 4 mM-glucose ($P < 0.001$).

δ -Haemolysin was also observed to induce a marked increase in the extent of $^{45}\text{Ca}^{2+}$ accumulation in islet cells relative to 4 mM-glucose (Table 4). In the presence of $10\text{ }\mu\text{g}$ of δ -haemolysin/ml the extent of $^{45}\text{Ca}^{2+}$ accumulation was increased by 56% relative to the control ($P < 0.001$), and was

Table 4. *Effect of δ -haemolysin on islet-cell ^{45}Ca accumulation*

Groups of ten isolated islets were incubated in medium supplemented as shown and containing $^{45}\text{Ca}^{2+}$ and $[^3\text{H}]\text{sucrose}$. After incubation for 1 h the islets were separated from the bulk medium by centrifugation through a phthalate layer and the extent of $^{45}\text{Ca}^{2+}$ accumulation was determined. Results are expressed as mean values for $^{45}\text{Ca}^{2+}$ accumulation $\pm$ S.E.M. in excess of the $[^3\text{H}]\text{sucrose}$ space. Significance: * $P < 0.001$ relative to 4 mM-glucose alone.

Glucose concn. (mM)	δ -Haemolysin concn. ($\mu\text{g}/\text{ml}$)	$^{45}\text{Ca}^{2+}$ accumulation (fmol/h per islet)
4	0	184 ± 20 (7)
20	0	329 ± 21 (7)*
4	5	238 ± 34 (5)
4	10	285 ± 17 (20)*
4	50	385 ± 22 (5)*

Table 5. *Effect of verapamil on δ -haemolysin-induced insulin release*

Groups of 100 isolated islets were perfused with medium containing 4 mM-glucose before stimulation with either 20 mM-glucose or $10\text{ }\mu\text{g}$ of δ -haemolysin/ml. After a 20-min period, verapamil ($10\text{ }\mu\text{M}$) was introduced concurrently with the stimuli, and perfusion continued for a final 20 min. The insulin content of samples of the perfusate was determined by radioimmunoassay. Results are expressed as integrated mean rates of insulin release $\pm$ S.E.M. relative to basal levels ($n = 10$). Significances: * $P < 0.005$ relative to 20 mM-glucose alone; ** $P < 0.001$ relative to δ -haemolysin alone.

Glucose concn. (mM)	δ -Haemolysin ($10\text{ }\mu\text{g}/\text{ml}$)	Verapamil ($10\text{ }\mu\text{M}$)	Insulin release (% of basal rate)
4	—	—	100 ± 8
4	—	+	96 ± 6
4	+	—	265 ± 21
4	+	+	400 ± 18 **
20	—	—	180 ± 14
20	—	+	120 ± 8 *

not significantly different from that seen in response to 20 mM-glucose.

The ability of δ -haemolysin to stimulate $^{45}\text{Ca}^{2+}$ accumulation in isolated islets was dose-dependent, with the extent of accumulation reaching a level which was some 110% higher than control levels in the presence of $50\text{ }\mu\text{g}$ of δ -haemolysin/ml.

Effect of verapamil on δ -haemolysin-induced insulin secretion

The results presented in Table 5 show the effect of the organic Ca^{2+} antagonist verapamil on insulin

Fig. 3. *Effect of δ -haemolysin on $^{45}\text{Ca}^{2+}$ efflux from preloaded islets*

Groups of 150 islets were preloaded with $^{45}\text{Ca}^{2+}$ then transferred to perfusion chambers and perfused with medium containing 4 mM-glucose. In controls (Δ), 4 mM-glucose was maintained throughout the remainder of perfusion. δ -Haemolysin ($10\text{ }\mu\text{g}/\text{ml}$; $\triangle$) or δ -haemolysin ($10\text{ }\mu\text{g}/\text{ml}$) plus verapamil ($10\text{ }\mu\text{M}$; $\bullet$) was introduced into test channels after 35 min of perfusion. The perfusate was collected and the $^{45}\text{Ca}^{2+}$ content of successive samples determined. Results are presented as mean rates ($n = 3$) of $^{45}\text{Ca}^{2+}$ efflux relative to that observed after 29 min of perfusion (100%). Arrow indicates when verapamil was introduced.

