The Interaction of Various Inhibitors and Stimuli of Insulin Release Studied with Rabbit Pancreas *in vitro*

By R. D. G. MILNER* AND C. N. HALES

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge

(Received 10 January 1969)

The effect of various inhibitors on insulin release from pieces of rabbit pancreas incubated *in vitro* was studied. Insulin release was stimulated by glucose (3 mg./ml.), leucine (5 mM), tolbutamide (200 µg./ml.), ouabain (10 µM), a raised extracellular K⁺ concentration (60 mM) and substitution of the Ca²⁺ content of the incubation medium by Ba²⁺ (2.5 mM). (a) Mannoheptulose (6 mg./ml.) inhibited glucose-stimulated insulin release only. (b) Anoxia abolished or inhibited insulin release stimulated by glucose, leucine, tolbutamide and K⁺, but had little or no effect on release stimulated by ouabain or Ba²⁺. (c) 2,4-Dinitrophenol (0.25 mM) abolished or inhibited insulin release stimulated by glucose, ouabain or Ba²⁺. (d) Diazoxide (250 µg./ml.) abolished or inhibited insulin release stimulated by glucose, leucine, tolbutamide, ouabain or Ba²⁺ (0.25 or 1 mM). Diazoxide had no effect on insulin release stimulated by Ba²⁺ (2.5 mM) and potentiated release stimulated by K⁺. (e) Adrenaline (1 µM) abolished insulin release stimulated by glucose, leucine, tolbutamide, ouabain or Ba²⁺. K⁺-stimulated release was inhibited by adrenaline. (f) Tetrodotoxin (1 µM) had no effect on insulin release stimulated by glucose, leucine, tolbutamide, ouabain, K⁺ or Ba²⁺. (g) Nupercaine (1 mM) abolished insulin release stimulated by glucose or Ba²⁺.

Our studies of the effects of ions on the release of insulin by pieces of rabbit pancreas incubated *in vitro* (Milner & Hales, 1967; Hales & Milner, 1968a,b) have led us to suggest that changes in ionic flux across cell membranes are a fundamental part of the mechanism by which a wide variety of stimuli of insulin release produce their effect. Glucose-stimulated insulin release is inhibited by mannoheptulose, adrenaline, anoxia, 2,4-dinitrophenol (Coore & Randle, 1964b) and diazoxide (Seltzer & Allen, 1965; Howell & Taylor, 1966). Tolbutamide-stimulated insulin release is inhibited by adrenaline and anoxia (Coore, Hellman, Idahl & Taljedal, 1967). To investigate the mechanism of action of various inhibitors we have studied their effects on different stimuli of insulin release from pieces of rabbit pancreas incubated *in vitro*.

MATERIALS AND METHODS

Animals. Rabbits (800–2000 g.) were used, one for each experiment. The animals, which were not of pure stock, were reared in the Laboratory animal house on a diet of Coney rabbit pellets daily with green stuff once a week. There was free access to food and water at all times.

* Present address: Medical Research Council Tropical Metabolism Research Unit, University of the West Indies, Mona, Kingston 7, Jamaica, W.I.

Procedure. After the rabbit had been killed by a sharp blow on the back of the head, the tail and body of the pancreas was dissected out, trimmed of mesentery, fat and blood vessels and divided into pieces weighing approx. 30 mg. Each piece was initially incubated in 4 ml. of medium in a 25 ml. conical flask covered with a skirted rubber cap (Britton Malcolm Ltd., London S.E.11) at 37° with shaking at 60–80 cyc./min. No measurement of insulin release was made in the first incubation, which lasted 60 min. Except where otherwise specified all subsequent incubations lasted 30 min. After each incubation each piece of pancreas was transferred to a new flask containing 2 or 4 ml. of incubation medium. At the end of the experiment each piece of pancreas was blotted carefully and weighed to the nearest 0.5 mg. on a Mettler balance. Insulin released by the pancreas into incubation media of different compositions was measured by immunoassay (Hales & Randle, 1963) by reference to standard solutions of ox insulin, by using an antiserum that had been demonstrated to cross-react identically with ox and rabbit insulin (Coore & Randle, 1964b).

Chemicals. Bovine plasma albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, L-leucine from Koch–Light Laboratories Ltd., Colnbrook, Bucks., sodium tolbutamide from Hoechst Pharmaceuticals Ltd., Slough, Bucks., ouabain from Martindale Moore Ltd., Norwich, Nupercaine from Ciba Pharmaceutical Co., Summit, N.J., U.S.A., and 2,4-dinitrophenol from British Drug Houses Ltd., Poole, Dorset. Tetrodotoxin, from Sankyo Ltd. (batch RTE-3-3,523093B), was kindly given by Dr P. F. Baker. Rabbit

anti-(guinea-pig serum) and ox insulin (21.6 i.u./g.) were kindly given by Dr B. A. L. Hurn of The Wellcome Research Laboratories, Beckenham, Kent. ^{125}I -labelled insulin was supplied by The Radiochemical Centre, Amersham, Bucks. Other chemicals were obtained from British Drug Houses Ltd. and were of the highest purity available.

