Calcium and pancreatic β -cell function

THE MECHANISM OF INSULIN SECRETION STUDIED WITH THE AID OF LANTHANUM

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La^{3+} was used to study the involvement of Ca^{2+} in insulin secretion in β -cell-rich pancreatic islets micro-dissected from non-inbred *ob/ob* mice. Ultrastructural studies revealed that the localization of La^{3+} was entirely restricted to the exterior of the cells. Consistent with a membrane action, exposure to La^{3+} failed to affect glucose oxidation and either the sucrose space or the general ultrastructure of the islets. In contrast, La^{3+} had marked effects on insulin release and ^{45}Ca fluxes. Exposure to La^{3+} resulted in pronounced inhibition of insulin release irrespective of the presence or absence of Ca^{2+} , 3-isobutyl-1-methylxanthine or glucose. Perfusion experiments revealed that the inhibitory action was prompt, sustained and readily reversible. Removal of La^{3+} was associated with a subsequent prolonged stimulatory phase of insulin release even in medium deficient in Ca^{2+} . This action could not be attributed to an increase in cyclic AMP, but was potentiated by 3-isobutyl-1-methylxanthine and abolished by L-adrenaline. La^{3+} displaced ^{45}Ca from superficially located binding sites and inhibited the uptake and efflux of ^{45}Ca . The stimulatory and inhibitory actions of glucose on ^{45}Ca efflux were also abolished in the presence of 2 mM- La^{3+} . Removal of La^{3+} was associated with the preferential mobilization of ^{45}Ca incorporated in response to glucose. The results indicate that binding of La^{3+} to superficial sites in the plasma membrane leads to inhibition of insulin release by suppression of transmembrane Ca^{2+} fluxes. It is suggested that accumulation of Ca^{2+} in the cytoplasm accounts for the stimulation of insulin release seen after removal of La^{3+} from inhibitory binding sites in the β -cell plasma membrane.

Although the precise nature of the molecular events leading to glucose stimulation of insulin release from the pancreatic β -cell remains to be established, there can be no doubt that Ca^{2+} plays a decisive role in coupling stimulus-recognition to the discharge of the secretory granules by exocytosis (Malaisse *et al.*, 1978; Hellman *et al.*, 1979). Indeed, exposure of the pancreatic β -cell to glucose and other stimulators of insulin release results in marked changes in the uptake, efflux and intracellular distribution of calcium (Hellman *et al.*, 1971a, 1980a,b; Malaisse-Lagae & Malaisse, 1971; Herman *et al.*, 1973; Malaisse *et al.*, 1973; Frankel *et al.*, 1978; Gylfe & Hellman, 1978; Kikuchi *et al.*, 1978; Sugden & Ashcroft, 1978; Klöppel & Bommer, 1979). It is envisaged that the interaction between these processes provides the appropriate

secretory response to a given stimulus by regulating the accumulation of Ca^{2+} in a stimulatory cytoplasmic pool adjacent to the β -cell plasma membrane (Malaisse *et al.*, 1978; Hellman *et al.*, 1979, 1980a,b).

In order to discriminate between intracellular uptake and the binding of Ca^{2+} to the exterior of the β -cell, advantage was taken of the La^{3+} -wash technique introduced for delineating differential Ca^{2+} movements in smooth-muscle membranes (Weiss & Goodman, 1969; Van Breemen *et al.*, 1972). In this procedure, it is assumed that La^{3+} both displaces superficial membrane Ca^{2+} and blocks transmembrane Ca^{2+} fluxes. These prerequisites appear to be satisfactorily fulfilled for the study of ^{45}Ca fluxes in isolated pancreatic islets (Hellman *et al.*, 1976a), especially when the wash procedure is performed at lower temperature (Hellman, 1978). Indeed, by using the La^{3+} -wash technique it has been possible to

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establish that glucose stimulates the intracellular uptake of ^{45}Ca into pancreatic islets of *ob/ob* mice (Hellman *et al.*, 1976*a,b*), as well as from suspensions of β -cells prepared from these animals (Sehlin & Täljedal, 1979).

