Regulation of insulin secretion from islets of Langerhans rendered permeable by electric discharge

M. Adel YASEEN, Kevin C. PEDLEY and Simon L. HOWELL

Department of Physiology, Queen Elizabeth College, University of London, Campden Hill Road,
London W8 7AH, U.K.

(Received 15 February 1982/Accepted 27 April 1982)

1. High-voltage electric discharge has been used to increase the permeability of B-cells of isolated islets of Langerhans to facilitate studies of the effects of normally impermeable substances on insulin secretion. 2. The application of an intense electric field increased the [^{14}C]sucrose space of the islets from $37.8 \pm 3.1\%$ to $86.2 \pm 5.2\%$ of their total volume as assessed by $^3\text{H}_2\text{O}$ content. The cells remained permeable for at least 40 min. 3. Ultrastructural studies showed no deleterious changes in the structure of the B-cells after discharge. 4. Insulin secretion from normal islets was unaffected by increasing the medium $[\text{Ca}^{2+}]$ from 10 nM to $10\text{ }\mu\text{M}$. In the islets that had been rendered permeable by discharge, insulin secretion was significantly increased under these conditions, without any alteration in the release of lactate dehydrogenase, a cytoplasmic marker enzyme. 5. Studies of the dynamics of insulin release during perfusion showed that the response to increased ($10\text{ }\mu\text{M}$) Ca^{2+} concentration was rapid and sustained over a period of at least 13 min. 6. Secretion responses to Ca^{2+} in perfusion established that maximum release in permeabilized islets occurs at approx. $1\text{ }\mu\text{M-Ca}^{2+}$ and half-maximum release occurs at approx. $0.6\text{ }\mu\text{M-Ca}^{2+}$. 7. The study of the effect of agents that interfere with the microtubular microfilamentous system in B-cells using a perfusion system revealed that cytochalasin B caused a considerable increase, whereas vinblastine sulphate caused a significant inhibition, in insulin release in response to $1\text{ }\mu\text{M-Ca}^{2+}$. 8. This technique should facilitate the study of the role of normally impermeable ions and metabolic intermediates in the regulation of insulin secretion.

One of the persistent problems in studying B-cell metabolism and the regulation of insulin secretion is the impermeability of the cells to certain ions and to metabolic intermediates that may be important in the regulation of the secretory process. This difficulty could be overcome if it were possible to render the cells permeable to such molecules without materially altering their structure or secretory characteristics. It has recently been suggested that the application of a high-voltage discharge across red cells (Riemann *et al.*, 1975), sea-urchin eggs (Baker *et al.*, 1980) or isolated adrenal medullary cells (Baker & Knight, 1978) will render them permeable to small molecules. Thus exposing the cells to intense electric fields results in the formation of pores by causing a localized breakdown of the plasma membrane (Kinosita & Tsong, 1977) but does not interfere with the integrity of subcellular organelles (Knight, 1981).

We have now applied this type of technique to isolated rat islets of Langerhans, which consist of groups of 4000–6000 cells, and have shown that

electric discharge will render the cells of the islets permeable, and that such cells will respond to increases in concentration of extracellular Ca^{2+} with a sustained insulin secretion via the microtubule and microfilamentous system. Preliminary reports of some of these findings have already appeared (Yaseen *et al.*, 1980, 1981) and use of an analogous system in islets of Langerhans was also reported by Pace *et al.* (1980).

Materials and methods

Apparatus

The apparatus for delivering the discharges consisted of a high-voltage power supply (model 4SP24; Hunting Hivolt, Shoreham-by-Sea, West Sussex, U.K.), two capacitors with total capacitance of $2\text{ }\mu\text{F}$ and a pulsation cell connected to the circuit as shown in Fig. 1. The pulsation cell comprised a plastic cuvette in which islets were suspended in 1.0 ml of pulsation medium between 1.0 cm -wide

Fig. 1. A circuit diagram of the high-voltage pulse former

Key: R1–R14, resistors; C1 and C2, capacitors; RL1, solenoid; SW1, mains switch; SW2, microswitch; D1, a diode; F1, fuse; PS1, 24 V power unit; PS2, 3.4 kV power unit; Ter. 1 (e.h.t. terminal) and Ter. 2, terminals for connecting the pulsation cell; Ter. 3–Ter. 5, monitoring terminals. All values for resistors and capacitors are in ohms and farads respectively.

stainless-steel electrodes placed in parallel and 1.0 cm apart. In these experiments islets were exposed to three to five discharges of 3.4 kV/cm and approx. 200 μ s duration (at 15 s intervals) and were resuspended between individual discharges. Preliminary experiments utilizing voltages in a range 0.6–3.6 kV/cm showed that 3.4 kV/cm elicited maximum increase in sucrose space with little damage to the cells.

