J. Physiol. (1976), **254**, *pp.* 639–656
With 6 text-figures
Printed in Great Britain

EFFECTS OF GLUCOSE ON $^{45}Ca^{2+}$ UPTAKE BY PANCREATIC ISLETS AS STUDIED WITH THE LANTHANUM METHOD

BY B. HELLMAN, J. SEHLIN AND I.-B. TÄLJEDAL

From the Department of Histology, University of Umeå, S-901 87 Umeå 6, Sweden

(*Received 21 February 1975*)

SUMMARY

1. Fluxes of $^{45}Ca^{2+}$ were studied in pancreatic islets from non-inbred *ob/ob*-mice. Because La^{3+} blocked the transmembrane fluxes of $^{45}Ca^{2+}$ in islet cells, incubations aimed at measuring glucose-induced changes of the intracellular Ca^{2+} were ended by washing the islets with 2 mM-La^{3+} for 60 min.

2. Uptake of $^{45}Ca^{2+}$ progressed for 2 hr; the intracellular concentration of exchangable Ca^{2+} was about 7 m-mole/kg dry wt., as estimated from the isotope distribution at apparent equilibrium in islets exposed to 3 mM D-glucose. Raising the D-glucose concentration to 20 mM enhanced the $^{45}Ca^{2+}$ uptake whether or not the islets had first been equilibrated with the isotope. The stimulatory effect of D-glucose was observed in Tris buffer containing no anions but Cl^- as well as in polyanionic bicarbonate buffer. The effect could not be reproduced with equimolar L-glucose.

3. The rate of $^{45}Ca^{2+}$ release was the same whether the islets had been pre-loaded in the presence of 3 or 20 mM D-glucose. Thus the $^{45}Ca^{2+}$ that had been taken up in response to 20 mM D-glucose appeared to be released much more slowly than the bulk of intracellular $^{45}Ca^{2+}$. The release of $^{45}Ca^{2+}$ was not significantly influenced by D-glucose during the release period. Incubation for 30 min was required for half of the radioactivity to be released.

4. The rates of insulin secretion were about the same in uni-anionic Tris buffer as in polyanionic bicarbonate buffer. A marked insulin secretory response to 20 mM D-glucose was observed in either buffer.

5. It is concluded that 20 mM D-glucose causes a net uptake of Ca^{2+} from the extracellular fluid into the interior of the β-cells. This uptake is probably not regulated at the level of the plasma membrane but more likely reflects an increased affinity of some intracellular phase or compartment for the ion. Because the observed uptake and release of intracellular $^{45}Ca^{2+}$ are slow processes in comparison with the rapid effects of extracellular Ca^{2+}

on insulin secretion, insulin secretion may also depend on a more superficial and La^{3+}-displacable Ca^{2+} pool.

INTRODUCTION

Glucose-stimulated insulin release is Ca^{2+}-dependent, perhaps because Ca^{2+} couples the process of stimulus recognition to that of insulin discharge (Douglas, 1968; Milner & Hales, 1970; Matthews, 1970; Malaisse, 1973; Malaisse & Pipeleers, 1974). Although several studies have indicated that glucose alters the state of Ca^{2+} in the pancreatic β-cells, the nature of the changes and the mechanisms by which they occur are poorly understood (see Täljedal, 1976, for a review). Electrophysiological experiments suggested that glucose increases Ca^{2+} influx into the β-cells (Dean & Matthews, 1970; Meissner & Schmelz, 1974). When mannitol was used as a marker of the extracellular space in isolated islets, it was observed that glucose enhanced the rate of $^{45}Ca^{2+}$ uptake by the islet cells (Hellman, Sehlin & Täljedal, 1971*a*). Studies on the release of $^{45}Ca^{2+}$ from preloaded islets indicated that glucose may inhibit Ca^{2+} exit (Malaisse, Brisson & Baird, 1973).

Because Ca^{2+} has a marked tendency to bind to various organic molecules, it is difficult to know whether the reported kinetics of $^{45}Ca^{2+}$ uptake and release reflect the transporting characteristics of the islet cell plasma membranes or the affinity of Ca^{2+} for the extracellular and intracellular phases. A method that might facilitate the analytical problem was presented by Breemen, Farinas, Gerba & McNaughton (1972), who used La^{3+} to displace the extracellular Ca^{2+} and to block transmembrane fluxes in $^{45}Ca^{2+}$ loaded smooth muscle. Chandler & Williams (1974) employed this method in experiments with mouse pancreatic fragments. We have applied it to microdissected pancreatic islets of non-inbred *ob/ob*-mice, in order to study whether glucose induces a net uptake of Ca^{2+} by the β-cells, and whether such an uptake is regulated at the level of the β-cell plasma membrane.

METHODS

Adult non-inbred *ob/ob*-mice of the Umeå colony were starved overnight. Fresh pancreatic islets were isolated by free-hand microdissection (Hellerström, 1964) with the pancreas immersed in basal medium at 2° C.

The basal medium used in microdissection and subsequent incubations was a salt-balanced buffer supplemented with 3 mM D-glucose. In some cases the buffer was a bicarbonate buffer equilibrated with O_2–CO_2 (95:5) and with the following composition, in mM: Na^+, 139·0; K^+, 5·9; Ca^{2+}, 2·6; Mg^{2+}, 1·2; Cl^-, 124·9; HCO_3^-, 24·0; SO_4^{2-}, 1·2 and $H_2PO_4^-$, 1·2. In most experiments, however, the buffer was equilibrated with ambient air and contained, in mM: 2-amino-2-hydroxymethylpropane-1,3-diol

(Tris), 5·0; Na^+, 139·0; K^+, 4·7; Ca^{2+}, 2·6; Mg^{2+}, 1·2; Cl^-, 151·2; as well as Cl^- added in the form of HCl to give a final pH of 7·4.