Incubation medium. The medium used in all incubations was a bicarbonate-buffered salt solution (Krebs, 1950) of the following final composition (m-equiv./l.): Na^+ , 136.1; K^+ , 5.9; Ca^{2+} , 5.1; Mg^{2+} , 2.4; phosphate, 2.2 (one P is taken as 1.8 equiv.); SO_4^{2-} , 2.4; HCO_3^- , 24.9; Cl^- , 104.8; pyruvate, 4.9; glutamate, 4.9; fumarate, 5.4; supplemented with 0.6 mg. of glucose/ml. and 1 mg. of bovine albumin/ml. Modifications of the medium are indicated where appropriate in the text.

Media containing glucose (3 mg./ml.), mannoheptulose (6 mg./ml.), leucine (5 mm), 2,4-dinitrophenol (0.25 mm), Nupercaine (1 mm) or ouabain (10 μM) were prepared by dissolving the solid in the medium. Medium containing 60 mm- K^+ was made by adding the appropriate amount of KCl to the normal medium. Medium containing 142 mm- K^+ was made by replacing the Na^+ salts in the normal medium with the appropriate K^+ salt. Medium containing tolbutamide (200 μg ./ml.) was prepared by diluting a therapeutic preparation of tolbutamide containing 200 mg./ml. in stock medium. In experiments to study the effect of Ba^{2+} on insulin release CaCl_2 and MgSO_4 were replaced by equivalent amounts of BaCl_2 and MgCl_2 respectively. Diazoxide was dissolved in approx. 0.1 M-NaOH at a concentration of 10 or 20 mg./ml. The appropriate volume of this solution was added to incubation medium to produce a final diazoxide concentration of 250 μg ./ml. An equal volume of the same approx. 0.1 M-NaOH was added to the control flasks in each experiment. A 100 μg . portion of tetrodotoxin was dissolved in water to make a 40 μM solution, which was stored at 4°. This was diluted in various media immediately before the experiment to give a final concentration of 1 μM .

Adrenaline was stored at 4° as a 1 mg./ml. solution in 0.1 M-L-ascorbic acid. Immediately before an experiment an aqueous solution (10 μg ./ml.) was prepared from the stock solution and diluted in various media to a final

concentration of 1 μM . All media except those for anoxic incubations were gassed with $\text{O}_2 + \text{CO}_2$ (95:5). Flasks were prepared for incubation of pancreas under anoxic conditions by blowing $\text{N}_2 + \text{CO}_2$ (95:5) vigorously over the surface of the incubation medium for 15 min. and then for a further 5 min. after the rubber caps had been fixed to the flasks.

Experimental design and analysis of results. In all experiments insulin release was expressed as ng. of insulin/g. of pancreas/min. In most experiments two groups of equal numbers of pieces of pancreas were incubated simultaneously under basal conditions. The test group was then incubated in the presence of stimulus and inhibitor whereas the control group was incubated in the presence of stimulus alone. The mean response to the stimulus by the control group was compared statistically with that of the test group by using Student's *t* test. If no significant increase in response occurred in the test group ($P > 0.05$) the inhibitor was judged to have abolished the effect of the stimulus. If a mean increase in response occurred in the test group that was significant ($0.05 > P$), but that was significantly less or greater than that occurring in the control group, the 'inhibitor' was said to have inhibited or augmented the effect of the stimulus. When the basal secretion of the test group was considerably lower than the basal secretion of the control group such that the absolute increase of secretion in the test group was decreased but the percentage increase of secretion was not, the difficulty of interpretation of the effect of the inhibitor has been indicated. In some experiments to study the effect of anoxia on different stimuli of insulin secretion, a group of pieces of pancreas was incubated first under test and then under control conditions. The two sets of observations were separated by a 30 min. incubation in oxygenated medium in which no measurement was made.

RESULTS

Effect of mannoheptulose (6 mg./ml.). The results of these experiments are given in Table 1. When mannoheptulose was present in the incubation

Table 1. *Effect of mannoheptulose (6 mg./ml.) on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ s.e.m. of the numbers of pieces in the control or the test group shown in parentheses.