Media

Islets were isolated in Krebs–Ringer bicarbonate buffer supplemented with 5 mM-glucose and 0.5 mg of bovine serum albumin/ml (Armour Pharmaceutical Co.). A pH of 7.4 was maintained by equilibration with O₂/CO₂ (19:1, v/v). The pulsation medium had the following composition (mM): Na⁺, 72; K⁺, 70; Mg²⁺, 1; Cl[−], 72; isethionate, 70; EGTA, 1; ATP, 1; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 16; glucose, 5; and bovine serum albumin (0.5 mg/ml). CaCl₂ was added and the pH was adjusted to 7.0 with NaOH. Free Ca²⁺ concentrations were calculated as described below.

The use of EGTA buffers and calculation of the concentration of Ca²⁺

Concentrations of Ca²⁺ and Mg²⁺ were controlled by the use of EGTA buffers (Portzehl *et al.*, 1964). EGTA (1 mM) was added to the pulsation medium followed by the addition of a calculated

amount of 1 M stock solution of CaCl₂. The pH was adjusted to 7.0 with NaOH.

Concentrations of free bivalent metal ions such as Mg²⁺ and Ca²⁺ in the presence of EGTA and ATP were computed using a ligand metal-binding programme of Feldman *et al.* (1972) kindly given to us by Dr. R. M. Denton, Department of Biochemistry, University of Bristol, Bristol, U.K., and was adapted for use with a Commodore PET 4032 micro-computer by Dr. I. C. H. Smith of this department.

Apparent dissociation constants at pH 7.0 used in these calculations are given in Table 1.

Isolation of islets

Islets were isolated from male Wistar rats weighing 200–250 g by collagenase digestion (Howell & Taylor, 1968). Collagenase was purchased from Sigma Chemical Co., Poole, Dorset, U.K., and Boehringer, Lewes, Sussex, U.K.

Determination of [¹⁴C]sucrose and ³H₂O spaces

Control islets, which were not subjected to the high-voltage discharge, were incubated for 30 min in 0.4 ml of pulsation medium, which contained 10 μ M-Ca²⁺ and a mixture of [U-¹⁴C]sucrose (25 μ Ci/ml; 0.93 MBq/ml) and ³H₂O (25 μ Ci/ml; 0.93 MBq/ml). Pulsated islets were transferred in 50 μ l of medium to 0.4 ml of the above radioactive mixture and incubated for 30 min.

Then both control and pulsated islets were transferred in groups of 20 in 50 μ l of medium to the

Table 1. *Apparent dissociation constants at pH 7.0*
The constants were calculated from reported stability constants and *pK* values (Martell & Sillén, 1964; Grzybowski *et al.*, 1970; Sillén *et al.*, 1971), as described by Portzehl *et al.* (1964).

Ligand	Bivalent metal ion ...	Apparent dissociation constant (M)	
		Ca ²⁺	Mg ²⁺
EGTA		2.07×10^{-7}	2.47×10^{-2}
ATP		1.73×10^{-4}	9.7×10^{-5}

top of an oil mixture in a polythene microtube (Alpha Laboratories, Greenford, Middx., U.K.). The microtube contained 200 μ l of di-*n*-butyl phthalate (BDH Chemicals, Poole, Dorset, U.K.)/benzene (9:1, v/v). The tubes were spun in a microfuge (Beckman Microfuge B; Beckman Instruments, Palo Alto, California, U.S.A.) for 15 s and then instantaneously frozen by immersion in liquid N₂ and the bottom 0.4 cm was cut off and placed in scintillation vials that contained 0.8 ml of 6% HClO₄. The vials were shaken for 30 min before adding 0.2 ml of 5 M-NaOH and 8.0 ml of scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole in toluene/Triton X-100 (2:1, v/v)]. Radioactivity was measured by using a double-label programme in a Beckman LS 7500 liquid-scintillation spectrometer.