Fig. 4. Effect of Ca^{2+} depletion on δ -haemolysin-induced $^{45}\text{Ca}^{2+}$ efflux from preloaded islets

Groups of 150 isolated islets were preloaded with $^{45}\text{Ca}^{2+}$ and transferred to perfusion chambers. They were perfused with medium containing 4 mM-glucose in the presence (●) or absence (○) of $^{40}\text{Ca}^{2+}$ (2 mM). Ca^{2+} -depleted media were supplemented with EGTA (1 mM). δ -Haemolysin (10 $\mu\text{g}/\text{ml}$) was introduced after 35 min of perfusion. Results are presented as mean rates ($n=3$) of $^{45}\text{Ca}^{2+}$ efflux into successive samples of the perfusate relative to that observed after 29 min of perfusion (100%). The arrow indicates when δ -haemolysin was introduced.

secretion from isolated islets. This agent was found to inhibit significantly the stimulation of insulin release induced by 20 mM-glucose ($P<0.005$). By contrast, the stimulant activity of δ -haemolysin was further accentuated in the presence of verapamil (10 μM) such that, in the presence of both agents, the observed rate of insulin secretion was increased by 51% ($P<0.001$) relative to that seen in response to δ -haemolysin alone.

Effect of δ -haemolysin on $^{45}\text{Ca}^{2+}$ efflux from pre-loaded islets

The effect of δ -haemolysin treatment on the rate of $^{45}\text{Ca}^{2+}$ efflux from islets preloaded with the isotope is shown in Fig. 3. In control islets, the rate of $^{45}\text{Ca}^{2+}$ efflux declined progressively with time, whereas addition of δ -haemolysin (10 $\mu\text{g}/\text{ml}$) resulted in a rapid, but short-lived, increase in efflux rate. This increase was evident within 2 min of the introduction of δ -haemolysin, and the maximum extent of stimulation was reached within a total of 4 min. Relative to control islets, δ -haemolysin induced a 34% increase in the extent of $^{45}\text{Ca}^{2+}$ efflux within 4 min of introduction ($P<0.005$).

When islets were perfused in medium depleted of extracellular Ca^{2+} the rate of $^{45}\text{Ca}^{2+}$ efflux under non-stimulating conditions was identical with that seen in the presence of 2 mM-extracellular Ca^{2+} (Fig.

Fig. 5. Comparison of the effects of δ -haemolysin and ionophore A23187 on $^{45}\text{Ca}^{2+}$ efflux from preloaded islets

Groups of 150 isolated islets were preloaded with $^{45}\text{Ca}^{2+}$ and transferred to perfusion chambers. They were perfused for 35 min with medium containing 4 mM-glucose, when either δ -haemolysin (10 $\mu\text{g}/\text{ml}$; ○) or ionophore A23187 (3 μM ; ●) was also introduced. The extent of $^{45}\text{Ca}^{2+}$ efflux into successive samples of the perfusate was determined. Results are expressed as mean rates ($n=3$) of $^{45}\text{Ca}^{2+}$ efflux, relative to that observed after 29 min of perfusion (100%). The arrow indicates when δ -haemolysin or A23187 was introduced.

4). However, addition of δ -haemolysin induced a markedly greater response than that seen in Ca^{2+} -containing medium. Under conditions of Ca^{2+} depletion, the rate of onset of the rise in $^{45}\text{Ca}^{2+}$ efflux and the time period for achievement of the maximal rate, were unchanged relative to controls. However, the extent of the increase under these conditions was markedly accentuated (Fig. 4), reaching a level which was 80% higher than that seen in controls perfused in Ca^{2+} -containing media. Indeed, an elevated rate of efflux was maintained throughout the period of exposure to δ -haemolysin under these conditions.

The results presented in Fig. 5 demonstrate that the $^{45}\text{Ca}^{2+}$ efflux response of perfused islets to δ -haemolysin treatment was similar to that evoked by the ionophore A23187. A rise in efflux rate was first detected within 2 min of the addition of either agent, was maximal within a further 2 min in both cases, and the duration of the response was the same with both agents.

