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CATIONS AND THE SECRETION OF INSULIN FROM RABBIT PANCREAS *IN VITRO*

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SUMMARY

1. Insulin secretion from pieces of rabbit pancreas incubated *in vitro* was studied in media of different ionic compositions.

2. Insulin secretion stimulated by glucose was abolished by removal of calcium from the incubation medium and inhibited by a twofold rise in the calcium concentration to 10·2 m-equiv/l. Removal of magnesium from the medium did not affect glucose-stimulated secretion but a tenfold rise in the extracellular magnesium concentration to 24 m-equiv/l. abolished secretion.

3. The replacement of calcium by an equivalent amount of barium (5·1 m-equiv/l.) stimulated insulin secretion. Barium stimulation declined with time and was inhibited by the presence of calcium, 5·1 m-equiv/l., or abolished by the presence of magnesium, 24 m-equiv/l., in the incubation medium.

4. The interactions of mono- and divalent cations on insulin secretion were studied by using ouabain or potassium as tools to raise intracellular sodium concentration and barium as a calcium analogue. Ouabain and potassium were effective stimuli only in the presence of calcium, and barium only stimulated insulin secretion in the presence of sodium.

5. The results of these experiments suggest that both calcium and sodium must act at the β cell membrane or enter the cell before insulin release can occur in response to a variety of stimuli.

INTRODUCTION

Extracellular cations play an important role in the release of storage products from a wide variety of 'secretory' cells. The release of acetylcholine at the synapse or neuro-muscular junction (Harvey & MacIntosh, 1940; del Castillo & Stark, 1952), catecholamines from the adrenal medulla or neurones (Douglas & Rubin, 1961; Kirkepar & Misu, 1967), vasopressin and oxytocin from the posterior pituitary (Douglas & Poisner,

1964; Dicker, 1966) and thyroid-stimulating hormone and luteinizing hormone from the anterior pituitary (Vale, Burgus & Guillemin, 1967; Samli & Geschwind, 1968) are all examples of calcium-dependent secretion.

Calcium has been shown to be necessary for the secretion of insulin *in vitro* (Grodsky & Bennett, 1966; Milner & Hales, 1967). Sodium and potassium also play a part in the release of insulin from the β cell (Hales & Milner, 1968). The experiments described in this paper were carried out to study the effects of calcium, magnesium and barium on insulin secretion and the possible interrelationship of these divalent cations with sodium in the genesis of insulin secretion. Preliminary reports of some of the findings have been published (Milner & Hales, 1967, 1968).

METHODS

Procedure. The preparation of pieces of rabbit pancreas and the method of studying insulin secretion from them in different incubation media have been described recently (Hales & Milner, 1968).

Incubation media. The medium for all experiments (subsequently called the normal medium) was a bicarbonate-buffered salt solution (Krebs, 1950) of the following final composition (m-equiv/l.): Na, 141·0; K, 5·9; Ca, 5·1; Mg, 2·4; Cl, 104·8; PO_4, 2·2 (one P is taken as 1·8 equivalent); SO_4, 2·4; HCO_3, 24·9; pyruvate, 4·9; glutamate, 4·9; and fumarate, 5·4; supplemented with 3·3 mM glucose and bovine albumin, 1 mg/ml., fraction V (Armour Pharmaceutical Co. Ltd., Hampden Park, Eastbourne, Sussex).

In some experiments calcium chloride or magnesium sulphate was omitted from the incubation medium (calcium or magnesium-free medium) and in others extra calcium chloride or magnesium sulphate was added to the medium. The calcium content of calcium-free media prepared in this way, measured with an atomic absorption flame photometer (Unicam S.P. 90), varied between 36 and 44 μ-equiv/l. In experiments to study the effect of barium on insulin secretion, calcium chloride and magnesium sulphate were replaced by equivalent amounts of barium chloride and magnesium chloride respectively. Other combinations of calcium, magnesium and barium in different media will be described in the text. In some experiments sodium in the incubation medium was completely replaced by potassium, choline or lithium as described previously (Hales & Milner, 1968).