Insulin secretion

Static incubations. After 30 min pre-incubation in Krebs–Ringer bicarbonate at 37°C, islets were washed five times with pulsation medium containing 10 nM-Ca²⁺. Groups of 20 islets were transferred to a pulsation cell containing 1.0 ml of buffer with the appropriate free Ca²⁺ concentration and were exposed to three high-voltage discharges. Islets were incubated for 15 min in a shaking water bath at 37°C after which time samples were withdrawn for insulin determination.

Perfusion. After 30 min pre-incubation in Krebs–Ringer bicarbonate, islets were washed five times with pulsation medium containing the lowest appropriate Ca²⁺ concentration. Then groups of 50 islets were transferred to pulsation cells containing 1.0 ml of medium supplemented with either 6 nM-, 10 nM-, 0.36 μ M-, 1 μ M- or 10 μ M-Ca²⁺ at 4°C and then subjected to five discharges. The islets were then placed on a 10 μ m-mesh nylon filter (Plastok, Birkenhead, Merseyside, U.K.) in a Swinnex filter holder (Millipore Corp.) and perfused by using a peristaltic pump (Gilson, Villiers-le-Bel, France). An additional channel contained 50 islets in 10 μ M-Ca²⁺ which had not been subjected to the high-voltage discharge. Perfusion started with a flow rate of 4.8 ml/min at 4°C for 5 min and then the flow rate

was decreased to 1 ml/min and the temperature brought up to 37°C. Perfusion continued for at least a further 10 min and 1 min fractions were collected.

In experiments with cytochalasin B (Sigma Chemicals, Poole, Dorset, U.K.) and vinblastine sulphate (Eli Lilly and Co., Basingstoke, Hants., U.K.), islets were discharged in 1 ml of pulsation medium containing 1 μ M-Ca²⁺ supplemented with either 13 μ M-vinblastine sulphate or 20 μ M-cytochalasin B, which was dissolved in dimethyl sulphoxide (1 mg/ml), and were perfused in identical media. Control channels contained islets discharged and perfused in medium containing 1 μ M-Ca²⁺.

Insulin assay

Insulin was determined by radioimmunoassay using ¹²⁵I-labelled ox insulin prepared in this laboratory by a chloramine-T procedure (Hunter & Greenwood, 1962) and an antiserum raised in guinea-pigs against bovine insulin (Wellcome Reagents, London, U.K.). Highly purified rat insulin (Novo, Denmark) was used as standard and antibody-bound radioactivity was precipitated in 12% polyethylene glycol 6000 (BDH Chemicals) (Desbuquois & Aurbach, 1971).

Lactate dehydrogenase determination

Lactate dehydrogenase (EC 1.1.1.27) activities of islet homogenates were determined fluorospectrophotometrically using an enzymic microassay (Lowry *et al.*, 1956). Portions of incubation media from the discharged islets were taken in parallel with samples for insulin determination, and were obtained after 15 min static incubation. NAD⁺ standards were treated in the same way as samples and were used as a reference to NAD⁺ produced in the incubations by enzymic conversion. The NAD⁺ (Sigma grade 1) standard solutions were standardized by an alcohol dehydrogenase (EC 1.1.1.1) spectrophotometric procedure (Lowry & Passonneau, 1972) by using a Pye–Unicam SP8-100 spectrophotometer.

Electron microscopy

Pulsated islets were fixed in 3.0% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, and post-fixed in 2% OsO₄ in a similar buffer. After dehydration and embedding by a standard procedure they were sectioned with an LKB Ultratome I (LKB Instruments, Croydon, Surrey, U.K.) and stained with a saturated solution of uranyl acetate in 50% ethanol before examination in an AEI EM6B electron microscope.