Table 6. *Effect of verapamil on $^{45}\text{Ca}^{2+}$ translocation in an artificial system*

The extent of $^{45}\text{Ca}^{2+}$ translocation from an aqueous phase to an immiscible organic phase containing δ -haemolysin or ionophore A23187 was assessed in the presence of increasing concentrations of verapamil. Tubes were vortex-mixed for 30 s and the organic phase sampled for determination of the extent of $^{45}\text{Ca}^{2+}$ translocation. Results are expressed as $^{45}\text{Ca}^{2+}$ (c.p.m.) translocated $\pm$ s.e.m. Significances: * $P < 0.05$ relative to δ -haemolysin alone; ** $P < 0.005$ relative to δ -haemolysin alone; *** $P < 0.005$ relative to absence of verapamil.

Verapamil concn. (μM)	$^{45}\text{Ca}^{2+}$ translocation (c.p.m.)			
	Control	δ -Haemolysin		Ionophore A23187 (20 μM)
		(10 $\mu\text{g}/\text{ml}$)	(50 $\mu\text{g}/\text{ml}$)	
0	18 $\pm$ 3 (11)	33 $\pm$ 5 (16)	257 $\pm$ 18 (5)	1641 $\pm$ 119 (5)
10	24 $\pm$ 1 (5)	52 $\pm$ 6 (6)*	473 $\pm$ 45 (5)**	1416 $\pm$ 41 (5)
100	18 $\pm$ 2 (6)	—	—	1061 $\pm$ 57 (5)***

Table 7. *Effect of phosphatidylcholine on δ -haemolysin-stimulated $^{45}\text{Ca}^{2+}$ translocation and insulin release*

$^{45}\text{Ca}^{2+}$ translocation was measured from an aqueous phase to an immiscible organic phase containing δ -haemolysin and phosphatidylcholine as shown. Insulin release was measured after incubation of isolated islets of Langerhans for 1 h in media supplemented with δ -haemolysin and phosphatidylcholine. Results are expressed as mean values $\pm$ s.e.m., with the numbers of observations in parentheses. Significance: * $P < 0.025$ relative to δ -haemolysin alone; ** $P < 0.005$ relative to δ -haemolysin alone.

δ -Haemolysin (10 $\mu\text{g}/\text{ml}$)	Phosphatidylcholine (25 $\mu\text{g}/\text{ml}$)	$^{45}\text{Ca}^{2+}$ translocated (c.p.m.)	Insulin release (% of basal rate)
—	—	23 $\pm$ 4 (6)	100 $\pm$ 19 (17)
—	+	26 $\pm$ 6 (6)	90 $\pm$ 10 (17)
+	—	57 $\pm$ 9 (6)	293 $\pm$ 38 (17)
+	+	31 $\pm$ 4 (6)*	153 $\pm$ 18 (17)**

Effect of δ -haemolysin on $^{45}\text{Ca}^{2+}$ translocation in an artificial system

δ -Haemolysin was found to stimulate the translocation of $^{45}\text{Ca}^{2+}$ from an aqueous phase to an immiscible organic phase in a dose-dependent manner (Table 6). The extent of translocation was directly proportional to the δ -haemolysin concentration over the range 0–100 $\mu\text{g}/\text{ml}$, with the highest concentration yielding a rate of translocation approx. 20-fold greater than that seen in controls. At the concentration routinely employed for insulin-secretion studies (10 $\mu\text{g}/\text{ml}$), δ -haemolysin increased the extent of $^{45}\text{Ca}^{2+}$ translocation by about 2-fold in this system.

Effect of agents that modulate δ -haemolysin-induced insulin secretion on δ -haemolysin-stimulated $^{45}\text{Ca}^{2+}$ translocation

(a) *Verapamil.* Verapamil was observed to antagonize the stimulatory effect of ionophore A23187 on $^{45}\text{Ca}^{2+}$ translocation in the present system (Table 6). This effect was dose-dependent, with 35% inhibition relative to the control seen at 100 μM -verapamil.

In contrast, the stimulatory influence of δ -haemolysin on $^{45}\text{Ca}^{2+}$ translocation was further

accentuated by the presence of verapamil (Table 6). Verapamil (10 μM) caused a significant elevation of $^{45}\text{Ca}^{2+}$ translocation in response to both 10 μg (58%) and 50 μg (84%) of δ -haemolysin/ml.