In studies of Ca^{2+} fluxes, batches of six islets were incubated at 37° C in 200 μl. basal medium kept in closed vials that were shaken with a frequency of 140 strokes/min and an amplitude of 2·5 cm. The division of experiments into periods of incubation in non-radioactive and $^{45}Ca^{2+}$-labelled media as well as the addition of test substances to the media are described in the legends to figs. and tables; $^{45}Ca^{2+}$ was added in trace amounts giving a specific radioactivity of about 7·8 c/mole. In a few experiments islets were loaded with $^{45}Ca^{2+}$ in the presence of 10 μM (6,6′-^{3}H)-sucrose (1450 c/mole), which was used as a marker of the extracellular space (Hellman, Sehlin & Täljedal, 1971*b*). Experiments were usually ended by 60 min of incubation in non-radioactive Tris-buffered basal medium to which had been added 2 mM-$LaCl_3$. This was done to wash out extracellular and superficially bound $^{45}Ca^{2+}$ without altering the intracellular concentration of the isotope; the rationale for this procedure is given in the text. In other cases the islet cell content of $^{45}Ca^{2+}$ was estimated by correcting for $^{45}Ca^{2+}$ occurring in the islet space occupied by sucrose. All islets were freeze-dried overnight (−40° C, 0·1 Pa) and weighed on a quartz-fibre balance. They were then dissolved in Hyamine and their content of $^{45}Ca^{2+}$, and in some cases ^{3}H, determined by liquid-scintillation counting. Samples of labelled medium (5 μl.) were used as external standards in the counting procedure. The radioactivity in the islets was expressed in terms of m-mole Ca^{2+} with the same specific labelling as in the medium. This way of expressing the data was used to correct for the unavoidable random variation in the labelling of media within a series of experiments; such a correction would not be obtained if the data were only expressed in terms of c.p.m. or d.p.m. To avoid misunderstanding, it should be emphasized that an uptake of labelled Ca^{2+}, as the term is used here, does not necessarily imply a net uptake of Ca^{2+} unless the islets are in isotopic equilibrium.

To measure insulin release, batches of two islets were incubated at 37° C in 300 μl. basal medium supplemented with serum albumin, 1 mg/ml., and D-glucose as required. At the end of the incubation, samples of medium were radio-immuno logically assayed for insulin with crystalline mouse insulin as standard. Islets were freeze-dried and weighed as above.

Radio-isotopes were obtained from The Radiochemical Centre, Amersham, Bucks.; $LaCl_3$ from British Drug Houses Ltd, Poole, Dorset; bovine serum albumin (fraction V) from Sigma Chemical Co., St Louis, Mo., U.S.A.; ^{125}I-insulin from Farbwerke Hoechst A.G., Frankfurt/Main, Germany, and crystalline mouse insulin from Novo A/S, Copenhagen, Denmark. All other conventional reagents were of analytical grade.

RESULTS

Effects of La^{3+} on the islet uptake of $^{45}Ca^{2+}$ and sucrose

Chandler & Williams (1974) showed that 10 mM-La^{3+} caused a marked reduction of the sucrose (extracellular) space in pancreatic fragments. The same observation was made in the present study of microdissected islets. The volume distribution of sucrose in control islets incubated for 2 min with 10 μM [6,6′-^{3}H]sucrose in Tris buffer was 3·57 ± 0·37 l./kg dry wt., while the sucrose space in islets incubated with 2 mM-La^{3+} was 2·70 ± 0·28 l./kg dry wt. (mean value ± S.E. of mean of four experiments). This difference was also seen after 30 and 60 min of incubation. Since sucrose does

not readily penetrate the islet cells (Hellman *et al.* 1971*b*), it is assumed that this effect is virtually confined to the extracellular space.

In the absence of La^{3+}, the uptake of $^{45}Ca^{2+}$ in excess of the sucrose space progressed quickly during the first 2 min and then continued at a lower rate for at least 1 hr (Fig. 1). La^{3+}, 2 mM, completely suppressed the uptake of $^{45}Ca^{2+}$ in excess of the sucrose space. These results are in agreement with previous studies on rabbit aorta (Breemen *et al.* 1972) and mouse exocrine pancreas (Chandler & Williams, 1974), and indicate that 2 mM-La^{3+} prevents $^{45}Ca^{2+}$ from binding to or entering the islet β-cells.

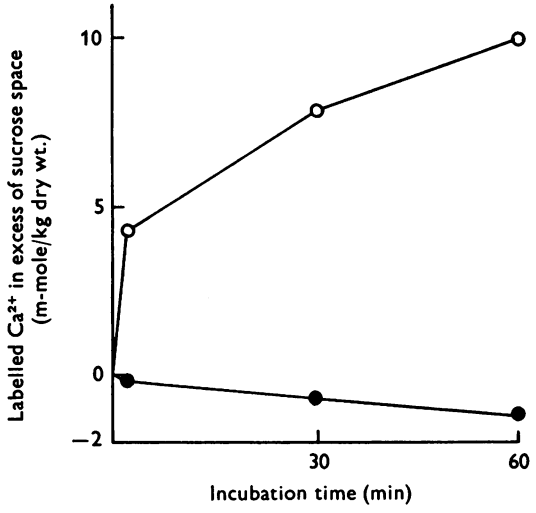

Fig. 1. Effect of La^{3+} on the islet uptake of $^{45}Ca^{2+}$. The points denote uptake of $^{45}Ca^{2+}$ in excess of the sucrose space in islets incubated for different periods of time with (●) or without (○) 2 mM-La^{3+}. Mean values of three or four experiments.

Effects of La^{3+} on the release of $^{45}Ca^{2+}$ from pre-loaded islets

To study the release of $^{45}Ca^{2+}$, islets were first incubated with the isotope for 90 min. The retention of $^{45}Ca^{2+}$ during subsequent incubations in non-radioactive media is shown in Fig. 2. When the non-radioactive medium contained no La^{3+}, the islets released $^{45}Ca^{2+}$ for at least 90 min; the rate was much greater during the first few min than at the end of the observation period. The rapid initial loss of radioactivity presumably reflected a wash-out of extracellular $^{45}Ca^{2+}$, while transmembrane flux and desorption from cell surfaces predominated toward the end of the incubation. The presence of 2 mM-La^{3+} did not greatly alter the initial release of $^{45}Ca^{2+}$, but the release was clearly inhibited at each time point studied. The inhibitory action of La^{3+} made the islet radioactivity

approach a stable plateau after about 60 min, indicating a block of $^{45}Ca^{2+}$ exit from the β-cells. Similar results have been reported for $^{45}Ca^{2+}$ efflux from pieces of rabbit aorta (van Breemen *et al.* 1972) and mouse pancreas (Chandler & Williams, 1974).