Stimuli. The final concentrations in the incubation medium of the following substances which were used to stimulate insulin secretion were: *glucose* 16·5 mM; *potassium* 60 mM, by dissolving potassium chloride in normal medium; *ouabain* 10^{-5} M and 5×10^{-5} M (Martindale Samoore Ltd., Norwich).

Experimental design. Four or more pieces of pancreas, incubated one piece in a flask, were treated identically, in each experiment. In experiments in which secretion from two groups of pieces of pancreas was studied in different media at the same time, all the pieces were cut from one pancreas and were allocated to different media alternately. All experiments started with a 30 or 60 min incubation, which was found to be necessary to establish a steady rate of insulin secretion, but in which no measurement was made. Subsequent incubations lasted 30 min and at the end of each incubation the piece of pancreas was transferred to a new flask, and insulin which had been secreted into the medium was measured. On changing the ionic composition of the medium no measurement was made in the first incubation (rinsing incubation), which was to establish a new steady ionic and secretory state.

In most experiments the effect of changing the ionic composition of the incubation medium was studied by measuring basal and stimulated insulin secretion from a group of

pieces first in the altered medium and then in a normal medium, or in normal, altered and then normal medium again. In some experiments two groups of pieces were used and were changed from normal to altered medium reciprocally. In the first type of experiment secretion from the same piece of pancreas in different media was studied sequentially; in the second type secretion from comparable pieces of pancreas in different media was studied simultaneously.

Measurement of insulin, calculation and expression of results. These were performed as described recently (Hales & Milner, 1968).

RESULTS

Effect of calcium and magnesium on insulin secretion. The change in insulin secretion caused by raising the glucose concentration from 3·3 to 16·5 mM was used to study the effect of alterations in the calcium or magnesium content of the medium.

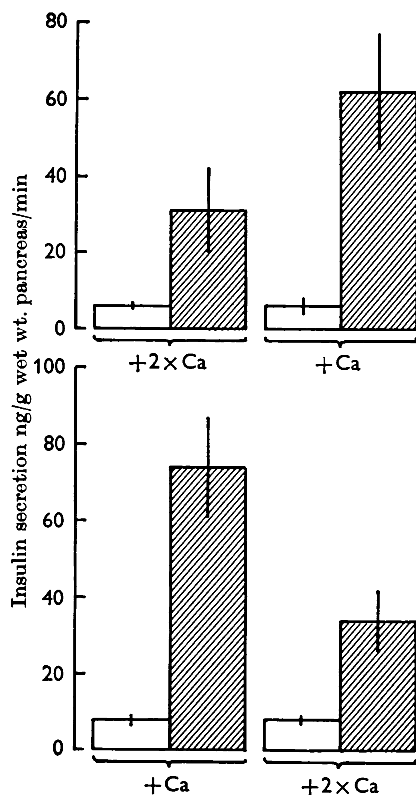

Fig. 1. Mean (±S.E. of mean) insulin secretion from two groups of four pieces from the same pancreas incubated in 3·3 □ or 16·5 mM ▨ glucose in normal medium (Ca) or a medium containing calcium at 10·2 m-equiv/l. (2×Ca). In this and all subsequent figures each incubation lasted 30 min. Spaces between adjacent histograms indicate incubations in which no measurement was made.

Glucose in a concentration of 16·5 mM failed to stimulate insulin secretion from pieces of pancreas incubated in calcium-free medium but did so equally in normal medium both before and after (Table 1). No difference was noted in the secretion of insulin in the presence of 3·3 mM glucose on omitting calcium chloride from the medium. The effect on insulin secretion of doubling the extracellular calcium concentration was studied by comparing secretion from two groups of pieces from the same pancreas. After an initial 60 min incubation, basal and glucose-stimulated insulin secretion was measured in normal medium or medium containing calcium, 10·2 m-equiv/l. After a 30 min rinsing incubation, the procedure was reversed (Fig. 1). The raised extracellular calcium concentration inhibited glucose-stimulated insulin reversibly.