In addition to these effect on $^{45}\text{Ca}^{2+}$ translocation in the artificial system, verapamil (10 μM) was also observed to accentuate the rate of $^{45}\text{Ca}^{2+}$ efflux from preloaded islets treated with δ -haemolysin (Fig. 3). In controls, addition of δ -haemolysin resulted in a rapid, short-lived increase in $^{45}\text{Ca}^{2+}$ efflux, whereas addition of δ -haemolysin together with verapamil resulted in a markedly accentuated response. This increase was, however, transient, and the temporal characteristics were identical with those seen in the absence of verapamil.

(b) *Phosphatidylcholine.* Phosphatidylcholine (25 $\mu\text{g}/\text{ml}$) was found to inhibit the ability of δ -haemolysin to stimulate the release of insulin from isolated islets of Langerhans (Table 7). In the presence of the phospholipid, the extent of insulin release induced by δ -haemolysin was decreased by 48% relative to controls. The basal rate of insulin secretion was unaffected by the presence of phosphatidylcholine.

The inhibitory effect of phosphatidylcholine on δ -haemolysin-induced insulin release was closely

paralleled by a similar effect on δ -haemolysin-stimulated ^{45}Ca translocation in the artificial system. Phosphatidylcholine caused a significant inhibition of $^{45}\text{Ca}^{2+}$ translocation in response to δ -haemolysin, with the extent of this inhibition (46%) being almost identical with that measured in the secretion experiments with isolated islets of Langerhans. No effect of the phospholipid was observed on the control rate of $^{45}\text{Ca}^{2+}$ translocation.

Discussion

δ -Haemolysin was termed a 'cytolytic toxin' (Bernheimer, 1968) in view of its ability to induce lesions in the membranes of a variety of cell types. However, more recent studies have indicated that, at low concentration, δ -haemolysin is capable of inducing more subtle permeability changes in the membranes of cells without causing lysis (O'Brien & Kapral, 1976; O'Brien *et al.*, 1978). It was important, therefore, to establish whether the effects of δ -haemolysin on islet cells were the result of gross plasma-membrane damage or whether they reflected a more specific interaction of the toxin with the plasma membrane of islet cells.

The release of lactate dehydrogenase from the cells of isolated islets has been used as a criterion to assess the extent of damage in these cells (Malaisse *et al.*, 1979), since it is a soluble cytoplasmic enzyme (Straub, 1940) which is not normally released from islet cells during insulin secretion (Dean *et al.*, 1979). Incubation of islets with low concentrations of δ -haemolysin (10 $\mu\text{g}/\text{ml}$) did not result in any significant increase in the extent of release compared with controls, suggesting that the major of islet cells remained intact during a 60-min incubation under these conditions.

In support of this evidence suggesting a lack of gross physical damage in δ -haemolysin-treated islet cells was the data obtained for the sucrose space of these cells. Sucrose is not transported into islet cells (McDaniel *et al.*, 1975) and has been widely employed as a marker for determination of the extracellular volume (Hellman *et al.*, 1971; McDaniel *et al.*, 1975; Wollheim *et al.*, 1977) of isolated islets. Damage to the cells during incubation would be expected to result in entry of sucrose into the cells, giving rise to an apparent increase in extracellular volume. This criterion has been previously employed to quantify rat islet-cell-membrane permeability changes (McDaniel *et al.*, 1975) and, since the intracellular volume of isolated islets is similar to the extracellular volume, the method is sufficiently sensitive to detect even subtle damage to the cell membrane (Wollheim *et al.*, 1977). When incubated under non-stimulating conditions, the islet extracellular volume was estimated to be 6.4 nl/islet and neither glucose (20 mM) nor

δ -haemolysin (10 $\mu\text{g}/\text{ml}$) stimulation were found to significantly alter this value. This provides strong evidence that the islets remained relatively undamaged throughout the period of stimulation.