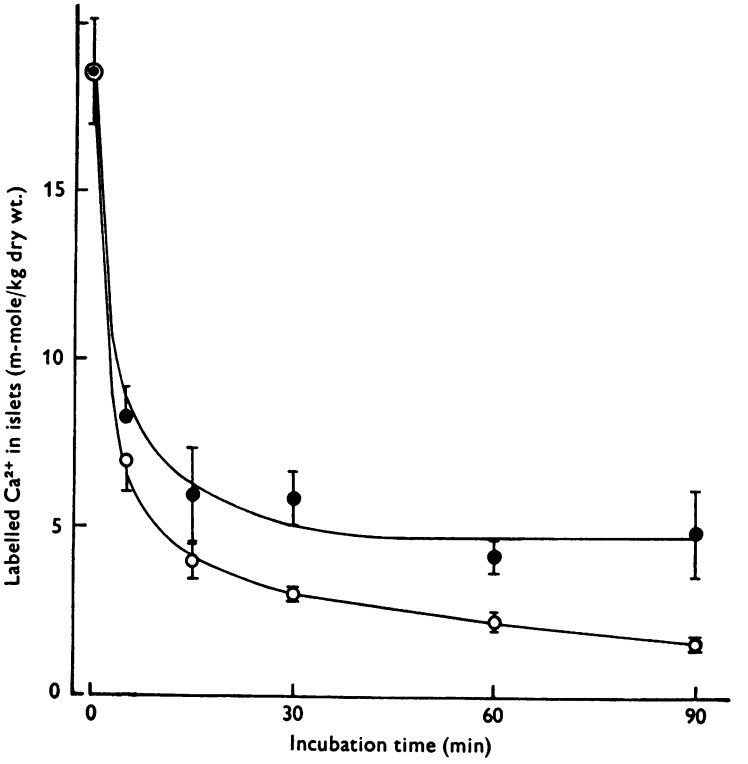

Fig. 2. Effect of La^{3+} on the release of $^{45}Ca^{2+}$ from pre-loaded islets. Tris buffer containing 3 mM D-glucose was used as basal medium. After loading with $^{45}Ca^{2+}$ for 90 min, islets were incubated for the indicated periods of time in non-radioactive medium containing (●) or lacking (○) 2 mM-La^{3+}. $^{45}Ca^{2+}$ retained by the islets is expressed in terms of m-mole Ca^{2+} with the same specific radioactivity as in the loading medium. Mean values ± S.E. of mean of four different experiments.

Since La^{3+} seemed to effectively inhibit both the uptake and release of $^{45}Ca^{2+}$ by the β-cells, La^{3+} was used to study the release of $^{45}Ca^{2+}$ from the interior of the islet cells. Fig. 3 shows the radioactivity of islets that had been pre-loaded with $^{45}Ca^{2+}$ as above, subsequently washed for various periods of time in La^{3+}-free, non-radioactive medium and finally incubated for 60 min in non-radioactive medium containing 2 mM-La^{3+}. The time curve for release of intracellular $^{45}Ca^{2+}$ seemed to asymptotically approach the time curve for release of $^{45}Ca^{2+}$ from islets not exposed to

La^{3+}: after 90 min of incubation in non-radioactive medium the islets contained about the same amount of $^{45}Ca^{2+}$ whether or not they were subsequently washed with La^{3+} (compare Figs. 2 and 3).

The difference between islets not exposed to La^{3+} and those finally washed with La^{3+} for 60 min are also plotted in Fig. 3; they show the

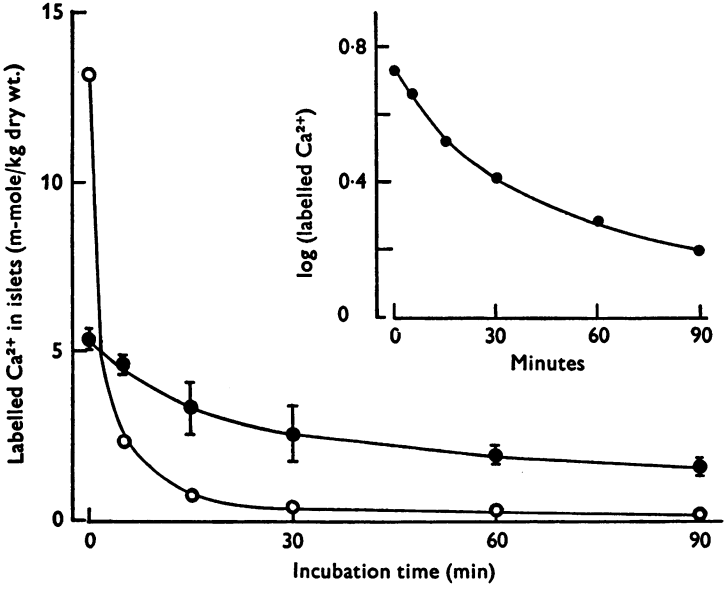

Fig. 3. Release of intracellular and extracellular $^{45}Ca^{2+}$ from islets with time. After loading with $^{45}Ca^{2+}$ as in Fig. 2, islets were incubated for the indicated periods of time in non-radioactive Tris buffer containing 3 mM D-glucose but lacking La^{3+}. They were then incubated for 60 min with 2 mM-La^{3+} in the Tris buffer; the radioactivity remaining in the islets probably represented intracellular $^{45}Ca^{2+}$. The intracellular content of $^{45}Ca^{2+}$ with time is given on linear scales in the main diagram (●) and as a semilogarithmic plot in the inset. The main diagram also shows the difference between total $^{45}Ca^{2+}$ in islets not exposed to La^{3+} (cf. Fig. 2) and those finally washed with La^{3+} to remove extracellular $^{45}Ca^{2+}$. The differential curve may be taken to estimate the content of extracellular $^{45}Ca^{2+}$ with time (○). Results are expressed in terms of m-mole Ca^{2+} with the same specific radioactivity as in the loading medium. Mean values of two to four experiments. Bars denote ± S.E. of mean.

amount of labelled Ca^{2+} that probably resided in the extracellular fluid or on cell surfaces after various periods of washing without La^{3+}. Although most of the intracellular $^{45}Ca^{2+}$ was removed by washing for 90 min in La^{3+}-free medium, the radioactivity remaining in the islets appeared to be located mainly within the islet cells.

The semilogarithmic plot in Fig. 3 shows that the release of $^{45}Ca^{2+}$ from intracellular (La^{3+}-resistant) stores did not follow first-order kinetics.

Thus the release of intracellular $^{45}Ca^{2+}$ to the extracellular space cannot be described in terms of simple diffusion or desorption from a mono-compartment store.