In view of the general ability of La^{3+} to serve as a specific Ca^{2+} substitute or antagonist at superficially located membrane binding sites (Weiss, 1974, 1977; Mikkelsen, 1976), it was decided to make a detailed investigation of the effects of this lanthanide on β -cell function. In addition to demonstrating the cellular localization of La^{3+} , the present study provides information about the effects of La^{3+} on insulin release and glucose-dependent ^{45}Ca fluxes. Although exposure to La^{3+} was associated with inhibition of secretion, pretreatment with the cation was associated with a subsequent stimulation of insulin release.

Materials and methods

Chemicals

Sigma Chemical Co., St. Louis, MO, U.S.A., supplied Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], L-adrenaline and bovine serum albumin (fraction V). Tris, D-glucose and $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. D-[U- ^{14}C]Glucose and [6,6'- ^3H]sucrose were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and $^{45}\text{CaCl}_2$ and Aquasol were from New England Nuclear Corp., Frankfurt/Main, Germany. Packard Instruments, Warrenville, IL, U.S.A., supplied Insta-fluor and Hyamine 10-X. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and ^{125}I -labelled pig insulin was provided by Farbwerke Hoechst A.G., Frankfurt/Main, Germany. Crystalline mouse insulin was given by Novo A/S, Copenhagen, Denmark, and insulin antibodies were prepared in guinea pigs by using crystalline pig insulin from the same source. Schwarz/Mann, Orangeburg, NY, U.S.A., supplied ^{125}I -labelled succinyl cyclic AMP tyrosine methyl ester and cyclic AMP antibodies. Dowex ion-exchange resin (AG 50W-X8; H^+ form; 100–200 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. For electron microscopy, glutaraldehyde was obtained from Taab Laboratories, Reading, Berks., U.K., osmium tetroxide from Drüfhout and Son, Amsterdam, The Netherlands, and Epon 812 from Fluka A.G., Buchs, Switzerland. All other reagents were of analytical grade, and distilled-deionized water was used throughout.

Animals and isolation of islets

Adult obese-hyperglycaemic mice (gene symbol *ob/ob*) of both sexes were taken from a local non-inbred colony (Hellman, 1965). Fresh pancreatic islets were isolated from overnight-starved mice by freehand micro-dissection (Hellerström,

1964). During this procedure, the pancreas was immersed in the same type of salt-balanced buffer as used during the subsequent incubation for measurement of insulin release, glucose oxidation or ^{45}Ca fluxes. The islets of these mice contain more than 90% β -cells, which respond appropriately to various stimulators and inhibitors of insulin secretion (Hellman, 1970; Hahn *et al.*, 1974). It should be emphasized that no collagenase was used in the isolation procedure.

General aspects of incubations

The basal medium used for incubation of islets for measurements of insulin release, ^{45}Ca efflux, glucose oxidation and electron microscopy consisted of a modified Krebs–Ringer buffer in which phosphate and sulphate were replaced with equimolar amounts of chloride, and the buffering capacity was provided by 25 mM-Hepes instead of bicarbonate (Hellman, 1975). Experiments involving the study of ^{45}Ca uptake were performed in a Tris buffer physiologically balanced in cations (139 mM- Na^+ , 4.7 mM- K^+ , 2.56 mM- Ca^{2+} , 1.2 mM- Mg^{2+}), but lacking anions other than chloride (Hellman *et al.*, 1976*a*). The usefulness of these two basic media for studying insulin release and ^{45}Ca fluxes of micro-dissected islets has previously been reported (Hellman, 1975; Hellman *et al.*, 1976*a,b*). It should be noted that phosphate, sulphate and bicarbonate must be omitted from the incubation media because their respective La^{3+} salts are extremely insoluble (Moeller, 1963). All incubations were performed at 37°C in buffer adjusted to pH 7.4. Except in the perfusion experiments, the islets were allowed to equilibrate during a preincubation period of 45–50 min in the basal medium supplemented with 3 mM-glucose. Further modifications to the basic composition of the media, regarding the use of albumin, La^{3+} , 3-isobutyl-1-methylxanthine, L-adrenaline or different concentrations of glucose and Ca^{2+} , are given in the legends to the Figures and Tables.