δ -Haemolysin was found to have a dramatic effect in stimulating the release of insulin from the β -cells of the islet (Fig. 1). This effect was rapid, with an increase in secretion rate being evident within 2 min. The maximum rate of secretion was achieved after 8 min and was maintained for at least 1 h in the continued presence of δ -haemolysin. When δ -haemolysin was removed from the medium, there was a rapid decrease in the rate of secretion. These results would not have been obtained if δ -haemolysin were acting simply by disrupting the plasma membrane of the β -cell. It therefore seems unlikely that the stimulation of insulin release from isolated islets by δ -haemolysin simply reflected leakage of insulin resulting from cell damage. However, since the δ -haemolysin molecule is markedly hydrophobic (Fitton *et al.*, 1980) and displays surface-active properties in a number of cell types (Kreger & Bernheimer, 1971), it is likely that it affects insulin secretion by interacting with the plasma membrane of the β -cell. Indeed, a number of membrane-active agents have been reported to stimulate the release of insulin from isolated islets, including 4-acet-amido-4'-isothiocyanostilbene-2,2'-disulphonic acid (Hellman *et al.*, 1973), chloromercuribenzenesulphonic acid (Hellman *et al.*, 1975) and mushroom lectin (Ewart *et al.*, 1975). None of these agents have been shown to stimulate insulin release simply by virtue of cytolytic actions, and all are reported to penetrate islet cells only poorly. It is therefore quite clear that an interaction with the β -cell surface membrane can result in the stimulation of insulin release.

It has recently been demonstrated that induction of islet-cell damage by cell-surface antibodies and complement resulted in total abolition of any secretory response to a subsequent glucose stimulus in perfused islet cells (Kanatsuna *et al.*, 1981). The observation that δ -haemolysin-treated islets remained responsive to glucose (Fig. 2) therefore also supports the idea that this agent stimulated insulin release by a mechanism that was independent of cell damage.

Effect of δ -haemolysin on islet glucose metabolism

Glucose is the major physiological stimulus for insulin release from isolated islets of Langerhans, and the weight of current evidence supports the suggestion that it stimulates insulin release by virtue of its metabolism in the pancreatic β -cell (Ashcroft, 1980). δ -Haemolysin was observed to stimulate insulin release markedly in the total absence of extracellular glucose, suggesting that an increased glucose metabolism was unlikely to account for this

stimulation. This was supported by measurement of the rate of [$U-^{14}C$]glucose metabolism in δ -haemolysin-treated islets, which demonstrated that stimulation of insulin release by the toxin was not accompanied by alterations in the rate of islet-cell glucose metabolism. This suggests that the early events in stimulus-secretion coupling may have been different when islet cells were stimulated with glucose as compared with δ -haemolysin.

Characterization of the insulin-secretory response to δ -haemolysin

Stimulation of insulin secretion by glucose and a variety of other secretagogues has long been known to depend on the presence of extracellular Ca^{2+} (Grodsky & Bennett, 1966; Milner & Hales, 1967). The results presented in Table 2 support these reports, since incubation of islets in Ca^{2+} -depleted media was observed to abolish the stimulant action of glucose. In addition, δ -haemolysin-induced insulin release was also significantly inhibited under these conditions. In the latter case, however, the secretory response was not totally abolished, since, even in the absence of extracellular Ca^{2+} , stimulation above the basal rate of secretion was observed. Similar effects have been reported for surface-active thiol reagents, which were capable of stimulating insulin release in the absence of extracellular Ca^{2+} , but whose stimulatory effects were accentuated by inclusion of the ion (Hellman *et al.*, 1974a).

The hormone adrenaline (epinephrine) is known to modulate the secretory response of isolated islets of Langerhans to a variety of stimuli, including glucose (Coore & Randle, 1964). In the present study, this hormone was found to inhibit markedly both glucose- and δ -haemolysin-stimulated insulin release (Table 2). This observation supports the concept that δ -haemolysin induced the release of insulin by true activation of some component of the insulin-secretory mechanism, since insulin release resulting largely from cell damage would not have been sensitive to adrenaline (epinephrine).

The results of the studies on the insulin-secretory response of islet cells to δ -haemolysin indicated that the response shared a number of features in common with the response induced by glucose and the toxin may therefore be of value for investigating the biochemical basis of the insulin-secretory response. It was therefore considered necessary to investigate the possible mechanism action of the toxin.