	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus
Glucose (3 mg./ml.)	10.4 $\pm$ 3.4 (5)	44.0 $\pm$ 7.2	5.8 $\pm$ 2.5 (5)	21.0 $\pm$ 6.0*
Glucose (3 mg./ml.)	1.0 $\pm$ 0.2 (4)	14.7 $\pm$ 0.9	1.0 $\pm$ 0.2* (4)	0.8 $\pm$ 0.1*
Leucine (5 mm)	0.9 $\pm$ 0.2 (4)	11.5 $\pm$ 1.4	1.3 $\pm$ 0.4* (4)	9.4 $\pm$ 1.9*
Tolbutamide (200 μg ./ml.)	1.2 $\pm$ 0.2 (4)	8.8 $\pm$ 0.2	2.1 $\pm$ 0.9* (4)	8.1 $\pm$ 1.8*
Ouabain (10 μM)	1.4 $\pm$ 0.4 (4)	34.7 $\pm$ 11.1	1.6 $\pm$ 0.3* (4)	18.6 $\pm$ 2.6*
K^+ (60 mm)	10.0 $\pm$ 2.4 (4)	54.8 $\pm$ 3.8	14.8 $\pm$ 2.0* (4)	58.0 $\pm$ 6.6*
Ba^{2+} (2.5 mm)	13.9 $\pm$ 2.7 (4)	41.1 $\pm$ 6.2	10.1 $\pm$ 3.2* (4)	57.7 $\pm$ 6.8*

* Mannoheptulose (6 mg./ml.) was present.

Table 2. *Effect of anoxia on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ S.E.M. of the numbers of pieces of pancreas in the control or the test group shown in parentheses.

	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus + anoxia
Glucose (3 mg./ml.)	8.0 $\pm$ 0.4 (4)	46.1 $\pm$ 13.5	3.4 $\pm$ 1.5	5.9 $\pm$ 1.9
Glucose (3 mg./ml.)*	5.7 $\pm$ 1.9 (4)	112.1 $\pm$ 16.6	6.9 $\pm$ 1.9	16.8 $\pm$ 8.8
Leucine (5 mM)	5.4 $\pm$ 1.2 (4)	71.8 $\pm$ 10.0	8.8 $\pm$ 1.5	23.4 $\pm$ 1.8
Leucine (5 mM)*	4.2 $\pm$ 1.7 (4)	30.6 $\pm$ 5.5	4.0 $\pm$ 2.0	1.7 $\pm$ 0.4
Tolbutamide (200 μ g./ml.)	0.8 $\pm$ 0.2 (6)	12.9 $\pm$ 3.6	1.6 $\pm$ 0.3	1.8 $\pm$ 0.8
Tolbutamide (200 μ g./ml.)*	2.8 $\pm$ 1.1 (4)	22.5 $\pm$ 4.4	1.6 $\pm$ 0.3	9.8 $\pm$ 0.7
Ouabain (10 μ M)*	1.6 $\pm$ 0.5 (4)	61.5 $\pm$ 10.7	4.7 $\pm$ 2.2	9.9 $\pm$ 3.2
Ouabain (10 μ M)*	2.5 $\pm$ 0.6 (4)	40.4 $\pm$ 3.1	2.4 $\pm$ 0.5	28.5 $\pm$ 5.4
K ⁺ (60 mM)	6.6 $\pm$ 1.6 (4)	70.8 $\pm$ 17.9	14.1 $\pm$ 2.2	55.7 $\pm$ 13.4
K ⁺ (60 mM)*	2.9 $\pm$ 1.2 (4)	50.4 $\pm$ 17.8	4.3 $\pm$ 1.2	65.2 $\pm$ 11.5
Ba ²⁺ (2.5 mM)	4.6 $\pm$ 1.7 (4)	29.7 $\pm$ 4.7	7.4 $\pm$ 1.0	17.6 $\pm$ 2.9
Ba ²⁺ (2.5 mM)*	5.8 $\pm$ 1.9 (4)	71.6 $\pm$ 9.6	3.3 $\pm$ 0.8	30.5 $\pm$ 5.8

* Basal secretion was measured in glucose-free medium.

Table 3. *Effect of anoxia on insulin release in response to various stimuli*

Experimental details are given in the text. Results are expressed as means $\pm$ S.E.M. In each experiment one group of pieces of pancreas was used (numbers shown in parentheses). Each incubation in which insulin release measured lasted 15 min. and after incubation under anoxic conditions pancreas was incubated in normal medium for 30 min. before measurements were resumed.