Insulin release

Two different techniques were used for studying insulin release. In some experiments, batches of two islets were incubated in 300 μl of medium for 60 min and insulin was determined in samples of medium taken at the end of incubation (Hellman, 1970). In the other series of experiments, the dynamics of insulin release were studied by using a perfusion system (Idahl, 1972). Two reservoirs were connected with the perfusion chamber so as to allow sudden alterations of the medium supplied to the islets. A specially constructed valve was used to prevent fluctuations of hydrodynamic pressure caused by switching the perfusion medium. Groups of five islets were placed in small polythene/nylon chambers (4.5 μl volume) and perfused at a con-

stant rate of 45–50 μ l/min. In each experiment, three different perfusion chambers were loaded with islets from a single animal and run in parallel.

Glucose oxidation

Groups of two islets were incubated for 60 min in liquid-scintillation vials equipped with a small glass centre well (Keen *et al.*, 1963) containing 100 μ l of medium supplemented with 3 or 20 mM-D-[U- 14 C]-glucose (5.67 or 0.85 Ci/mol). Blank values were obtained from incubations without islets. Metabolism was stopped with 100 μ l of 0.1 M-HCl, and 14 CO₂ was collected in 100 μ l of Hyamine and determined as previously described (Hellman *et al.*, 1971b).

Uptake of 45 Ca and [3 H]sucrose

Groups of two islets were incubated for 20 min in 200 μ l of medium containing 2.56 mM- 45 Ca (7.81 Ci/mol) and 7 μ M-[6,6'- 3 H]sucrose (3000 Ci/mol). Previous studies have demonstrated the suitability of sucrose as a marker for the extracellular space of these islets (Hellman *et al.*, 1973). After incubation, the islets were processed for measurement of radioactivity either directly or after washing for 60 min at 1°C in non-radioactive medium supplemented with 2 mM-LaCl₃ to remove extracellular radioactivity. The application of this technique to the measurement of transmembrane 45 Ca fluxes in pancreatic islets has been described in detail elsewhere (Hellman *et al.*, 1976a). However, it is important to note that the present La³⁺-wash procedure differs from that originally employed for islet studies (Hellman *et al.*, 1976a,b; Hellman & Gylfe, 1978) in being performed at lower temperature. This modified procedure provides a much better estimate of both the superficial and intracellular 45 Ca (Hellman, 1978). After being freeze-dried overnight and weighed, the islets were dissolved in 100 μ l of Hyamine. After addition of 5 ml of Instafluor, the radioactivity was analysed by liquid-scintillation spectrometry. Samples of the labelled incubation medium were used as external standards in the radioactivity-counting procedure. The 45 Ca contents of islets, after correction for radioactivity in the sucrose space, were expressed in terms of mmol of calcium with the same specific radioactivity as in the medium. In accordance with previous nomenclature (Hellman *et al.*, 1976b), the 45 Ca content of unwashed islets is called total 45 Ca, that of lanthanum-washed islets is termed La³⁺-non-displaceable 45 Ca, and the difference between the two groups is referred to as La³⁺-displaceable 45 Ca.

Efflux of 45 Ca

Groups of ten islets were loaded with 45 Ca during incubation for 90–120 min in 100 μ l of medium containing 3 or 20 mM-glucose and either 1.28 or

2.56 mM- 45 Ca (780 or 390 Ci/mol). After two 5 min washes in non-radioactive medium, the islets were transferred to 10 μ l chambers and perfused at a constant rate of approx. 40 μ l/min (Gylfe & Hellman, 1978). The use of two reservoirs connected to each micro-chamber made it possible to make sudden alterations in the composition of the medium supplied to the islets. It was checked that changing reservoirs did not in itself modify the efflux of 45 Ca. In each experiment, two different chambers were loaded with islets from the same animal and run in parallel. The perfusate was collected over successive periods of 2 or 5 min. Samples of the perfusate or loading medium were mixed with Aquasol, and analysed for radioactivity by liquid-scintillation counting. The 45 Ca-efflux rate was expressed in terms of μ mol of calcium, assuming that the Ca²⁺ released had the same specific radioactivity as in the loading medium.