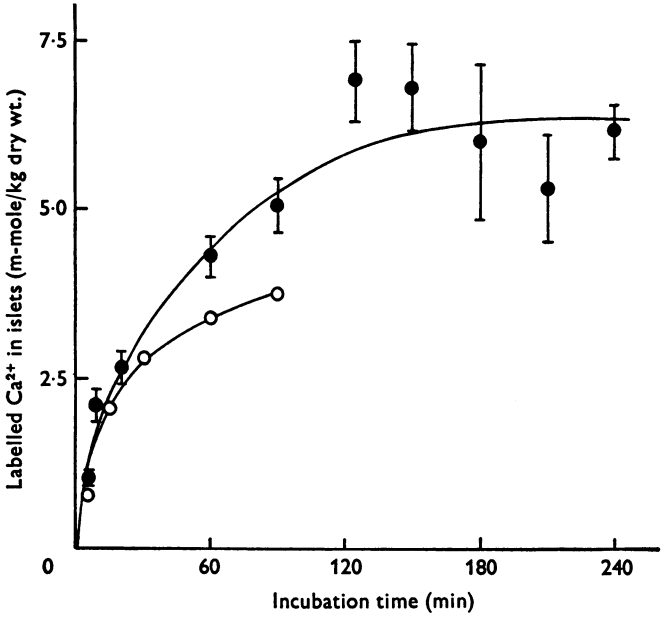

Fig. 4. Intracellular uptake of $^{45}Ca^{2+}$ with time. Tris buffer containing 3 mM D-glucose was used as basal medium. After preliminary incubation for 30 min in the basal medium only, islets were incubated for the indicated periods of time in basal medium labelled with $^{45}Ca^{2+}$. The diagram shows the radioactivity remaining in the islets after a final incubation for 60 min in non-radioactive buffer containing 2 mM-La^{3+} (●); mean values ± S.E. of mean of four to twenty-two experiments are given. To facilitate comparisons of the initial rates of $^{45}Ca^{2+}$ uptake with those of $^{45}Ca^{2+}$ release from pre-loaded islets, data from Fig. 3 have also been replotted to show the amounts of intracellular $^{45}Ca^{2+}$ released with time (○). Results are expressed in terms of m-mole Ca^{2+} with the same specific radioactivity as in medium.

The intracellular uptake of $^{45}Ca^{2+}$ with time

Islets were incubated with $^{45}Ca^{2+}$ for various periods of time followed by 60 min of washing with 2 mM-La^{3+}. The intracellular uptake of $^{45}Ca^{2+}$ is shown in Fig. 4. Although the uptake was clearly the fastest at the beginning of the incubation, it was slow enough to permit estimation of the initial uptake rate during the first 5–10 min. A comparison of the uptake curve with the time curve for release of intracellular $^{45}Ca^{2+}$ from pre-loaded islets shows that the initial rate of transmembrane $^{45}Ca^{2+}$ flux was about the same in the inward as in the outward direction (Fig. 4).

As the net flux of Ca^{2+} was zero, this symmetry suggests that loading with $^{45}Ca^{2+}$ for 90 min was sufficient to obtain a near-maximum labelling of the most labile cytoplasmic Ca^{2+} pool(s). This conclusion is reinforced by the uptake curve in Fig. 4 showing the isotope equilibrium to be nearly complete after 90–120 min. In view of the apparent isotopic equilibrium, the concentration of exchangable intracellular Ca^{2+} in the β-cells can be assumed to be about 6–7 m-mole/kg dry wt. of islets when the extracellular Ca^{2+} concentration is 2·6 mM.

Relationship between the extracellular and intracellular Ca^{2+} concentrations

Experiments were performed to investigate the relationship between the extracellular Ca^{2+} concentration and the concentration of readily exchangable Ca^{2+} within the islet cells. Since Fig. 4 suggested that isotopic equilibrium was obtained after 120 min of incubation with 2·6 mM-Ca^{2+}, islets were incubated for that length of time in media containing

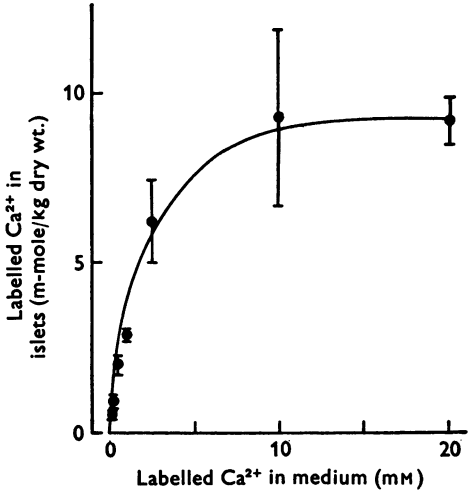

Fig. 5. Relationship between extracellular and intracellular concentrations of labelled Ca^{2+}. Experiments were performed as in Fig. 4 except that all islets were incubated with labelled Ca^{2+} for 120 min and that the concentration of Ca^{2+} in medium was varied as indicated. Mean values of three or four experiments.

various concentrations of labelled Ca^{2+}; they were finally washed with La^{3+} as before. Fig. 5 shows that the estimated intracellular Ca^{2+} concentration rose almost linearly with the extracellular Ca^{2+} concentration below 2·6 mM. At higher concentrations the curve levelled off, indicating that the readily exchangeable intracellular stores could accommodate, at the most, about 10 m-mole Ca^{2+}/kg dry wt. of islets.

Effects of glucose on the intracellular uptake of $^{45}Ca^{2+}$

Table 1 shows that the intracellular (La^{3+}-resistant) uptake of $^{45}Ca^{2+}$ was greater in the presence of 20 mM D-glucose than in the presence of 3 mM D-glucose. This effect of 20 mM D-glucose was statistically significant after, 5, 20 and 60 min of incubation with isotope and sugar. During the first 5 min, the stimulation amounted to as much as 50 % of the uptake recorded in the presence of 3 mM D-glucose.

TABLE 1. Effects of D-glucose on $^{45}Ca^{2+}$ uptake from Tris buffer

Incubation time (min)	Uptake of labelled Ca^{2+} (m-mole/kg dry islet)		
	3 mM D-glucose (*a*)	20 mM D-glucose (*b*)	(*b*) − (*a*)
	Intracellular (La^{3+}-resistant) uptake		
5	1·04 ± 0·11 (15)	1·57 ± 0·10 (15)	0·54 ± 0·11*** (15)
20	2·65 ± 0·24 (10)	3·88 ± 0·24 (10)	1·24 ± 0·29** (10)
60	4·18 ± 0·35 (7)	5·96 ± 0·50 (7)	1·78 ± 0·30** (7)
90	4·92 ± 0·69 (6)	7·15 ± 0·70 (6)	2·24 ± 1·04 (6)
120 + 5	6·88 ± 0·62 (8)	7·51 ± 0·44 (8)	0·63 ± 0·62 (8)
120 + 30	7·16 ± 0·57 (7)	8·49 ± 0·75 (7)	1·33 ± 0·38* (7)
	Uptake in excess of the sucrose space		
5	5·72 ± 1·17 (8)	6·91 ± 1·02 (8)	1·20 ± 0·78 (8)