	Insulin release (ng./g. of pancreas/min.)			
	N ₂ + CO ₂ (95:5)		O ₂ + CO ₂ (95:5)	
	Basal	+ Stimulus	Basal	+ Stimulus
Glucose (3 mg./ml.)	1.4 $\pm$ 0.1 (5)	1.4 $\pm$ 0.1	1.4 $\pm$ 0.1	10.5 $\pm$ 2.0
Leucine (5 mM)	1.3 $\pm$ 0.1 (5)	8.6 $\pm$ 1.0	1.4 $\pm$ 0.1	32.8 $\pm$ 1.7
Tolbutamide (200 μ g./ml.)	2.4 $\pm$ 0.6 (6)	1.0 $\pm$ 0.2	1.4 $\pm$ 0.3	4.4 $\pm$ 1.0
K ⁺ (60 mM)	1.5 $\pm$ 0.1 (5)	2.1 $\pm$ 0.4	1.7 $\pm$ 0.2	18.9 $\pm$ 5.4
Ba ²⁺ (2.5 mM)	2.3 $\pm$ 0.5 (6)	20.4 $\pm$ 5.7	1.2 $\pm$ 0.2	32.3 $\pm$ 9.2

medium containing 3 mg. of glucose/ml. only, the stimulation of insulin release was inhibited if the results were expressed as absolute increases but not if expressed as percentage increases over the basal release. If mannoheptulose was also present in the test basal incubation containing 0.6 mg. of glucose/ml. the subsequent stimulation by 3 mg. of glucose/ml. was abolished. In all experiments to study the effect of mannoheptulose on other stimuli, the inhibitor was present in both the basal and stimulated incubations. In these experiments no effect of mannoheptulose was found.

Effect of anoxia. Initially, basal and stimulated insulin release from the control group of pieces of pancreas incubated in oxygenated medium through-

out was compared with that from the test group of pieces incubated in oxygenated medium during the basal period but in the absence of oxygen during the stimulated period (Table 2). Anoxia abolished insulin release stimulated by glucose. Insulin release stimulated by leucine and tolbutamide was abolished or inhibited in the absence of oxygen. In the experiment in which tolbutamide was tested in the absence of glucose an inhibitory effect of anoxia was not apparent if the results were expressed as percentage increases over basal release. A variable effect of anoxia in inhibiting ouabain- or Ba²⁺-stimulated insulin release was found and under the conditions of these experiments anoxia had no effect on the release of insulin stimulated by K⁺.

Table 4. *Development of insulin release in response to various stimuli*

Experimental details are given in the text. Results are expressed as means $\pm$ s.e.m. Six pieces of pancreas were used in each experiment and after the preincubation period (60 min.) were subjected to two 30 min. incubations followed by three 10 min. incubations.

Period of incubation (min.) Stimulus	Insulin release (ng./g. of pancreas/min.)				
	Basal		+ Stimulus		
	61-90	91-120	121-130	131-140	141-150
Glucose (3 mg./ml.)	1.2 $\pm$ 0.2	1.0 $\pm$ 0.2	56.9 $\pm$ 8.0	51.1 $\pm$ 5.9	43.9 $\pm$ 6.0
Leucine (5 mM)	9.2 $\pm$ 1.2	13.9 $\pm$ 4.7	30.6 $\pm$ 5.7	31.0 $\pm$ 5.6	39.9 $\pm$ 6.6
Tolbutamide (200 μ g./ml.)	2.9 $\pm$ 0.4	4.2 $\pm$ 1.9	40.9 $\pm$ 10.8	21.7 $\pm$ 4.1	24.1 $\pm$ 5.5
Ouabain (10 μ M)	1.2 $\pm$ 0.2	0.6 $\pm$ 0.1	20.9 $\pm$ 2.5	53.1 $\pm$ 11.9	62.7 $\pm$ 17.9
K ⁺ (60 mM)	4.0 $\pm$ 1.0	5.7 $\pm$ 1.4	62.5 $\pm$ 14.7	37.4 $\pm$ 5.7	27.7 $\pm$ 5.4
Ba ²⁺ (2.5 mM)	4.1 $\pm$ 0.9	9.0 $\pm$ 2.0	34.0 $\pm$ 3.7	16.7 $\pm$ 3.5	19.2 $\pm$ 5.1

Table 5. *Effect of 0.25 mM-2,4-dinitrophenol on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ s.e.m. Six pieces of pancreas were used in the control or the test group in each experiment.