Weighing of islets and assays of cyclic AMP and insulin

After incubation, the islets were plunged into melting isopentane (–160°C), freeze-dried overnight (–40°C, 0.1 Pa) and subsequently weighed on a quartz-fibre balance. HClO₄ extracts of freeze-dried islets were purified by ion-exchange chromatography, and cyclic AMP was determined by radioimmunoassay (Hellman *et al.*, 1974b). The concentration of insulin in diluted samples of incubation media was measured radioimmunologically by using crystalline mouse insulin as the reference. Separation of free and antibody-bound hormone was achieved by precipitation with ethanol (Heding, 1966). It was established that the highest concentration of La³⁺ encountered in the assay diluent did not affect the recovery of immunoreactive insulin or the radioimmunoassay.

Statistical evaluation of results

The biochemical data were expressed per unit dry islet weight. In studies of the effects of altered medium composition, notably the addition of La³⁺, parallel control incubations were routinely performed by using islets isolated from the same animals. This experimental design made it possible to use each pancreas as its own control. Unless otherwise indicated, statistical significances were estimated by Student's *t* test of the differences between paired test and control incubations over a series of repeated experiments.

Electron microscopy

Islets were taken for ultrastructural examination after 60 min incubation with or without 2 mM-LaCl₃ in medium supplemented with 3 mM-glucose and 2.56 mM-Ca²⁺. The specimens were fixed in 2.5% (w/v) glutaraldehyde in 0.36 M-veronal acetate buffer

(pH 7.4) and post-fixed in 1% (w/v) osmium tetroxide dissolved in the acetate buffer. After rinsing and stepwise dehydration in ethanol, the fixed tissue was embedded in Epon 812 and ultrathin sections were cut with an LKB Ultratome. Both unstained sections and sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiskop 1A or 101 electron microscope.

Results

Effects of La^{3+} on the amounts of insulin released at different concentrations of glucose, Ca^{2+} and 3-isobutyl-1-methylxanthine

The effects of La^{3+} on insulin release during batch incubations of islets with different concentrations of glucose, Ca^{2+} and 3-isobutyl-1-methylxanthine are shown in Table 1. In the absence of La^{3+} , glucose stimulation of insulin release was markedly potentiated after the addition of 3-isobutyl-1-methylxanthine. At a concentration of 0.5 mM, La^{3+} exerted clear inhibitory effects on insulin release under all experimental conditions. Inhibition was consequently seen both at the non-stimulatory glucose concentration and when the Ca^{2+} concentration was decreased to 0.26 mM in a medium supplemented with 1 mM-3-isobutyl-1-methylxanthine and 20 mM-glucose. Whereas the percentage inhibition was similar at both glucose concentrations, it became more pronounced in the presence of 3-isobutyl-1-methylxanthine. Consequently, the relative magnitude of the glucose effect remained unchanged in the presence of La^{3+} . It was possible to demonstrate an inhibitory effect on glucose-stimulated insulin release even when La^{3+} was added at a concentration of 0.03 mM to a medium containing 3-isobutyl-1-methylxanthine and 0.26 mM- Ca^{2+} .

Effects of La^{3+} on the dynamics of insulin release at different concentrations of glucose, Ca^{2+} and 3-isobutyl-1-methylxanthine

Perfusion of islets was used to study the dynamics of insulin release in response to concentrations between 0.5 and 2.0 mM- La^{3+} . The effects are illustrated in Figs. 1 and 2. There was a rapid onset of inhibition, which persisted throughout the exposure to La^{3+} . Other experiments also indicated that consecutive 2 min pulses of 0.8 mM- La^{3+} produced repeated inhibitions, followed by prompt reversal (results not shown). In addition to the inhibitory action of La^{3+} , it was possible to demonstrate a stimulation of insulin release above the initial rate after excluding La^{3+} from the perfusion medium. This stimulatory effect was particularly pronounced when combining media deficient in Ca^{2+} with measures intended to sensitize

Fig. 1. Dynamics of insulin release in response to La^{3+} during perfusion with glucose-free medium containing 2.56 mM- Ca^{2+}

After perfusion with medium lacking glucose but supplemented with albumin (1 mg/ml), 0.8 mM- La^{3+} was included from 70 to 90 min, as indicated by the horizontal black bar. The rate of insulin release is given as mean values $\pm$ S.E.M. for four different experiments.