The four top lines give data on islets that were incubated for 30 min in non-radioactive Tris buffer containing 3 mM D-glucose, subsequently incubated for 5, 20, 60, or 90 min in the same buffer labelled with $^{45}Ca^{2+}$ and containing 3 or 20 mM D-glucose, and finally washed for 60 min with 2 mM-La^{3+} in non-radioactive buffer. The next two lines give data on islets that were incubated for 120 min in $^{45}Ca^{2+}$-labelled Tris buffer containing 3 mM D-glucose, subsequently incubated for 5 or 30 min in the same type of labelled buffer containing 3 or 20 mM D-glucose, and finally washed for 60 min with La^{3+}. In the bottom line, islets were incubated for 30 min in non-radioactive Tris buffer containing 3 mM D-glucose, subsequently incubated for 5 min in Tris buffer labelled with $^{45}Ca^{2+}$ and containing 10 μM (6,6′-^{3}H)sucrose as well as 3 or 20 mM D-glucose; those islets were taken for radioactivity measurements without prior washing with La^{3+}. $^{45}Ca^{2+}$ remaining in islets after washing with La^{3+}, or in excess of the sucrose space in unwashed islets, is given as m-mole Ca^{2+} with the same specific radioactivity as in the medium. Mean values ± S.E. of the mean are shown for each group of islets as well as for the differences between parallel incubations with high and low glucose concentrations; the numbers of experiments are given in parentheses. Statistical significances by *t* test: * $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$.

In other experiments, islets were first loaded with $^{45}Ca^{2+}$ for 2 hr in the presence of 3 mM D-glucose and then exposed to 20 mM D-glucose. The stimulatory effect of 20 mM D-glucose was clearly discernible against the background of rather high radioactivity that had accumulated during

the preceding loading period (Table 1). Moreover, the absolute values of the D-glucose-induced uptake were very nearly the same whether the islets were exposed to 20 mM D-glucose and $^{45}Ca^{2+}$ simultaneously, or 20 mM D-glucose was included in the medium only after 2 hr of exposure to the radio-isotope. Under both experimental conditions the effect of 20 mM D-glucose was non-linear with time. Thus, D-glucose did not merely enhance the rate of $^{45}Ca^{2+}$ uptake by a compartment that would finally equilibrate with the isotope whatever the sugar concentration, but probably induced a net movement of Ca^{2+} from the medium to some intracellular compartment(s).

In an attempt to see whether D-glucose also stimulated the uptake of $^{45}Ca^{2+}$ by some superficial cell compartment, uptake of $^{45}Ca^{2+}$ in excess of the sucrose space was studied. In agreement with the results obtained with the La^{3+}-wash method, 20 mM D-glucose increased the mean value for early uptake of $^{45}Ca^{2+}$ in excess of the sucrose space, although the effect was not statistically significant. In view of the magnitude and random error of the observed increase, it probably reflected the same intracellular change as that demonstrable by the La^{3+}-wash method.

In all of the above experiments, the islets were incubated in Tris-buffered media devoid of anions other than Cl^-. Since insulin release is usually studied in bicarbonate buffers containing sulphate and phosphate, the islet uptake of $^{45}Ca^{2+}$ from such a buffer was also investigated; after incubation in bicarbonate buffer, the islets were washed with Tris-buffered La^{3+} as above. A comparison of Tables 1 and 2 shows that islets incubated in the bicarbonate buffer exhibited a considerably greater uptake of $^{45}Ca^{2+}$ than those incubated in the Tris buffer. However, the stimulatory effect of D-glucose was clearly reproducible in the bicarbonate buffer. Table 2 also shows that the stimulatory effect of 20 mM glucose was restricted to the D isomer; equimolar L-glucose had no effect.

Effects of D-glucose on the release of intracellular $^{45}Ca^{2+}$

Experiments were designed to study the release of that portion of the islet $^{45}Ca^{2+}$ uptake which had been stimulated by D-glucose. Islets were loaded with the isotope in the presence of 3 or 20 mM D-glucose, subsequently incubated in non-radioactive medium containing the low glucose concentration only, and finally washed with La^{3+}. From the difference between the release curves for islets loaded in the presence of high and low glucose concentrations, the release of the glucose-stimulated $^{45}Ca^{2+}$ load was estimated. Fig. 6 shows that the two release curves seemed to be parallel throughout a release period as long as 90 min. This result suggests that the glucose-enhanced load of $^{45}Ca^{2+}$ was more firmly associated with the cells than was the bulk of the intracellular $^{45}Ca^{2+}$.

TABLE 2. Effects of D-glucose and L-glucose on $^{45}Ca^{2+}$ uptake from bicarbonate buffer

Sugar tested	Intracellular uptake of labelled Ca^{2+} (m-mole/kg dry islet)	
	Primary data	Test minus control
Incubation for 20 min		
D-glucose, 3 mM (control)	6·08 ± 0·25	—
D-glucose, 20 mM	9·01 ± 0·50	2·93 ± 0·33***
D-glucose, 3 mM, plus L-glucose, 17 mM	5·89 ± 0·56	−0·19 ± 0·71
Incubation for 60 min		
D-glucose, 3 mM	13·21 ± 1·98	7·13 ± 2·02*

All islets were incubated for 30 min in bicarbonate buffer containing 3 mM D-glucose. Most of them were then incubated for 20 min in $^{45}Ca^{2+}$-labelled buffer containing 3 or 20 mM D-glucose or 3 mM D-glucose plus 17 mM L-glucose; one group of islets was incubated for 60 min in $^{45}Ca^{2+}$-labelled buffer containing 3 mM D-glucose. The islets were washed for 60 min with 2 mM-La^{3+} in Tris buffer. The radioactivity retained by the islets is given as m-mole Ca^{2+} with the same specific radioactivity as in the medium. Mean values ± S.E. of the mean are shown for each group of islets as well as for the differences between parallel test and control incubations; there were six different experiments in each case. Statistical significances by *t* test: * $P < 0·02$; *** $P < 0·001$.