Stimulus	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus + dinitrophenol
Glucose (3 mg./ml.)	5.5 $\pm$ 0.9	64.9 $\pm$ 9.9	5.3 $\pm$ 0.4	4.9 $\pm$ 1.1
Ouabain (10 μ M)	2.6 $\pm$ 0.2	11.5 $\pm$ 2.5	3.5 $\pm$ 0.8	4.5 $\pm$ 0.3
Ba ²⁺ (2.5 mM)	3.5 $\pm$ 0.8	43.6 $\pm$ 3.0	4.3 $\pm$ 0.5	11.5 $\pm$ 2.0

Table 6. *Effect of diazoxide (250 μ g./ml.) on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ s.e.m. Five pieces of pancreas were used in the control or the test group in each experiment. An equal amount of NaOH soln. was added to both the control and test media containing the stimulus.

	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus + diazoxide
Glucose (3 mg./ml.)	3.4 $\pm$ 0.5	21.8 $\pm$ 3.3	1.5 $\pm$ 0.3	2.8 $\pm$ 0.5
Leucine (5 mM)	4.6 $\pm$ 0.7	43.8 $\pm$ 8.0	7.7 $\pm$ 1.5	6.3 $\pm$ 1.2
Tolbutamide (200 μ g./ml.)	3.4 $\pm$ 0.5	28.8 $\pm$ 2.9	4.7 $\pm$ 0.7	9.3 $\pm$ 0.8
Ouabain (10 μ M)	10.5 $\pm$ 2.6	36.7 $\pm$ 2.3	8.0 $\pm$ 1.9	8.4 $\pm$ 1.8
K ⁺ (60 mM)	1.5 $\pm$ 0.3	26.9 $\pm$ 3.6	2.2 $\pm$ 0.2	44.9 $\pm$ 5.8
Ba ²⁺ (2.5 mM)	4.2 $\pm$ 0.8	39.1 $\pm$ 8.3	6.8 $\pm$ 0.8	42.2 $\pm$ 8.1

To ensure complete anoxia from the beginning of the stimulated incubation period further experiments were performed in which one group of pieces of pancreas was subjected to basal and stimulated incubations first in the absence and then in the

presence of oxygen (Table 3). The results of these experiments confirmed the earlier findings (Table 2) on the effect of anoxia on glucose-, leucine-, tolbutamide- and Ba²⁺-stimulated insulin release.

In these experiments K⁺-stimulated insulin

Table 7. *Effect of diazoxide (250 μ g./ml.) on insulin release by different concentrations of Ba^{2+}*

Experimental details are given in the text. Results are expressed as means $\pm$ S.E.M. of the numbers of pieces of pancreas shown in parentheses.

	Insulin release (ng./g. of pancreas/min.) in successive 30 min. incubation periods				
	Basal	With Ba^{2+} $\pm$ diazoxide			
Ba^{2+} (2.5 mm)	7.2 $\pm$ 1.3 (6)	72.6 $\pm$ 12.0	35.0 $\pm$ 4.2	21.2 $\pm$ 2.6	
Ba^{2+} (2.5 mm) + diazoxide	7.4 $\pm$ 1.8 (6)	86.9 $\pm$ 12.7	39.1 $\pm$ 4.9	26.1 $\pm$ 3.7	
Ba^{2+} (1 mm)	15.4 $\pm$ 4.2 (5)	53.1 $\pm$ 8.0	23.2 $\pm$ 3.8	15.7 $\pm$ 0.9	13.9 $\pm$ 1.0
Ba^{2+} (1 mm) + diazoxide	16.1 $\pm$ 3.8 (5)	17.7 $\pm$ 4.1	26.8 $\pm$ 5.5	34.3 $\pm$ 9.0	26.9 $\pm$ 6.3
Ba^{2+} (0.25 mm)	4.4 $\pm$ 0.9 (6)	6.8 $\pm$ 1.1	5.3 $\pm$ 0.6	6.7 $\pm$ 1.2	9.2 $\pm$ 1.3
Ba^{2+} (0.25 mm) + diazoxide	5.2 $\pm$ 0.7 (6)	3.1 $\pm$ 0.8	1.9 $\pm$ 0.3	3.7 $\pm$ 0.7	5.1 $\pm$ 1.0

Table 8. *Effect of adrenaline (1 μ M) on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ S.E.M. Five pieces of pancreas were used in the control or test group in each experiment.