Table 1. Effects of La^{3+} on insulin release at different concentrations of glucose, calcium and 3-isobutyl-1-methylxanthine

After preincubation in media containing albumin (1 mg/ml) and either 0.26 or 2.56 mM- Ca^{2+} , the islets were incubated for 60 min in the presence and absence of La^{3+} in the same type of media containing 3 or 20 mM-glucose with or without 1 mM-3-isobutyl-1-methylxanthine (IBMX). The results represent insulin release presented as mean values $\pm$ S.E.M. for n experiments. Statistical significances were judged from the differences between paired incubations performed in the presence and absence of La^{3+} : * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$.

Glucose (mM)	La^{3+} (mM)	Ca^{2+} (mM)	IBMX (mM)	n	Insulin release (ng/ μ g dry wt. of islets)		
					Control (a)	La^{3+} (b)	Effect of La^{3+} (b) - (a)
3	0.5	2.56	0	9	0.33 $\pm$ 0.06	0.17 $\pm$ 0.03	-0.16 $\pm$ 0.06*
3	0.5	2.56	1	12	2.51 $\pm$ 0.68	0.20 $\pm$ 0.07	-2.32 $\pm$ 0.66***
3	0.5	0.26	1	8	1.30 $\pm$ 0.48	0.09 $\pm$ 0.04	-1.21 $\pm$ 0.46*
3	0.03	0.26	1	8	1.30 $\pm$ 0.48	0.67 $\pm$ 0.24	-0.63 $\pm$ 0.38
20	0.5	2.56	0	9	0.78 $\pm$ 0.10	0.37 $\pm$ 0.10	-0.41 $\pm$ 0.09***
20	0.5	2.56	1	12	9.97 $\pm$ 1.32	0.93 $\pm$ 0.28	-9.04 $\pm$ 1.21****
20	0.5	0.26	1	8	10.30 $\pm$ 1.88	0.82 $\pm$ 0.44	-9.48 $\pm$ 1.60****
20	0.03	0.26	1	8	10.30 $\pm$ 1.88	6.57 $\pm$ 1.34	-3.73 $\pm$ 1.32**

Fig. 2. Dynamics of insulin release in response to La^{3+} in the presence and absence of glucose during perfusion with Ca^{2+} -deficient medium ($<50\text{ }\mu\text{M-Ca}^{2+}$) supplemented with 3-isobutyl-1-methylxanthine. After perfusion with medium supplemented with 1 mM-3-isobutyl-1-methylxanthine and albumin (1 mg/ml), 0.5 mM- La^{3+} was included from 60 to 90 min and from 120 to 150 min as indicated by the horizontal black bars. Experiments were performed in the presence (●) and absence (○) of 20 mM-glucose. The rate of insulin release is given as mean values $\pm$ S.E.M. for five and four different experiments respectively.

Table 2. Effects of pretreatment with La^{3+} on insulin release and the islet content of cyclic AMP

After preincubation in the presence and absence of 0.5 mM- La^{3+} in Ca^{2+} -deficient media ($<50\text{ }\mu\text{M-Ca}^{2+}$) containing albumin (1 mg/ml), the islets were incubated for 60 min without La^{3+} in the same type of media containing 1 mM-3-isobutyl-1-methylxanthine and supplemented with L-adrenaline (2 $\mu\text{g/ml}$) or 20 mM-glucose as indicated. The results represent insulin release and islet cyclic AMP content, presented as mean values $\pm$ S.E.M. for eight different experiments. Statistical significances were judged from the differences between paired incubations of La^{3+} -pretreated and control islets: * $P < 0.05$; ** $P < 0.02$.