Results

Most previous studies using this type of methodology have employed isolated cells as their starting

Table 2. *The effect of high-voltage discharge on the permeability of islets to [^{14}C]sucrose in pulsation medium containing a mixture of [^{14}C]sucrose and $^3\text{H}_2\text{O}$*

In treatment I, pulsated islets were transferred to the doubly labelled radioactive medium immediately after their exposure to the high-voltage discharge, whereas in treatment II the pulsated islets were incubated at 37°C for 40 min before their transfer to the radioactive medium. The number of observations is shown in parentheses. Values are means $\pm$ S.E.M. *, $P < 0.001$, compared with control, as calculated by using Student's t test.

	Islet volume (nl)	Extracellular space (%)	
		Control islets	Pulsated islets
Krebs-Ringer bicarbonate	3.2 ± 0.28 (6)	41.6 ± 2.9 (6)	—
Pulsation medium			
Treatment I	3.0 ± 0.3 (6)	37.8 ± 3.1 (5)	$86.2 \pm 5.2^*$ (6)
Treatment II	3.1 ± 0.54 (5)	50.0 ± 3.6 (3)	$84.4 \pm 4.1^*$ (5)

material, rather than groups of cells, as was the case in the present study. It was therefore important to establish at the outset that the technique could be used successfully to access large numbers of cells in a single islet in this way. This was achieved by estimating the [^{14}C]sucrose and $^3\text{H}_2\text{O}$ spaces in isolated islets that had been exposed to the electric discharge. Preliminary investigations with various combinations of high-voltage and capacitance established the optimal values that were used for all subsequent experiments. Attempts to further increase the number of cells accessed by exposure to ten or more discharges resulted in a large increase in lactate dehydrogenase release from the islets and considerable ultrastructural damage.

The sucrose space (extracellular space) was $37.8 \pm 3.1\%$ of the total volume of the control islets, consistent with previous estimates (Kawazu *et al.*, 1978), whereas, after discharge, the proportion increased to $86.2 \pm 5.2\%$ of total volume (Table 2). Thus exposure of the islets to discharge caused a large increase in the sucrose space, consistent with the majority of the cells having been made permeable. Ultrastructural studies (see Plate 1) and estimations of Trypan Blue exclusion (not shown) confirmed that the majority of cells in the islet were accessed by the discharge procedure. It was also apparent that the cells do not reseal spontaneously in these conditions, since the sucrose space remained at close to 80% of the total islet volume even after a further 40 min of incubation (Table 2).

By analogy with results using adrenal medullary cells (Baker & Knight, 1978) and in accordance with current concepts of the regulation of insulin secretion, it seemed worthwhile to test the effects of altering the Ca^{2+} concentration in the incubation medium from 10 nM to $10\text{ }\mu\text{M}$ during a static incubation. In these experiments it was shown that insulin secretion measured in static incubations

increased from 0.8 ± 0.13 to 1.4 ± 0.2 ng/islet per h ($n = 89$, $P < 0.001$) in pulsation media containing 10 nM- and $10\text{ }\mu\text{M}$ - Ca^{2+} respectively. In parallel experiments lactate dehydrogenase levels in the incubation media measured under identical conditions after pulsation of the islets were similar, i.e. 0.26 ± 0.05 nmol of NADH oxidized/islet per h ($n = 7$) in 10 nM- and 0.16 ± 0.013 ($n = 7$) in $10\text{ }\mu\text{M}$ - Ca^{2+} . The total islet content was 2.10 ± 0.22 nmol of NADH oxidized/islet, which, assuming that the dry weight of a rat islet (Beckman *et al.*, 1980) is $1.4\text{ }\mu\text{g}$, is equivalent to 1.5 ± 0.15 ($n = 5$) mol/kg dry wt./h, in close agreement with published values (Kissane & Brolin, 1963; Hellman & Täljedal, 1967). The incubation media of control islets incubated in identical conditions contained 0.16 ± 0.01 nmol of NADH oxidized/islet per h in 10 nM- and 0.092 ± 0.01 nmol/islet per h in $10\text{ }\mu\text{M}$ - Ca^{2+} . About 90% of the additional lactate dehydrogenase released after the discharge occurs in the first 5 min. Thus the stimulation of insulin secretion in the presence of $10\text{ }\mu\text{M}$ - Ca^{2+} was not due to non-specific leakage as judged by the inability of macromolecular components to leak out of the cells.