	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus + adrenaline
Glucose (3 mg./ml.)	3.4 $\pm$ 0.5	21.8 $\pm$ 3.3	3.3 $\pm$ 0.4	3.3 $\pm$ 0.6
Leucine (5 mm)	4.6 $\pm$ 0.7	43.8 $\pm$ 8.0	6.3 $\pm$ 1.3	7.4 $\pm$ 1.6
Tolbutamide (200 μ g./ml.)	3.4 $\pm$ 0.5	28.8 $\pm$ 2.9	4.3 $\pm$ 1.3	3.1 $\pm$ 0.9
Ouabain (10 μ M)	10.5 $\pm$ 2.6	36.7 $\pm$ 2.3	6.6 $\pm$ 1.2	5.7 $\pm$ 1.7
K^+ (60 mm)	1.5 $\pm$ 0.3	26.9 $\pm$ 3.6	2.0 $\pm$ 0.2	9.5 $\pm$ 0.8
K^+ (142 mm)	6.7 $\pm$ 0.7	50.4 $\pm$ 10.9	2.8 $\pm$ 0.6	16.1 $\pm$ 1.9
Ba^{2+} (2.5 mm)	4.2 $\pm$ 0.8	39.1 $\pm$ 8.3	5.0 $\pm$ 1.0	7.9 $\pm$ 0.7

release was inhibited in the absence of oxygen. The discrepancy between the two sets of observations on the effects of anoxia on K^+ -stimulated insulin release may be explained by the fact that the stimulatory effect of K^+ is greatest during the first 10 min. of exposure to the stimulus (Table 4) and that in the experiments described in Table 2 anoxia was not complete during this period.

Effect of dinitrophenol. The effect of 0.25 mm-dinitrophenol was tested against glucose-, ouabain- and Ba^{2+} -stimulated insulin release and the results of these experiments are given in Table 5. Dinitrophenol abolished insulin release stimulated by glucose and ouabain and inhibited release stimulated by Ba^{2+} .

Effect of diazoxide (250 μ g./ml.). The results of these experiments are given in Table 6. Diazoxide inhibited or abolished insulin release stimulated by glucose, leucine, tolbutamide and ouabain. K^+ -stimulated insulin release was augmented by diazoxide. Diazoxide had no effect on the stimulation of insulin release by 2.5 mm- Ba^{2+} .

The effect of diazoxide on Ba^{2+} -stimulated insulin release was studied further in experiments in which two groups of pieces of pancreas were incubated under basal conditions and then in

medium containing Ba^{2+} for three or four successive incubations. In each experiment a different concentration of Ba^{2+} was employed, but the concentration of diazoxide in the test group remained constant. The results of these experiments are given in Table 7. In this type of experiment also diazoxide had no effect on the stimulation of insulin release by 2.5 mm- Ba^{2+} . The stimulation by Ba^{2+} (1 mm) was inhibited in the first period of incubation. In the three subsequent periods there was no significant difference in Ba^{2+} -stimulated insulin release in the presence or absence of diazoxide. Ba^{2+} (0.25 mm) stimulated insulin release in the fourth period of incubation. No stimulation of insulin release was found in a similar experiment in which diazoxide was also present.

Effect of adrenaline (1 μ M). The results of these experiments are given in Table 8. Adrenaline (1 μ M) abolished insulin release stimulated by glucose, leucine, tolbutamide, ouabain or Ba^{2+} . K^+ -stimulated insulin release was inhibited by adrenaline. In the experiment in which K^+ (142 mm) was tested the inhibitory effect of adrenaline was not apparent if the results were expressed as percentage increases over the basal release.

Table 9. *Effect of tetrodotoxin (1 μ M) on insulin release in response to various stimuli*

Experimental details are given in the text. Two groups of pieces of pancreas were incubated simultaneously for 30 min. under basal conditions. The test group was then incubated for 30 min. in the presence of stimulus and inhibitor whereas the control group was incubated for 30 min. in the presence of stimulus alone. Results are expressed as means $\pm$ S.E.M. of the number of pieces of pancreas in the control or the test group shown in parentheses.

	Insulin release (ng./g. of pancreas/min.)			
	Control		Test	
	Basal	+ Stimulus	Basal	+ Stimulus + tetrodotoxin
Glucose (3 mg./ml.)	10.4 $\pm$ 3.4 (4)	44.0 $\pm$ 7.2	5.1 $\pm$ 1.4 (5)	47.8 $\pm$ 11.0
Leucine (5 mM)	1.5 $\pm$ 0.5 (5)	65.2 $\pm$ 7.5	5.0 $\pm$ 1.7 (5)	51.8 $\pm$ 7.3
Tolbutamide (200 μ g./ml.)	11.2 $\pm$ 4.3 (4)	42.4 $\pm$ 6.4	13.1 $\pm$ 1.6 (4)	73.2 $\pm$ 14.4
Oubain (10 μ M)	6.5 $\pm$ 1.5 (4)	64.2 $\pm$ 2.6	10.4 $\pm$ 2.1 (4)	55.4 $\pm$ 6.2
K ⁺ (60 mM)	7.2 $\pm$ 3.4 (4)	62.1 $\pm$ 8.4	14.4 $\pm$ 5.7 (4)	69.4 $\pm$ 14.7
Ba ²⁺ (2.5 mM)	9.4 $\pm$ 1.5 (4)	84.2 $\pm$ 5.0	9.5 $\pm$ 2.2 (4)	76.5 $\pm$ 9.2

Table 10. *Insulin release in response to glucose and Ba²⁺ in the presence of 1 mM-Nupercaine*

Experimental details are given in the text. Results are expressed as means $\pm$ S.E.M. Six pieces of pancreas were used in each experiment.