Modification of test medium	Insulin release (ng/ μg dry wt. of islets)		
	Control (a)	Pretreatment with La^{3+} (b)	Effect of La^{3+} (b) - (a)
None	0.75 $\pm$ 0.11	1.51 $\pm$ 0.22	+0.76 $\pm$ 0.22**
L-Adrenaline (2 $\mu\text{g/ml}$)	1.28 $\pm$ 0.50	1.02 $\pm$ 0.24	-0.27 $\pm$ 0.58
Glucose (20 mM)	0.79 $\pm$ 0.21	1.52 $\pm$ 0.48	+0.74 $\pm$ 0.30*
	Islet cyclic AMP (fmol/ μg dry wt. of islets)		
	(a)	(b)	(b) - (a)
None	10.2 $\pm$ 1.6	8.3 $\pm$ 0.8	-2.0 $\pm$ 1.1
L-Adrenaline (2 $\mu\text{g/ml}$)	8.7 $\pm$ 1.8	7.7 $\pm$ 1.0	-1.0 $\pm$ 1.3
Glucose (20 mM)	13.2 $\pm$ 2.6	8.9 $\pm$ 1.9	-3.6 $\pm$ 2.7

the secretory machinery to Ca^{2+} , namely the inclusion of methylxanthines or high concentrations of glucose (Hellman *et al.*, 1979).

Effects of pretreatment with La^{3+} on insulin release and the islet content of cyclic AMP

The observation that removal of La^{3+} was associated with an increased rate of insulin release prompted studies to examine the mechanism in-

involved. For this purpose, isolated islets were pre-treated for 40 min with 0.5 mM- La^{3+} before a test incubation for 60 min in a Ca^{2+} -deficient medium supplemented with 1 mM-3-isobutyl-1-methylxanthine. The amounts of insulin released in the presence and absence of 20 mM-glucose or 2 μg of L-adrenaline/ml and the final islet content of cyclic AMP are shown in Table 2. It is evident that pretreatment with La^{3+} significantly increased in-

sulin release in both the presence and the absence of glucose. Addition of L-adrenaline to the glucose-free medium abolished this stimulatory effect. The enhanced release of insulin after exposure to La^{3+} was not associated with an increase in the islet content of cyclic AMP. The stimulatory effect of pretreatment with La^{3+} was observed under a variety of experimental conditions irrespective of whether Ca^{2+} was present or absent during exposure to the lanthanide. Despite a relatively great experimental variation, the data in Table 3 indicate persistence of the stimulatory effect after an intervening rinsing period of at least 15 min before test incubation in a glucose-free medium supplemented with 2.56 mM- Ca^{2+} . There was no further enhancement of the stimulatory action after the La^{3+} concentration was increased from 0.5 to 2.0 mM.

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

The volume distribution of sucrose in islets incubated for 20 min in the presence of 3 and 20 mM-glucose was 3.82 ± 1.04 and 3.45 ± 0.26 litre/kg dry wt. respectively (mean $\pm$ S.E.M. for six observations). There was no significant change after exposure to 0.5 mM- La^{3+} . Slightly lower values were observed, corresponding to a difference of 0.51 ± 0.21 and 0.26 ± 0.20 litre/kg dry wt. respectively. The corresponding ^{45}Ca uptake in excess of the sucrose space is shown in Table 4. The introduction of 0.5 mM- La^{3+} to media containing 3 or 20 mM-glucose resulted in marked inhibition of total ^{45}Ca uptake, as well as uptake into both the constituent intracellular and superficial pools revealed by using the La^{3+} -wash technique to

Table 3. *Latency of the La^{3+} -induced stimulatory effects on insulin release*

After preincubation in the presence and absence of La^{3+} in media containing 2.56 mM- Ca^{2+} , the islets were rinsed for different periods of time in 2 ml of glucose-free medium devoid of La^{3+} but supplemented with albumin (1 mg/ml). Each rinsing period comprised three successive changes of the medium. After this procedure, the islets were incubated for 60 min in the same type of medium as was used for rinsing. The results represent insulin release presented as mean values $\pm$ S.E.M. for eight different experiments. Statistical significances were judged from the differences between paired incubations of La^{3+} -pretreated and control islets: * $P < 0.05$.