TABLE 1. The effect of changes in the divalent cation content of the incubation medium on glucose-stimulated insulin secretion. Values indicate mean ± S.E. of mean insulin secretion in 3·3 (basal) or 16·5 mM glucose (stimulated). The first measurement of basal secretion was preceded by a 30 min incubation in which no measurement was made. The test and control periods were separated by 30 min incubations in which no measurements were made.

Change in ionic condition in test period	Number of observations	Insulin secretion (ng/g wet wt. pancreas/min)					
		Control		Test		Control	
		Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
−Ca	4	12·4 ± 1·4	99·0 ± 7·8	16·0 ± 4·4	9·7 ± 2·4	14·0 ± 1·4	92·0 ± 11·7
−Mg	10	8·7 ± 1·9	94·4 ± 11·5	5·1 ± 2·2	104·0 ± 24·0	3·8 ± 1·2	120·0 ± 14·7
+Mg, 24 m-equiv/l.	10	2·9 ± 0·5	24·8 ± 4·1	3·2 ± 0·6	2·5 ± 0·2	3·1 ± 0·5	17·0 ± 4·6

Omission of magnesium sulphate from an otherwise normally constituted incubation medium had no effect on the stimulation of insulin secretion by 16·5 mM glucose. If, however, the magnesium concentration was raised tenfold to 24 m-equiv/l. there was complete suppression of glucose-stimulated insulin secretion (Table 1). The possibility that inhibition of insulin secretion by omission of calcium or increasing the magnesium concentration of the incubation could have been due to an increased rate of destruction of insulin was tested. Insulin recoveries in different ionic media varied previously between 71 and 107 % (Hales & Milner, 1968). The mean insulin recovery (± S.E. of mean), similarly determined in calcium-free medium, was 89·6 ± 3·5 % ($n = 6$) and, in medium containing magnesium, 24 m-equiv/l., 81·5 ± 5·2 % ($n = 6$). The experiments shown in Table 1 also demonstrate the reproducibility of this method for studying insulin release *in vitro* and the viability of pieces of pancreas for 270 min, which is the longest period that insulin secretion has been studied under these conditions.

Effect of barium on insulin secretion. Two groups of pieces of pancreas

were incubated in normal medium for 60 min and then one group which served as a control was incubated for four 30 min periods in normal medium while the other group was incubated in a medium in which calcium chloride had been replaced by an equivalent amount of barium chloride (barium medium) (Fig. 2). Insulin secretion was stimulated by exposure to barium, maximally in the first 30 min and progressively less thereafter.

An attempt to stimulate insulin secretion twice with barium was next made. After measurement of basal secretion in normal medium, a group of pieces of pancreas was incubated in barium medium for 30 min. After a

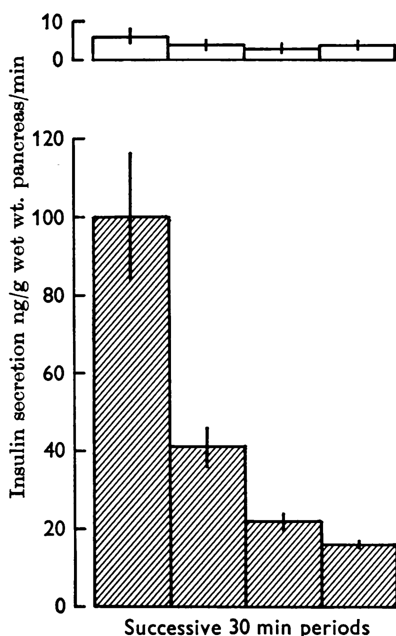

Fig. 2. Mean (± S.E. of mean) insulin secretion from two groups of six pieces from the same pancreas incubated in normal medium □ or in medium in which calcium had been replaced by an equivalent amount of barium ▨. Each piece had been incubated for 60 min in normal medium before measurements commenced.