Stimulus	Insulin release in successive 30 min. incubation periods (ng./g. of pancreas/min.)			
	Basal	Basal + 1 mM-Nupercaine	Basal + 1 mM-Nupercaine	+ Stimulus + 1 mM-Nupercaine
Glucose (3 mg./ml.)	1.6 $\pm$ 0.1	2.2 $\pm$ 0.3	2.1 $\pm$ 0.2	2.0 $\pm$ 0.3
Ba ²⁺ (2.5 mM)	2.1 $\pm$ 0.2	2.5 $\pm$ 0.2	1.7 $\pm$ 0.2	2.0 $\pm$ 0.4

Effect of tetrodotoxin (1 μ M). The results of these experiments are given in Table 9. Tetrodotoxin had no effect on insulin release in response to any of the stimuli used.

Effect of Nupercaine (1 mM). Nupercaine had no effect on basal insulin release but abolished the stimulation of insulin release by glucose or Ba²⁺ (Table 10).

DISCUSSION

Previous work on the mechanism by which glucose stimulates insulin release is consistent with there being three stages in the process: (i) metabolism of glucose by the β -cell (see Grodsky & Forsham, 1966; Hellman, 1969) to produce a compound that (ii) signals a change in the transmembrane Na⁺ and Ca²⁺ flux leading to an increase in the membrane-bound or intracellular Ca²⁺ that (iii) activates insulin release from the cell (Hales & Milner, 1968*b*). Studies of the requirements for the stimulation of insulin release *in vitro* suggest that a wide variety of stimuli increase insulin release by a final common ionic pathway (Hales & Milner, 1968*a,b*). If this is so for all stimuli, it is likely that inhibitors will fall into three groups: those affecting the final common pathway will be non-specific and inhibitory for all

stimuli; those affecting the transport or metabolism of a stimulus will be specific for that stimulus; those inhibitors that affect a step in the sequence of events leading to stimulated insulin release may be common to a number of, but not all, stimuli. A study of the interaction of a number of stimuli and inhibitors of insulin release may thus further an understanding of the mode of action of both a stimulus or an inhibitor. For example, an inhibitor of the proposed first type must act relatively late in the sequence of events leading to insulin release. Conversely, any stimulus that is blocked only by this type of inhibitor must be acting later in the sequence of events leading to insulin release than a stimulus that is affected by a wider range of inhibitors. Although one may be confident about an experimental result that shows the effectiveness of a certain inhibitor, results that demonstrate failure of inhibition must be interpreted cautiously. A lack of inhibition could be due to the relative concentrations of stimulus and inhibitor or the time-course of stimulation or inhibition. The present results are discussed in the light of these concepts.

The specificity of mannoheptulose in inhibiting glucose stimulation only is in accord with the proposal that this inhibitor acts by specifically blocking glucose phosphorylation (Coore & Randle,

1964a). The failure of mannoheptulose to act on any other stimulus demonstrates also that extracellular glucose is not a requirement for the action of the other stimuli studied. It is unlikely that our failure to observe a glucose requirement for these stimuli is due to the synthesis of glycolytic intermediates from the pyruvate, fumarate and glutamate in the incubation medium since stimulation of insulin release has been observed in the absence of these compounds (R. D. G. Milner & C. N. Hales, unpublished work).

Anoxia and dinitrophenol increase the rate of glycolysis and alter the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate in muscle (see Randle & Morgan, 1962). Inhibition of glucose-stimulated insulin release by these conditions might therefore be related to a specific effect on glucose metabolism. The findings that anoxia or dinitrophenol inhibited ionic stimuli as well as glucose indicate that these inhibitors operate at a late stage in the release mechanism. They may act by causing a fall in cellular ATP concentration, which may be required for Ca^{2+} uptake or insulin release. It has been suggested that the Ca^{2+} -dependent release of catecholamine granules from adrenal chromaffin cells also requires ATP (Poisner & Trifaró, 1967).