The release of insulin from islets that had been exposed to the high-voltage discharge was also examined in perfusion (Fig. 2). For this purpose, islets were exposed to the discharge in the presence of either 10 nM- or $10\text{ }\mu\text{M}$ - Ca^{2+} and then perfused in identical media. An initial flow rate of 4.8 ml/min and temperature of 4°C allowed a basal rate of insulin release to be established within 3–5 min. At 5 min, the flow rate and temperature of the perfusion media were adjusted to 1 ml/min and 37°C respectively. In the presence of $10\text{ }\mu\text{M}$ - Ca^{2+} , peak insulin release was achieved within 4 min, and the rate of release remained significantly higher than that from islets that had been pulsed and perfused in medium containing 10 nM- Ca^{2+} (integrated values

EXPLANATION OF PLATE 1

Electron micrographs of B cells in isolated islets that had been exposed to three discharges while incubating in media containing 10 nM-Ca²⁺ in the conditions described in the text

As illustrated in (a) the cells and organelles are essentially intact, although there is some evidence of vacuolation of elements of the endoplasmic reticulum (v). Other abbreviations: N, nucleus; m, mitochondria; g, insulin storage granules. In (b) arrows indicate probable sites of insulin secretion by exocytosis in a B-cell that had been incubated in 10 μM-Ca²⁺ after pulsation. The bar in (a) and (b) represents 0.1 μm.

Fig. 2. The dynamics of insulin release in pulsated islets in perfusion

Islets were pulsated in media containing either 10 nM- (○) or 10 μ M- Ca^{2+} (●) and subsequently perfused. Islets were kept at 4°C during their exposure to the high-voltage discharge and at the beginning of the perfusion where the flow rate was 4.8 ml/min. After 5 min, the temperature was brought to 37°C and the flow rate adjusted to 1 ml/min; 1 min fractions were collected throughout the perfusion and these were assayed for insulin. Values are means $\pm$ S.E.M. (represented by the bars) of four observations. Islets that had not been exposed to the discharge perfused in 10 μ M- Ca^{2+} are shown as (---●---).

being 611 ± 64 versus 224 ± 62 pg/islet in 10 min; $P < 0.001$). Indeed, the profile and magnitude of insulin release from pulsated islets in the presence of 10 nM- Ca^{2+} were indistinguishable from those of the control islets.

We have examined the response to Ca^{2+} more thoroughly to establish a Ca^{2+} -dose-response curve. For this purpose islets were subjected to the discharge procedure and were then perfused in pulsation media supplemented with 6 nM-, 10 nM-, 0.36 μ M-, 1 μ M- or 10 μ M- Ca^{2+} . The integrated areas under the curve in the perfusion profiles were calculated for each condition from at least three different experiments. The results of these experiments are shown in Fig. 3. The maximum stimulatory concentration was found to be 1 μ M, with half-maximum stimulation at approx. 0.6 μ M.

At very low Ca^{2+} concentrations (6 nM and 10 nM) insulin release appeared to undergo a paradoxical slight stimulation (Fig. 3). To investigate this further, we carried out experiments which showed that ATP- and temperature-dependent secretion from intact islets is identical at 6 nM- and 10 nM- Ca^{2+} , suggesting that the rise in insulin release seen at 6 nM- Ca^{2+} occurs by some other mechanism, perhaps as a consequence of destabilization of membranes or loss of islet integrity in the presence of very low concentrations of Ca^{2+} .

Fig. 3. Secretory response in discharge islets to Ca^{2+} . Points on the curve represent means $\pm$ S.E.M. of the integrated area under the curve of at least three perfusion experiments between 5 min and 15 min of the perfusion. Maximum stimulation of release in pulsated islets was achieved at a Ca^{2+} concentration of 1 μ M and this value (879 ± 171.66 pg/islet per 10 min) was taken as 100%, to which all other values on the curve were compared. The release from intact islets (control) perfused in pulsation medium containing 1 μ M- Ca^{2+} was 200 pg/islet per 10 min. *, $P < 0.05$; **, P is not significant, as calculated by Student's t test.

Perfusion of discharged islets in the presence of 13 μ M-vinblastine sulphate or 20 μ M-cytochalasin B (Fig. 4) showed that the first agent caused a significant inhibition, whereas the latter caused a significant increase in insulin release, in comparison with the secretion seen with permeated islets in the presence of 1 μ M- Ca^{2+} .