30 min rinsing incubation in normal medium, the procedure was repeated (Table 2). The second basal secretion was as high as the first barium-stimulated secretion and although there was a rise in the mean insulin secretory rate on exposure to barium a second time this was very variable and not significant. It was concluded that barium stimulated insulin secretion, but that it showed tachyphylaxis, was not repeatedly effective, and differed from stimuli such as glucose, glucagon, leucine and tolbutamide which were repeatedly effective (Milner & Hales, 1967).

Since a rise in the extracellular concentration of calcium or magnesium

inhibited glucose-stimulated insulin secretion, the effect of similar changes in the concentrations of these ions on barium-stimulated secretion was studied. The medium for the initial and rinsing incubations and the measurement of basal secretion in these experiments contained neither calcium nor barium. After the measurement of basal secretion,

TABLE 2. The effect of ionic changes in the incubation medium on barium-stimulated insulin secretion. Values indicate mean ± S.E. of mean insulin secretion in normal medium (basal) or medium in which calcium has been replaced by an equivalent amount of barium (stimulated). Incubations were preceded by a 60 min incubation and the test and control incubations were separated by a 30 min incubation in which no measurements were made

		Insulin secretion (ng/g wet wt. pancreas/min)			
		Test		Control	
Change in ionic condition in test period	Number of observations	Basal	Stimulated	Basal	Stimulated
None	6	17·5 ± 5·3	43·6 ± 13·6	50·4 ± 15·1	80·0 ± 36·2
+ Ca, 5·1 m-equiv/l.	6	11·0 ± 2·0	26·7 ± 5·3	14·5 ± 3·0	44·1 ± 3·0
+ Mg, 24·0 m-equiv/l.	5	7·8 ± 2·7	14·4 ± 4·3	9·8 ± 2·9	50·8 ± 9·7
− Na	6	11·8 ± 3·5	10·8 ± 1·5	5·6 ± 0·9	100·4 ± 11·0

secretion was measured in medium containing both barium and calcium, 5·1 m-equiv/l. After a rinsing incubation the procedure was repeated except that in the final incubation the medium contained barium alone, 5·1 m-equiv/l. (Table 2). The effect of calcium on barium stimulation resembled that seen in glucose-stimulated secretion when the calcium concentration was doubled (Fig. 1). The effect of a tenfold rise in the extracellular magnesium concentration was studied similarly (Table 2). This abolished barium-stimulated secretion as it had glucose-stimulated secretion (Table 1). It was also noted that when pancreas was exposed to barium in the presence of calcium or a high concentration of magnesium, subsequent measurements of basal and barium-stimulated secretion were normal.

Interaction of monovalent and divalent cations on insulin secretion. Extracellular sodium has been shown to be necessary for a wide variety of stimuli of insulin secretion to be effective (Hales & Milner, 1968). Many of these stimuli also depend on the presence of extracellular calcium (Milner & Hales, 1967). Ouabain and potassium are thought to stimulate insulin secretion by causing a rise in the intracellular concentration of sodium. If barium is considered as a calcium analogue, use can be made of these stimuli to investigate indirectly a possible interdependence of sodium and calcium in the stimulation of insulin secretion.

Insulin secretion was measured first in a medium in which all sodium had been replaced by potassium (for details see Hales & Milner, 1968) and then in medium with a nomal sodium content. In each case basal secretion was measured in the presence of calcium followed by a measurement of

secretion stimulated by barium. Barium was an effective stimulus only in the presence of extracellular sodium (Table 2). Similar results were obtained in experiments where sodium was replaced by choline or lithium. In an analogous experiment, insulin secretion was measured first in a calcium-free medium and then in normal medium, using 60 mM potassium as the stimulus in each case (Fig. 3). Potassium only stimulated insulin secretion in the presence of extracellular calcium.