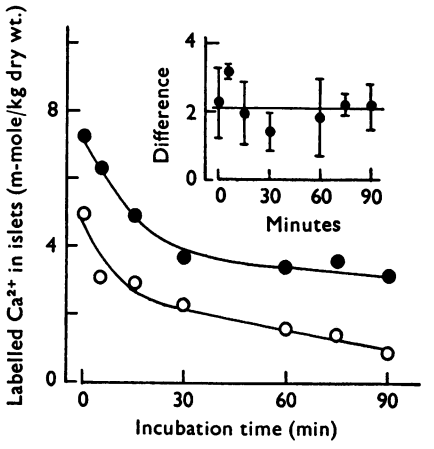

Fig. 6. Effect of glucose during pre-loading with $^{45}Ca^{2+}$ on the subsequent release of intracellular isotope. Islets were incubated for 90 min in Tris buffer labelled with $^{45}Ca^{2+}$ and containing 3 (○) or 20 (●) mM D-glucose; parallel incubations were performed with the two glucose concentrations. All islets were then incubated for the indicated periods of time in non-radioactive Tris buffer containing 3 mM D-glucose. The main diagram shows the radioactivity remaining in the islets after a final incubation for 60 min in buffer containing 2 mM-La^{3+}. The inset shows the differences between the two curves. Mean values of two to six experiments. For reasons of clarity, bars denoting ± S.E. of the mean are only shown in the inset. Results are expressed in terms of m-mole Ca^{2+} with the same specific radioactivity as in the loading medium.

In other experiments it was tested whether the rate of $^{45}Ca^{2+}$ release differed between islets exposed to 3 or 20 mM D-glucose during the release period. After the islets had been pre-loaded as in Fig. 6 and then incubated for 5 min in non-radioactive medium, they contained the following amounts of La^{3+}-resistant Ca^{2+} (m-mole labelled $^{45}Ca^{2+}$/kg dry wt.; mean values ± S.E. of mean): 4·14 ± 0·43 (7) after pre-loading and release in 3 mM D-glucose; 4·17 ± 0·41 (7) after pre-loading in 3 mM and release in 20 mM D-glucose; 5·56 ± 0·55 (10) after pre-loading in 20 mM and release in 3 mM D-glucose; 5·59 ± 0·34 (10) after pre-loading and release in 20 mM D-glucose. Similarly, no effect of D-glucose was observed during release periods of 15 or 45 min. Thus, there was no evidence for 20 mM D-glucose inhibiting the unidirectional efflux of Ca^{2+} from the β-cells.

Insulin release in uni-anionic Tris buffer and polyanionic bicarbonate buffer

Because islets incubated in the bicarbonate buffer exhibited a greater intracellular uptake of $^{45}Ca^{2+}$ than islets incubated in the Tris buffer (Tables 1 and 2), it was decided to measure insulin release in the two buffers. Table 3 shows that the secretory rates at 3 and 20 mM D-glucose were probably greater in the bicarbonate buffer than in the Tris buffer. However, this difference was small, and the secretory response induced by raising the D-glucose concentration from 3 to 20 mM was the same in the two buffers.

TABLE 3. Effect of D-glucose on insulin release in Tris buffer and bicarbonate buffer

Concentration of glucose (mM)	Insulin release (ng/μg dry islet)		
	Tris buffer (*a*)	Bicarbonate buffer (*b*)	Effect of buffer (*b*) − (*a*)
3	0·30 ± 0·07	0·59 ± 0·12	0·30 ± 0·12*
20	2·58 ± 0·35	3·07 ± 0·50	0·50 ± 0·20*
Effect of glucose	2·28 ± 0·40**	2·49 ± 0·44**	

Parallel incubations were performed in Tris and bicarbonate buffers containing 1 mg serum albumin/ml. After 30 min of preliminary incubation in the presence of 3 mM D-glucose, insulin release was measured during 60 min of incubation with 3 or 20 mM D-glucose in either buffer. Mean values ± S.E. of the mean are given for each group of islets as well as for the differences between buffers and concentrations of sugar; eight different experiments were performed. Statistical significances by *t* test: * $P < 0·05$; ** $P < 0·01$.

DISCUSSION

General

In previous studies of ^{45}Ca-labelled islets, ^{45}Ca in the extracellular space was compensated for by washing without La^{3+} (Malaisse-Lagae & Malaisse, 1971; Malaisse & Pipeleers, 1974) or by using radioactive mannitol as an extracellular space marker (Hellman *et al.* 1971*a*). Both techniques are attended by weaknesses with regard to the possibility of estimating the intracellular content of $^{45}Ca^{2+}$. The mannitol method does not compensate for $^{45}Ca^{2+}$ bound to the surface of cells and non-cellular tissue components; washing without La^{3+} permits a fraction of the intracellular $^{45}Ca^{2+}$ to escape before the islet radioactivity is measured. The idea of including La^{3+} in the washing buffer to displace extracellular $^{45}Ca^{2+}$ and to prevent the loss of intracellular $^{45}Ca^{2+}$ is due to van Breemen *et al.* (1972), who used the method in studies of stimulus–excitation coupling in smooth muscle. Chandler & Williams (1974) have shown that La^{3+} can be used successfully with fragments of mouse pancreas, both to displace extracellular Ca^{2+} and to inhibit Ca^{2+} movement out of cells. The results reported here document the usefulness of the La^{3+}-wash method in experiments with the endocrine pancreatic islets and allow new conclusions to be drawn about the effect of glucose on Ca^{2+} in the islet cells. Since the islets used in these studies contain more than 90% β-cells, the results are presumably representative of that cell type and may shed some light on the role of Ca^{2+} in insulin secretion.

With the mannitol technique we previously observed that D-glucose increased the initial rate of $^{45}Ca^{2+}$ uptake by pancreatic islets (Hellman *et al.* 1971*a*). The present results indicate that the effect of glucose concerns an uptake of $^{45}Ca^{2+}$ into the interior of the β-cells. Because the glucose-induced uptake of $^{45}Ca^{2+}$ in excess of the sucrose space was about the same as the La^{3+}-resistant uptake, it seems reasonable to assume that glucose had little, if any, effect on the binding of $^{45}Ca^{2+}$ to the β-cell surfaces; however, direct measurements of the surface-located Ca^{2+} pool were not performed. The results raise two important questions: what mechanism is responsible for the glucose-induced uptake of $^{45}Ca^{2+}$ and what relevance does this uptake have for insulin secretion?