Ultrastructural studies showed that the cells of the islets that had been subjected to discharge were intact, although a tendency to slight vacuolation in the cytoplasm (Plate 1), was consistently observed. The significance of this vacuolation is unclear. There were some indications that the insulin released in these conditions was secreted by exocytosis (Plate 1).

Discussion

Pace *et al.* (1980) have used a similar electrical-discharge technique to study insulin secretion from rat islets of Langerhans. These authors have used a

Fig. 4. The effect of cytochalasin B and vinblastine sulphate on insulin release from discharged islets in perfusion

■, Islets discharged and perfused in $1\mu\text{M-Ca}^{2+}$ (control channel); □, islets discharged and perfused in $1\mu\text{M-Ca}^{2+}$ supplemented with $20\mu\text{M-cytochalasin B}$; ●, islets discharged and perfused in $1\mu\text{M-Ca}^{2+}$ supplemented with $13\mu\text{M-vinblastine sulphate}$. Points on the curve represent the mean and bars the S.E.M. of at least three perfusions with cytochalasin B and vinblastine sulphate and six experiments with the control channel. Integrated areas under the curve from 5 min to 15 min were compared with control and P was <0.05 , calculated by Student's t test.

voltage of 2.5 kV/cm and pulse duration of $250\mu\text{s}$, in a buffer containing $140\text{ mM-sodium glutamate}$. In these conditions they obtained values of 0.05 and $0.35\mu\text{M-Ca}^{2+}$ for half-maximal and maximal stimulation of insulin secretion. Of particular interest was the fact that the permeated islets appeared to reseal within 30 min in their experiments, whereas in our conditions no resealing (as indicated by reduction of sucrose space) was observed, even after discharge at 37°C in glutamate buffer (i.e. in closely comparable conditions). The use of glutamate buffer may not be optimal for studies of the role of metabolic intermediates in regulating insulin secretion in view of the known fuel function of glutamine in the B-cell (Malaisse & Sener, 1980) and the high permeability of discharged islets to glutamic acid. In the present experiments we therefore substituted chloride/isethionate for glutamate. In our hands 70 mM-Cl^- did not have any inhibitory effect on insulin secretion from permeated islets, nor did 70 mM-

isethionate have any deleterious osmotic effects, whereas intact islets incubated in this medium respond to 20 mM-glucose stimulation in a normal way (results not shown). In general, however, both the study of Pace *et al.* (1980) and the present work indicate the potential of this method for the investigation of insulin secretion by normally impermeable substances.

The results above demonstrate that islets of Langerhans that are subjected to electrical discharge undergo a considerable increase in their sucrose (apparent extracellular) space, and are capable of responding to an increase in Ca^{2+} concentration. The values obtained for half-maximal stimulation are consistent with a key role for free cytosolic Ca^{2+} concentrations in the regulation of secretion (see Wollheim & Sharp, 1981). By analogy with systems in which direct measurement has been possible free cytosolic Ca^{2+} in B-cells may be expected to be of the order of approx. $0.1\mu\text{M}$ (see Ashley & Campbell, 1979; Tsien, 1981). The observed inhibition of Ca^{2+} -induced stimulation by vinblastine (Fig. 4), which is known to disrupt microtubule function (Devis *et al.*, 1974), and stimulation by cytochalasin B, which alters microfilament function (Malaisse *et al.*, 1972), both closely parallel the effects observed in intact islets under such conditions. It is of interest that the effects of these two agents are exerted very rapidly in this system, presumably because, in contrast with normal islets, the drugs can obtain rapid access to the cell interior. Thus it seems likely that the Ca^{2+} -induced secretion in permeabilized islets closely reflects the normal insulin secretory mechanism insofar as it involves both microtubules and microfilaments and probably granule release by exocytosis.

This experiment also provides important evidence that Ca^{2+} stimulation involves both the intracellular transport step of secretion as well as the final granule-membrane-plasma-membrane fusion of exocytosis. We have recently discussed the possible involvement of Ca^{2+} -induced actomyosin contraction in the mechanism of granule movement via the microtubule-microfilamentous system (Howell & Tyhurst, 1982).