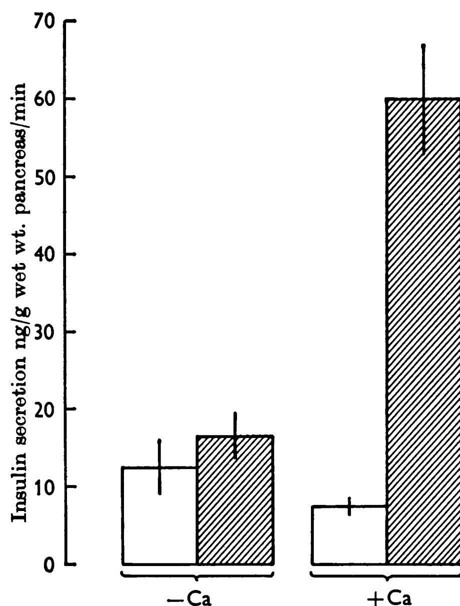

Fig. 3. Mean (±S.E. of mean) insulin secretion from five pieces of pancreas incubated in medium containing potassium at 5·9 m-equiv/l. □ or potassium at 60 m-equiv/l. ▨. In the first two periods the medium contained no calcium and in the second two 5·1 m-equiv/l.

It was not possible to study the dependence of ouabain on calcium in the same way, for, when basal secretion in normal medium was measured on pieces of pancreas which had been earlier exposed to ouabain in calcium-free medium, it was very high (Fig. 4). Therefore an experiment was performed in which two groups of pieces of pancreas were studied simultaneously. The effect of ouabain in a normal medium was studied on one group and in a calcium-free medium on the other (Fig. 5). Ouabain, like potassium, was only effective in the presence of extracellular calcium.

These experiments were thought to provide indirect evidence for an interdependent role of sodium and calcium at the β cell in the secretion of insulin.

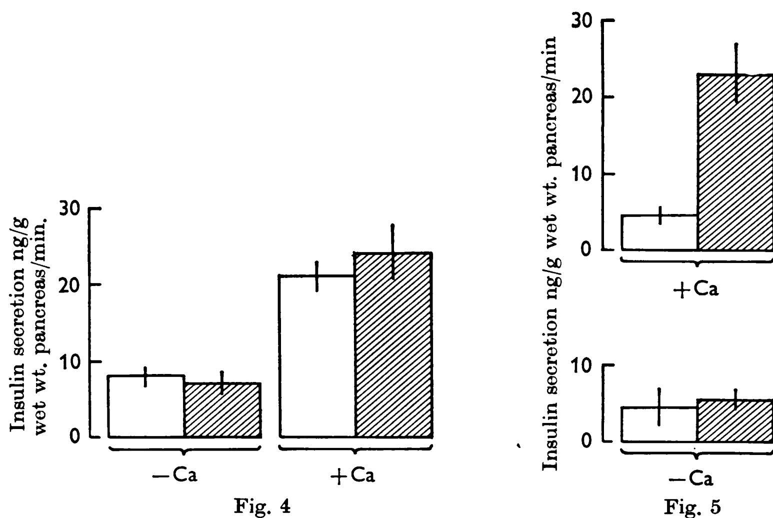

Fig. 4. Mean (± S.E. of mean) insulin secretion from five pieces of pancreas incubated in medium containing 10^{-5} M ouabain ▨ or no ouabain □. The medium contained no calcium in the first two periods and 5·1 m-equiv/l. in the second two.

Fig. 5. Mean (± S.E. of mean) insulin secretion from two groups of five pieces from one pancreas in medium containing 5×10^{-5} M ouabain ▨ or no ouabain □. One group was incubated in normal medium (+Ca) and the other in calcium-free medium (−Ca).

DISCUSSION

The use of pieces of rabbit pancreas for the study of the effects of ions on insulin secretion *in vitro* has been validated (Hales & Milner, 1968). The inhibitory effects on insulin secretion of a calcium-free medium or a medium with a high magnesium content in the present experiments was shown not to be due to increased insulin degradation. Conversely the stimulatory effect of barium on insulin secretion could not be the result of decreased insulin destruction which could result in a maximum apparent increase in secretion of 30%, for barium stimulation was many times greater than this.