Using a lower concentration of diazoxide, Howell & Taylor (1966) found no inhibition of tolbutamide stimulation of insulin release. However, the interaction of tolbutamide and diazoxide has been shown to be dose-dependent both *in vitro* and *in vivo* (Loubatieres, Mariani, Alric & Chapal, 1967) and the diazoxide concentration chosen for the present study was inhibitory for all stimuli except K^+ . The concentration-dependence of the diazoxide inhibition of Ba^{2+} stimulation suggests that the drug may interfere with Ba^{2+} or Ca^{2+} movements across the cell membrane. Diazoxide decreases both the K^+ - and the Ca^{2+} -dependent components of the Na^+ efflux from squid axons (P. F. Baker, personal communication) and inhibits competitively the action of Ca^{2+} or Ba^{2+} to induce contraction of vascular smooth muscle (Wohl, Hausler & Roth, 1967, 1968). High Ca^{2+} concentrations, or prolonged exposure of pancreas to Ba^{2+} , cause a decrease in stimulated insulin release (Hales & Milner, 1968b). The potentiation of K^+ -induced insulin release by diazoxide could be explained by a degree of inhibition of Ca^{2+} uptake that permitted optimum insulin release.

Adrenaline was the most potent inhibitor of insulin release in our experiments, inhibiting the action of K^+ and abolishing other stimuli. This suggests a site of action late in the release mechanism. Adrenaline inhibition of smooth-muscle contraction has been related to an increased membrane permeability to K^+ (Jenkinson & Morton,

1967a,b). However, adrenaline inhibits the contraction of smooth muscle (Jenkinson & Morton, 1967b) and secretion of insulin (Table 8) stimulated by the total replacement of extracellular Na^+ by K^+ . It is therefore unlikely that the effects of adrenaline on either of these systems can be completely explained by an effect on K^+ permeability.

Tetrodotoxin was studied because this compound is known to block specifically the inward movement of Na^+ that accompanies depolarization of many types of neurons and muscle cells (see Kao, 1966). The failure to inhibit insulin release by tetrodotoxin provides evidence that none of the stimuli used was mediated by nerves but does not exclude their mediation by an inward movement of Na^+ . Abolition of glucose- or Ba^{2+} -stimulated insulin secretion by Nupercaine is consistent with an involvement of transmembrane ion flux in these processes.

We are grateful to Professor F. G. Young for his advice and encouragement and are indebted to Miss J. Adams for technical assistance and Mr J. Brittain for help with the animals. This work was supported by a grant from the British Diabetic Association. R. D. G. M. holds the Stanley Elmore Senior Research Fellowship, Sidney Sussex College, Cambridge.

REFERENCES

- Coore, H. G., Hellman, B., Idahl, L.-Å. & Taljedal, I.-B. (1967). *Opusc. Med.* **7**, 285.
- Coore, H. G. & Randle, P. J. (1964a). *Biochem. J.* **91**, 56.
- Coore, H. G. & Randle, P. J. (1964b). *Biochem. J.* **93**, 66.
- Grodsky, G. M. & Forsham, P. H. (1966). *Annu. Rev. Physiol.* **28**, 347.
- Hales, C. N. & Milner, R. D. G. (1968a). *J. Physiol.* **194**, 725.
- Hales, C. N. & Milner, R. D. G. (1968b). *J. Physiol.* **199**, 177.
- Hales, C. N. & Randle, P. J. (1963). *Biochem. J.* **88**, 137.
- Hellman, B. (1969). *Proc. 6th Congr. International Diabetes Federation* (in the Press).
- Howell, S. L. & Taylor, K. W. (1966). *Lancet*, *i*, 128.
- Jenkinson, D. H. & Morton, I. K. M. (1967a). *J. Physiol.* **188**, 373.
- Jenkinson, D. H. & Morton, I. K. M. (1967b). *J. Physiol.* **188**, 387.
- Kao, C. Y. (1966). *Pharmacol. Rev.* **18**, 997.
- Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
- Loubatieres, A., Mariani, M. M., Alric, R. & Chapal, J. (1967). *Excerpta med. int. Congr. ser. no. 149: Proc. Brook Lodge Symp.* p. 100.
- Milner, R. D. G. & Hales, C. N. (1967). *Diabetologia*, **3**, 47.
- Poisner, A. M. & Trifaró, J. M. (1967). *Molec. Pharmacol.* **3**, 561.
- Randle, P. J. & Morgan, H. E. (1962). *Vitam. & Horm.* **20**, 199.
- Seltzer, H. S. & Allen, E. W. (1965). *Diabetes*, **14**, 439.
- Wohl, A. J., Hausler, L. M. & Roth, F. E. (1967). *J. Pharmacol.* **158**, 531.
- Wohl, A. J., Hausler, L. M. & Roth, F. E. (1968). *Life Sci.* **7**, 381.