Effect of δ -haemolysin on islet cyclic AMP metabolism

It has become evident that the cyclic AMP concentration in the β -cell plays an important role in modulation of the secretory response to glucose (see Sharp, 1979). The results in Table 2 show that,

under conditions when the cyclic AMP content of islets cells was increased by 3-isobutyl-1-methylxanthine, the secretory response of isolated islets to a δ -haemolysin stimulus was also markedly enhanced. It would therefore appear that cyclic AMP levels in the islet cells may exert a modulatory influence on the response to δ -haemolysin treatment, in a similar manner to that seen with glucose.

Treatment of isolated rat islets of Langerhans with δ -haemolysin (10 μ g/ml) was observed to induce a 2-fold increase in the intracellular cyclic AMP accumulation of islet cells after a 30-min incubation period. Many agents which stimulate the secretion of insulin from islets have been reported to elevate intracellular cyclic AMP levels. These include a variety of metabolizable sugars and sugar derivatives, such as glucose (Charles *et al.*, 1973), D-glyceraldehyde (Hellman *et al.*, 1974b) and mannose (Grill & Cerasi, 1976). Several lines of evidence have, however, demonstrated that cyclic AMP is unlikely to represent the primary intracellular 'trigger' molecule for insulin release (see Sharp, 1979, for review). It is unlikely, therefore, that this ability to elevate intracellular cyclic AMP could, itself, have represented the mechanism by which δ -haemolysin stimulated the release of insulin from islets of Langerhans.

Ultrastructural studies (Howell & Whitfield, 1972) have demonstrated that the islet-cell adenylate cyclase was located in the plasma membranes of both α - and β -cells of islets of Langerhans. An increase in intracellular cyclic AMP concentration in islet cells, caused by δ -haemolysin, might therefore have resulted from activation of adenylate cyclase on interaction of the toxin with the cell surface. However, δ -haemolysin did not stimulate the adenylate cyclase activity of islet homogenates.

The response of the islet-cell cyclic AMP system to δ -haemolysin is similar to that of the system to glucose, since glucose has been reported to elevate islet-cell cyclic AMP levels (Charles *et al.*, 1975), an effect not attributable to direct activation of adenylate cyclase (Davis & Lazarus, 1972), but dependent on the presence of extracellular Ca^{2+} (Zawalich *et al.*, 1975; Katada & Ui, 1979). It has been proposed that the glucose-induced rise in islet-cell cyclic AMP accumulation represents an effect which was secondary to a glucose-induced change in the cytosolic Ca^{2+} concentration and the consequent activation of adenylate cyclase by formation of the Ca^{2+} -calmodulin complex (Valverde *et al.*, 1979; Sharp *et al.*, 1980). It was therefore considered necessary to investigate the effect of δ -haemolysin on islet-cell calcium metabolism.

Effect of δ -haemolysin on islet-cell calcium metabolism

It is generally accepted that an increase in the

cytosolic Ca^{2+} concentration of the β -cell is a crucial event in triggering the insulin-secretory mechanism (Malaisse *et al.*, 1978). In the case of stimulation by glucose it is thought that the metabolism of the sugar is somehow linked to a net flux of Ca^{2+} into the β -cell from the extracellular medium. If this suggestion is correct, then agents which alter the permeability of the plasma membrane to Ca^{2+} may affect the secretory activity of the β -cell. Since δ -haemolysin appears to interact with the plasma membrane of the β -cell, and its effect on insulin secretion are Ca^{2+} -dependent, it was necessary to study the effect of δ -haemolysin on calcium metabolism by the β -cell.

The results presented in Table 6 demonstrate that stimulation of islet cells by raising the glucose concentration induced a net increase in $^{45}\text{Ca}^{2+}$ uptake by these cells after incubation for 1 h. This is in agreement with a number of other reports (Malaisse *et al.*, 1978; Wollheim *et al.*, 1977) and supports the concept that β -cell stimulation is associated with an increase in the rate of Ca^{2+} influx (see Malaisse *et al.*, 1978). In addition, treatment of isolated islets of Langerhans with δ -haemolysin ($10\text{ }\mu\text{g/ml}$) was also observed to give rise to a net accumulation of $^{45}\text{Ca}^{2+}$ (Table 4). The extent of this effect was very similar to that induced by glucose in the present study.