Among the wide variety of secretory cells that are calcium-dependent, catecholamine release from the adrenal medulla has been extensively investigated (see Banks, 1967; Douglas, Kanno & Sampson, 1967; Poisner & Trifáro, 1967). Calcium, magnesium and barium have many similar effects on granule release from the chromaffin cell and the β cell. In both, stimulated secretion can only occur in the presence of extracellular calcium, a rise in the extracellular magnesium concentration inhibits secretion, the replacement of calcium by barium causes a stimulation of secretion, and barium stimulation can be antagonized by calcium or magnesium

(Douglas & Rubin, 1961; Douglas & Rubin, 1964*a*, *b*; Douglas *et al.* 1967). The stimuli of catecholamine release, acetylcholine and a high extracellular potassium concentration, cause cell membrane depolarization and calcium influx (Douglas & Poisner, 1962; Douglas *et al.* 1967). Nothing is known, however, of the ionic fluxes associated with a wide variety of stimuli of insulin secretion (glucose, glucagon, leucine, tolbutamide, ouabain and potassium) which are known to be calcium-dependent (Milner & Hales, 1967), although it has been suggested that ouabain and potassium may act by causing a rise in the intracellular sodium concentration (Hales & Milner, 1968). A difference between the chromaffin cell and the β cell is that stimulated secretion from the adrenal medulla rises with the external calcium concentration up to 17·6 mM (Douglas & Rubin, 1961) whereas glucose-stimulated insulin secretion is less with a calcium concentration of 10·2 m-equiv/l. than of 5·1 m-equiv/l. (Fig. 1), and previous work suggests that the optimal calcium concentration for insulin secretion is in the region of 2·5 mM (Milner & Hales, 1967). The optimal extracellular calcium concentration for secretion may depend as importantly on experimental conditions as on the cell studied. An optimal calcium concentration of 4·4 mM existed for the release of vasopressin from neurohypophyses incubated *in vitro* (Douglas & Poisner, 1964), whereas insulin secretion studied with a perfused rat pancreas preparation increased with the extracellular calcium concentration between 0 and 11 m-equiv/l. (Curry, Bennett & Grodsky, 1968).

Douglas & Rubin (1964*b*) have suggested that barium stimulates secretion by displacing membrane-bound calcium with a resulting increase in membrane permeability to calcium or barium. The results of the present experiments are partly in accord with this hypothesis. If barium acts intracellularly as a calcium analogue, its stimulatory action could be due to more rapid influx into or slower efflux of the ion from the cell. The high basal secretion 31–60 min after removal from barium medium suggests that barium is slow to leave the cell. The tachyphylaxis seen on prolonged exposure to barium could be the consequence of intracellular or cell membrane barium accumulation. On the other hand, the normal basal secretion which occurs after the exposure of pancreas to barium in the presence of magnesium, 24 m-equiv/l., suggests a reduction of barium influx by magnesium. It is possible that barium stimulates secretion by acting as a calcium analogue, entering the cell in the same way but being removed from the cytoplasm more slowly.

The demonstration that barium stimulates insulin secretion only in the presence of extracellular sodium and that ouabain and potassium are only effective in the presence of extracellular calcium suggests that sodium and calcium have an interrelated role in the genesis of insulin secretion. This

view is supported by the observation that either ion is necessary for a variety of stimuli to be effective *in vitro*. It contrasts with the finding that catecholamine release can be stimulated in the absence of extracellular sodium (Douglas & Rubin, 1963). It is possible that the lack of dependence of catecholamine secretion on sodium may reflect more a point of experimental technique than the ionic fluxes physiologically associated with secretion. Banks (1967) has demonstrated that catecholamine secretion from the adrenal medulla can be stimulated by ouabain and that this stimulation is calcium-dependent although the fall in intracellular potassium accompanying it is not. Secretion *in vivo* is probably accompanied by an influx of sodium and calcium ions (Douglas *et al.* 1967).

Our working hypothesis for the sequence of ionic events following glucose, glucagon, leucine, tolbutamide, ouabain and potassium stimulation of the β cell is that these substances cause an increase in the intracellular sodium concentration. This could then cause an increase in the intracellular calcium concentration, possibly by a mechanism similar to that described in squid axons by Baker & Blaustein (1968). A rise in the intracellular concentration of calcium provides the signal for the release of insulin from β cell granules.

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