The mechanism of glucose-induced $^{45}Ca^{2+}$ uptake

Although previous studies have indicated that D-glucose alters the state of Ca^{2+} in the islet cells, they have not conclusively shown that a net uptake occurs. The mannitol technique revealed an enhanced rate of isotope uptake in glucose-stimulated islets but did not detect any significant change of equilibrium (Hellman *et al.* 1971*a*). Washing the

islets without La^{3+}, Malaisse-Lagae & Malaisse (1971) observed that the retention of $^{45}Ca^{2+}$ depended on the D-glucose concentration to which the islets were exposed during pre-loading. Although those results are compatible with a glucose-induced net uptake of Ca^{2+}, they do not rule out that glucose only altered the intracellular distribition of Ca^{2+} resulting in a slower wash-out of the isotope (Hellman *et al.* 1971*a*; Malaisse & Pipeleers, 1974). D-glucose was also reported to rapidly inhibit the release of $^{45}Ca^{2+}$ from pre-loaded islets (Malaisse *et al.* 1973), but inferences concerning the net flux of Ca^{2+} cannot be drawn from knowledge of the unidirectional efflux of isotope alone. Although the present study, too, has only used a tracer method for studying fluxes of Ca^{2+}, the kinetics of $^{45}Ca^{2+}$ movements to and from the La^{3+}-resistant pool do indicate that D-glucose induces a net uptake of Ca^{2+} from the extracellular fluid to the β-cell interior. This conclusion is based on the following observations in glucose-stimulated islets: an enhanced influx, an unchanged efflux, and an increased content of the isotope under apparent equilibrium conditions.

Because Ca^{2+} binds to a great variety of molecules, fluxes of $^{45}Ca^{2+}$ do not allow safe conclusions to be drawn about the structural level at which the fluxes are regulated. Still, it seems important to analyse whether the present results can be explained by assuming that glucose exerts a direct or indirect influence on the transporting properties of the β-cell plasma membrane. According to a common theory of membrane function, a net uptake of unmetabolizable solutes may occur as the result of the plasma membrane being changed to facilitate influx or hamper efflux. The theory envisages the inward and outward fluxes as being mechanistically separated and treats the situation of zero net flux as a circular steady state. The paradigm is the interpretation of zero net flux of Na^+ as being due to the rate of passive influx equalling the rate of 'active' extrusion by the Na^+/K^+-pump. In keeping with this theory, one might perhaps postulate that the β-cells contain an outward Ca^{2+} pump whose inhibition by glucose could account for the present results. The assumption of such a Ca^{2+} pump seems to be implicit in the suggestion (Malaisse, 1973; Malaisse & Pipeleers, 1974) that glucose stimulates insulin release by increasing the cytosolic Ca^{2+} concentration through inhibition of Ca^{2+} efflux. However, in the present study we did not observe any effect of glucose on $^{45}Ca^{2+}$ efflux and therefore have no evidence for either an inhibited pump or an increased permeability of the plasma membrane. Moreover, a comparison of the efflux curves for islets pre-loaded in the presence of high and low glucose concentrations suggests that the glucose-stimulated islets had incorporated $^{45}Ca^{2+}$ into a compartment with a particularly high affinity for the ion. Such a result would not be expected if glucose only enhanced the total inflow of Ca^{2+} by

altering the transporting properties of the β-cell plasma membrane. It seems more probable that glucose, in effect, created a new Ca^{2+}-storing compartment in the β-cells. Such a change could involve an increase in the number of intracellular Ca^{2+}-binding sites, an increase in the affinity of pre-existing sites, a sequestering of Ca^{2+} within membranes and so forth. Histochemical studies at the ultrastructural level have shown that glucose promotes the deposition of Ca^{2+} near the insulin secretory granules and the plasma membranes of β-cells (Herman, Sato & Hales, 1973; Schäfer & Klöppel, 1974). This deposition could result from a glucose-induced increase in the reactivity with Ca^{2+} in the regions in question.

Glucose-stimulated Ca^{2+} uptake and insulin release

Our results are in agreement with the general hypothesis that Ca^{2+} plays a role in stimulus-secretion coupling (Douglas, 1968). The comparisons of uni-anionic Tris buffer with polyanionic bicarbonate buffer showed a qualitative correlation between $^{45}Ca^{2+}$ uptake and insulin secretion; note, however, that the stimulatory actions of glucose did not require the addition of anions other than Cl^-. In support of the idea that the glucose-induced Ca^{2+} uptake is a physiological phenomenon, L-glucose had no effect on the $^{45}Ca^{2+}$ uptake and does not stimulate insulin release. An increased incorporation of $^{45}Ca^{2+}$ into glucose-stimulated islets was also reported by Malaisse-Lagae & Malaisse (1971). Although those authors washed their islets without La^{3+}, and although Malaisse (1973) and Malaisse & Pipeleers (1974) stressed the interpretation that glucose primarily alters the transporting activity of the β-cell plasma membrane, it seems likely that we are here dealing with the same basic phenomenon. Malaisse-Lagae & Malaisse (1971) concluded that the increased $^{45}Ca^{2+}$ incorporation was not the result of stimulated secretion, since it could be diminished by mannoheptulose even when the glucose-stimulated insulin secretion was already blocked due to a low extracellular Ca^{2+} concentration. This finding suggests, but does not establish, that the insulin-releasing action of glucose is somehow mediated by the observed changes in the state of β-cell Ca^{2+}.

Like many forms of secretion, insulin release requires the presence of Ca^{2+} in the extracellular fluid. The question arises whether the inhibitory effect of withdrawing the extracellular Ca^{2+} is due to depletion of those intracellular Ca^{2+} stores which normally change their concentration in response to glucose. Such an interpretation has the attraction of not presupposing that Ca^{2+} plays more than one regulatory role in the secretory process. However, because the insulin-releasing action of some thiol reagents is less inhibitible by Ca^{2+} deficiency than is the action of glucose, we proposed that Ca^{2+} serves at least two distinct functions (Hellman,

Idahl, Lernmark, Sehlin & Täljedal, 1973*a*). In addition to being probably essential for some distal step in secretion, Ca^{2+} was envisaged to be involved in the recognition of some, but not all, insulin secretagogues. The present data on the time course of $^{45}Ca^{2+}$ uptake and release provide another argument for the same idea. The release of $^{45}Ca^{2+}$ from pre-loaded islets was so slow that the islets had to be incubated in non-radioactive medium for about 30 min before their radioactivity had fallen by 50%; such a long incubation did not seem to diminish the apparently glucose-sensitive Ca^{2+} store, i.e. the difference between islets pre-loaded in the presence of high and low glucose concentrations.