The use of this high-voltage technique (see also Pace *et al.*, 1980) has provided the first demonstration that insulin secretion can be modulated directly by Ca^{2+} concentrations in the anticipated physiologically important range. It should now be possible to extend the use of the method to the study of a variety of the aspects of stimulus-secretion coupling in the pancreatic β -cell.

We thank Dr. D. Knight (Department of Physiology, King's College, London, U.K.) for advice and stimulating discussions and Mrs. M. Tyhurst for skilled work in the ultrastructural studies. Financial assistance

from the Medical Research Council, Wellcome Trust and the British Diabetic Association is gratefully acknowledged. M. A. Y. is supported in part by a British Council scholarship and is very grateful for support from Mr. Naji Al Mufti.

References

- Ashley, C. C. & Campbell, A. K. (eds.) (1979) *Detection and Measurement of Free Calcium in Cells*, Elsevier/North-Holland, Amsterdam
- Baker, P. F. & Knight, D. E. (1978). *Nature (London)* **276**, 620–622
- Baker, P. F., Knight, D. E. & Whitaker, M. J. (1980) *Proc. R. Soc. London Ser. B* **207**, 149–161
- Beckmann, J., Holze, S., Lenzen, S. & Panten, U. (1980) *Acta Diabetol. Lat.* **18**, 51–57
- Desbuquois, G. & Aurbach, G. D. (1971) *J. Clin. Endocrinol. Metab.* **33**, 732–738
- Devis, G., Van Obberghen, E., Somers, G., Malaisse-Lagae, F., Orci, L. & Malaisse, W. J. (1974) *Diabetologia* **10**, 53–59
- Feldman, H., Rodbard, D. & Levine, D. (1972) *Anal. Biochem.* **45**, 530–556
- Grzybowski, A. K., Tate, S. S. & Datta, S. P. (1970) *J. Chem. Soc. A* 241–245
- Hellman, B. & Täljedal, I. B. (1967) *Endocrinology* **81**, 125–131
- Howell, S. L. & Taylor, K. W. (1968) *Biochem. J.* **108**, 17–24
- Howell, S. L. & Tyhurst, M. (1982) *Diabetologia* **22**, 301–308
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
- Kawazu, S., Boschero, A. C., Delcroix, C. & Malaisse, W. J. (1978) *Pflügers Arch.* **375**, 197–206
- Kinosita, K. & Tsong, T. Y. (1977) *Nature (London)* **268**, 438–441
- Kissane, J. M. & Brolin, S. E. (1963) *J. Histochem. Cytochem.* **11**, 197–203
- Knight, D. E. (1981) *Techniques in Life Sciences*, Elsevier Press, Amsterdam, in the press
- Lowry, O. H. & Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis*, pp. 16–18, Academic Press, London and New York
- Lowry, O. H., Roberts, N. R. & Kapphahn, J. I. (1956) *J. Biol. Chem.* **224**, 1047–1064
- Malaisse, W. J. & Sener, A. (1980) *Diabetologia* **19**, 296–297
- Malaisse, W. J., Hager, D. L. & Orci, L. (1972) *Diabetes* **21**, Suppl. 2, 594–604
- Martell, A. E. & Sillen, L. G. (eds.) (1964) *Stability Constants*, The Chemical Society, London
- Pace, C. S., Tarvin, J. T., Neighbors, A. S., Pirkel, J. A. & Greider, M. H. (1980) *Diabetes* **29**, 911–918
- Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964) *Biochim. Biophys. Acta* **70**, 581–591
- Riemann, F., Zimmermann, U. & Pilwat, G. (1975) *Biochim. Biophys. Acta* **394**, 449–462
- Sillén, L. G., Martell, A. E., Högfeldt, E. & Smith, R. M. (ed.) (1971) *Stability Constants: Suppl. No. 1*, The Chemical Society, London
- Tsien, R. Y. (1981) *Nature (London)* **290**, 527–528
- Wollheim, C. B. & Sharp, G. W. G. (1981) *Physiol. Rev.* **61**, 914–973
- Yaseen, M. A., Pedley, K. C. & Howell, S. L. (1980) *Diabetologia* **19**, 565
- Yaseen, M. A., Pedley, K. C. & Howell, S. L. (1981) *Diabetologia* **21**, 345