The pancreatic β -cell displays electrical activity under the influence of secretagogues, and electrophysiological studies have demonstrated that glucose-induced electrical activity carries a Ca^{2+} -dependent component (Dean & Matthews, 1970). One route by which Ca^{2+} enters the β -cell is a voltage-dependent channel characterized by its sensitivity to a variety of Ca^{2+} antagonists, including various bivalent cations (Malaisse *et al.*, 1976; Somers *et al.*, 1976) and organic Ca^{2+} antagonists (Devis *et al.*, 1975). The importance of this channel in the regulation of Ca^{2+} entry during insulin release was demonstrated by the observation that specific inhibition by verapamil and its analogue D600 exerted a profound inhibitory effect on glucose-stimulated insulin release (Devis *et al.*, 1975; Malaisse *et al.*, 1976). These observations were confirmed in the present study (Table 5). In contrast, however, the elevated rate of insulin release seen in response to δ -haemolysin was further increased on addition of verapamil (Table 5). These data suggests that the observed stimulation of $^{45}\text{Ca}^{2+}$ uptake in the presence of δ -haemolysin was probably not mediated by gating of the voltage-dependent channel if the crucial event for toxin-stimulated insulin release was an increase in Ca^{2+} entry. It appears, therefore, that the routes of Ca^{2+} entry into the islet cells, in response to glucose and δ -haemolysin stimulation, may have been different.

In addition to its ability to stimulate $^{45}\text{Ca}^{2+}$

accumulation in isolated islets of Langerhans, δ -haemolysin ($10\text{ }\mu\text{g/ml}$) was also observed to stimulate the efflux of this cation from islets preloaded with $^{45}\text{Ca}^{2+}$. This effect was rapid in onset, but relatively modest in size (Fig. 3). This is in contrast with the effect of glucose on $^{45}\text{Ca}^{2+}$ efflux, which produces an initial inhibition over a period of some 4 min, followed by a large secondary rise (Malaisse *et al.*, 1978). This rise in $^{45}\text{Ca}^{2+}$ efflux has been reported to be completely abolished in Ca^{2+} -depleted medium (Hellman *et al.*, 1979), suggesting that the increase may result from a process of $^{40}\text{Ca}^{2+}$ – $^{45}\text{Ca}^{2+}$ exchange (Malaisse *et al.*, 1978). Such a mechanism could not, however, entirely account for the ability of δ -haemolysin to stimulate Ca^{2+} efflux from islet cells, since, rather than showing inhibition in Ca^{2+} -depleted medium, the effect was dramatically enhanced (Fig. 4). In view of the lack of initial inhibition of $^{45}\text{Ca}^{2+}$ efflux in response to δ -haemolysin and the increase in efflux in Ca^{2+} -depleted medium, it appears that the mechanism for $^{45}\text{Ca}^{2+}$ efflux from islets must have been different in δ -haemolysin-treated islets compared with those stimulated with glucose.

The response of preloaded islets to δ -haemolysin did display a number of similarities to that induced by the Ca^{2+} ionophore A23187. Thus the ionophore, like δ -haemolysin, did not inhibit the initial $^{45}\text{Ca}^{2+}$ efflux from perfused islets (Fig. 5), and the temporal changes in efflux were identical in response to both agents.

In addition, ionophore A23187 has also been reported to cause a rapid and marked increase in $^{45}\text{Ca}^{2+}$ loss from islets incubated in Ca^{2+} -depleted medium (Tamarit-Rodriguez, 1978; Hellman *et al.*, 1979). This would be expected for an ionophore-mediated Ca^{2+} efflux, since, in the absence of extracellular Ca^{2+} , the electrochemical gradient would favour loss of ^{45}Ca , leading to an increase in the extent of efflux. The profiles of ^{45}Ca efflux in response to δ -haemolysin therefore suggest that an ionophoretic mechanism might account, at least in part, for the effects of δ -haemolysin on islet-cell Ca^{2+} handling. In support of this, δ -haemolysin was found to display potent ionophoretic activity in the artificial system, the toxin being able to translocate $^{45}\text{Ca}^{2+}$ from a bulk aqueous phase to a bulk organic phase in a dose-dependent manner (Table 6).