The sluggish behaviour of the $^{45}Ca^{2+}$ release is in striking contrast to the rapidity with which the glucose-stimulated insulin secretion is affected by changes in the extracellular Ca^{2+} concentration. When the rat pancreas was perfused with about 17 mM D-glucose in the absence of Ca^{2+}, the sudden inclusion of 2·5 mM Ca^{2+} in the perfusion medium caused a prompt secretory response that reached its maximum within a couple of min (Grodsky, 1972). Conversely, the renewed withdrawal of Ca^{2+} from the medium resulted in a quick drop of the secretory rate to basal values within 2 min (Grodsky, 1972). Studying the same type of microdissected islets as used here we observed that the withdrawal of Ca^{2+} from islets perfused with 17 mM D-glucose and 0·1 mM iodoacetamide caused a 50% drop of the secretory rate within about 7 min (Hellman, Idahl, Lernmark, Sehlin & Täljedal, 1973*b*). Against this background it seems unlikely that the inhibition of insulin release in Ca^{2+}-deficient medium is solely due to a decrease of the intracellular Ca^{2+} concentration. It is particularly difficult to envisage that the inhibition is due to depletion of that same Ca^{2+} store, whose content was found to be glucose-dependent in the present study. The rapidity with which the glucose-induced insulin release is inhibited on withdrawal of extracellular Ca^{2+} rather suggests that the secretory responsiveness is also dependent on a fairly labile Ca^{2+} pool that is perhaps located in the β-cell plasma membrane. A critical amount of Ca^{2+} in the membrane may be a condition for its proper function in secretion whether or not this Ca^{2+} is subject to regulation by insulin secretagogues. To study specifically the membrane-located cation stores would require techniques other than those employed in the present investigation. Dean & Matthews (1970), Matthews & Sakamoto (1975) and Meissner & Schmelz (1974) have shown that insulin-releasing agents induce fast oscillations of the β-cell membrane potential. Those authors have also adduced indirect evidence for the view that the 'action potentials' signify rapid transmembrane fluxes of Ca^{2+} of significance for secretion. It is difficult to evaluate the relationship between the 'action potentials' and the fluxes of $^{45}Ca^{2+}$ reported here. The number of ions

responsible for one 'action potential' is likely to be exceedingly small in comparison with the amount of Ca^{2+} occurring in the β-cells, and the ionic mechanisms underlying the oscillating behaviour of the membrane potential resulting in repetitive firing are so far obscure.

This work was supported by the Swedish Medical Research Council grant 12X-2288.

REFERENCES

BREEMEN, C. VAN, FARINAS, B. R., GERBA, P. & MCNAUGHTON, E. D. (1972). Excitation–contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. *Circulation Res.* **30**, 44–54.

CHANDLER, D. E. & WILLIAMS, J. A. (1974). Pancreatic acinar cells: effects of lanthanum ions on amylase release and calcium ion fluxes. *J. Physiol.* **243**, 831–846.

DEAN, P. M. & MATTHEWS, E. K. (1970). Electrical activity in pancreatic islet cells: effect of ions. *J. Physiol.* **210**, 265–275.

DOUGLAS, W. W. (1968). Stimulus–secretion coupling: the concept and clues from chromaffine and other cells. *Brit. J. Pharmac. Chemother.* **34**, 451–474.

GRODSKY, G. M. (1972). A threshold distribution hypothesis for packet storage of insulin. II. Effect of calcium. *Diabetes* **21**, suppl. 2, 584–593.

HELLERSTRÖM, C. (1964). A method for the microdissection of intact pancreatic islets of mammals. *Acta endocr., Copenh.* **45**, 122–132.

HELLMAN, B., IDAHL, L.-Å., LERNMARK, Å., SEHLIN, J. & TÄLJEDAL, I.-B. (1973*a*). Role of thiol groups in insulin release: studies with poorly permeating disulphides. *Molec. Pharmacol.* **9**, 792–801.

HELLMAN, B., IDAHL, L.-Å., LERNMARK, Å., SEHLIN, J. & TÄLJEDAL, I.-B. (1973*b*). Iodoacetamide-induced sensitization of the pancreatic β-cells to glucose stimulation. *Biochem. J.* **132**, 775–789.

HELLMAN, B., SEHLIN, J. & TÄLJEDAL, I.-B. (1971*a*). Calcium uptake by pancreatic β-cells as measured with the aid of ^{45}Ca and mannitol-^{3}H. *Am. J. Physiol.* **221**, 1795–1801.

HELLMAN, B., SEHLIN, J. & TÄLJEDAL, I.-B. (1971*b*). Transport of α-aminoisobutyric acid in mammalian pancreatic β-cells. *Diabetologia* **7**, 256–265.

HERMAN, L., SATO, T. & HALES, C. N. (1973). The electron microscopic localization of cations to pancreatic islets of Langerhans and their possible role in insulin secretion. *J. Ultrastruct. Res.* **42**, 298–311.

MALAISSE, W. J. (1973). Insulin secretion: multifactorial regulation for a single process of release. *Diabetologia* **9**, 167–173.

MALAISSE, W. J., BRISSON, G. R. & BAIRD, L. E. (1973). Stimulus-secretion coupling of glucose-induced insulin release. X. Effect of glucose on ^{45}Ca efflux from perifused islets. *Am. J. Physiol.* **224**, 389–394.

MALAISSE, W. J. & PIPELEERS, D. G. (1974). The role of cations in insulin synthesis and release. *8th Congr. Int. Diabetes Fed.*, pp. 95–103.

MALAISSE-LAGAE, F. & MALAISSE, W. J. (1971). Stimulus–secretion coupling of glucose-induced insulin release. III. Uptake of ^{45}calcium by isolated islets of Langerhans. *Endocrinology* **88**, 72–80.

MATTHEWS, E. K. (1970). Electrical activity in islet cells and insulin secretion. *Acta Diab. Latina* **7**, suppl. 1, 83–89.

MATTHEWS, E. K. & SAKAMOTO, Y. (1975). Electrical characteristics of pancreatic islet cells. *J. Physiol.* **246**, 421–437.

MEISSNER, H. P. & SCHMELZ, H. (1974). Membrane potential of beta-cells in pancreatic islets. *Pflügers Arch. ges. Physiol.* **351**, 195–206.

MILNER, R. D. G. & HALES, C. N. (1970). Ionic mechanisms in the regulation of insulin secretion. In *The Structure and Metabolism of the Pancreatic Islets*, ed. HELLMAN, B., FALKMER, S. & TÄLJEDAL, I.-B., pp. 489–493. Oxford: Pergamon Press.

SCHÄFER, H.-J. & KLÖPPEL, G. (1974). The significance of calcium in insulin secretion. *Virchows Arch. path. Anat. Physiol.* **362**, 231–245.

TÄLJEDAL, I.-B. (1976). Problems and issues in insulin secretion. In *Topics in Diabetes Mellitus*, ed. HOCKADAY, T. D. & ALBERTI, K. G. M. M. London: William Heinemann Medical Books (in the Press).