Verapamil, an organic molecule which inhibits Ca^{2+} entry into cells (Kohlhardt *et al.*, 1972), has been reported to inhibit the translocation of $^{45}\text{Ca}^{2+}$ from a bulk aqueous phase to a bulk organic phase induced by ionophore A23187 (Malaisse *et al.*, 1977). A similar effect of verapamil was observed in the present system (Table 6). Verapamil, however, did not inhibit δ -haemolysin-stimulated insulin release from isolated islets of Langerhans (Table 5), but, rather, accentuated the effect of the toxin. The

results presented in Table 6 demonstrate that this effect of verapamil was paralleled by a similar effect on δ -haemolysin-stimulated $^{45}\text{Ca}^{2+}$ translocation in the artificial system. This observation could be interpreted to suggest that the direct interaction of verapamil with δ -haemolysin did not lead to hindered binding of Ca^{2+} as with ionophore A23187, but, rather, produced a change at the Ca^{2+} -binding site which facilitated the interaction with Ca^{2+} . Under these conditions the toxin may then have developed an increased capacity to act as a Ca^{2+} ionophore. This concept is supported by the results presented in Fig. 3, where verapamil was observed to enhance greatly the rate of δ -haemolysin-stimulated $^{45}\text{Ca}^{2+}$ efflux from preloaded islets. Since verapamil was observed to facilitate the ionophoretic properties of δ -haemolysin in the artificial system, an enhanced rate of efflux from preloaded islets in response to both agents suggests that δ -haemolysin may also have altered islet-cell Ca^{2+} handling by virtue of its ionophoretic properties.

Verapamil has been reported to decrease glucose-stimulated $^{45}\text{Ca}^{2+}$ efflux from isolated islets (Malaisse *et al.*, 1978), and it was suggested that this inhibition occurred indirectly, being the consequence of a decreased rate of $^{40}\text{Ca}^{2+}$ entry into the cells. A slower rate of $^{40}\text{Ca}^{2+}$ entry would have led to a less rapid process of $^{40}\text{Ca}^{2+}$ - $^{45}\text{Ca}^{2+}$ exchange, and hence to inhibition $^{45}\text{Ca}^{2+}$ efflux.

If δ -haemolysin acts as a Ca^{2+} ionophore, the simultaneous presence of verapamil and δ -haemolysin could have induced a more rapid rate of $^{40}\text{Ca}^{2+}$ entry into the islet cells, which, in turn, may have caused an increase in the extent of $^{40}\text{Ca}^{2+}$ - $^{45}\text{Ca}^{2+}$ exchange. This would then have resulted in the observed rise in $^{45}\text{Ca}^{2+}$ efflux. Alternatively, the enhanced ionophoretic properties of δ -haemolysin in combination with verapamil could have directly facilitated the increase in $^{45}\text{Ca}^{2+}$ efflux rate. However, this is less likely to account for the total increase, since the electrochemical gradient would favour Ca^{2+} influx. A combination of these two effects might, perhaps, have contributed to the observed increase in $^{45}\text{Ca}^{2+}$ efflux rate.

The results presented in Table 7 demonstrate that phosphatidylcholine (25 $\mu\text{g}/\text{ml}$) was a potent inhibitor of δ -haemolysin-induced insulin secretion. This is in accord with the effect of this phospholipid on the haemolytic activity of the toxin (Kapral, 1972). In addition, the ability of δ -haemolysin to translocate $^{45}\text{Ca}^{2+}$ in the artificial system was also inhibited by phosphatidylcholine. The decreased rate of insulin release in the presence of phosphatidylcholine could reflect a decrease in the extent of islet-cell Ca^{2+} accumulation, as evidenced by the decreased capacity of δ -haemolysin to translocate $^{45}\text{Ca}^{2+}$ in the artificial system under these conditions. In this respect it is noteworthy that the extent of

inhibition of $^{45}\text{Ca}^{2+}$ translocation in the artificial system was almost identical with the extent of inhibition of insulin release in isolated islets of Langerhans, when the same concentrations of δ -haemolysin and phosphatidylcholine were employed.

Since the ionophoretic properties of δ -haemolysin in the artificial system were observed to correlate well with changes in Ca^{2+} handling and insulin secretion in islet cells, and since a rise in cytosolic Ca^{2+} concentration is believed to be a critical factor in β -cell activation (Malaisse *et al.*, 1978), the expression of such properties in islet cells could have accounted, at least in part, for the ability of δ -haemolysin to act as an insulin secretagogue.

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