Pretreatment with La^{3+} (mM)	Rinsing after exposure to La^{3+} (min)	Insulin release (ng/ μ g dry wt. of islets)	
		Primary data	Effect of La^{3+}
0	6	0.15 ± 0.04	—
0.5	6	0.86 ± 0.33	$+0.71 \pm 0.35$
2.0	6	0.47 ± 0.11	$+0.32 \pm 0.12^*$
0	15	0.13 ± 0.04	—
0.5	15	0.49 ± 0.12	$+0.36 \pm 0.13^*$
2.0	15	0.47 ± 0.12	$+0.34 \pm 0.12^*$
0	30	0.13 ± 0.01	—
0.5	30	0.59 ± 0.21	$+0.46 \pm 0.21$
2.0	30	0.38 ± 0.11	$+0.25 \pm 0.11$

Table 4. *Effects of La^{3+} on ^{45}Ca uptake into different calcium pools*

After preincubation in medium containing 2.56 mM- Ca^{2+} , the islets were incubated in the presence and absence of 0.5 mM- La^{3+} in the same type of medium containing 2.56 mM- ^{45}Ca (7.8 Ci/mol), 7 μM -[6,6- ^3H]sucrose (3000 Ci/mol) and either 3 or 20 mM-glucose. Radioactivity in the islets was measured with or without subsequent washing for 60 min with 2 mM- La^{3+} at $+1^\circ\text{C}$. The amount of ^{45}Ca in each pool after subtraction of label in the sucrose space is expressed as mmol of calcium, assuming the same specific radioactivity as in the loading medium. Mean values $\pm$ S.E.M. are given for six different experiments. Statistical significances were judged from the differences between paired incubations performed in the presence and absence of La^{3+} : * $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

	Glucose (mM)	^{45}Ca uptake (mmol/kg dry wt. of islets)		
		Control (a)	La^{3+} (b)	Effect of La^{3+} (b) - (a)
Total ^{45}Ca	3	19.68 ± 1.04	11.25 ± 0.35	$-8.43 \pm 1.11^{****}$
Non-displaceable ^{45}Ca	3	3.53 ± 0.27	2.13 ± 0.28	$-1.40 \pm 0.30^{***}$
Displaceable ^{45}Ca	3	16.12 ± 0.99	9.13 ± 0.39	$-7.00 \pm 1.02^{****}$
Total ^{45}Ca	20	20.47 ± 1.31	14.25 ± 0.59	$-6.22 \pm 1.43^{***}$
Non-displaceable ^{45}Ca	20	5.34 ± 0.62	3.46 ± 0.49	$-1.89 \pm 0.43^{***}$
Displaceable ^{45}Ca	20	15.13 ± 1.42	10.80 ± 0.84	$-4.33 \pm 1.47^*$

displace extracellular calcium. A stimulatory effect of glucose on ^{45}Ca uptake into the non-displaceable pool was also observed in the presence of La^{3+} , the difference being 1.33 ± 0.51 , compared with 1.81 ± 0.56 for control islets.

Effects of La^{3+} on glucose-dependent ^{45}Ca efflux

Perfusion of islets loaded with ^{45}Ca in the presence of 20mM-glucose was used to study the effects of La^{3+} on the efflux of Ca^{2+} . When introduced at concentrations between 0.8 and 2.0mM, La^{3+} decreased efflux to a low but constant rate within 10min (Figs. 3 and 4). This effect was often preceded by a transient stimulatory phase, which was particularly pronounced when the islets were perfused with a Ca^{2+} -deficient medium (Fig. 4).

It is well established that glucose has dual effects on the efflux of ^{45}Ca from preloaded islets. The prominent stimulatory phase of ^{45}Ca efflux observed on exposure to 20mM-glucose in the presence of 2.56mM- Ca^{2+} was almost completely sup-

pressed by 2mM- La^{3+} (Fig. 3). Since this effect of glucose is believed to represent an increased turnover of ^{45}Ca incorporated in response to glucose (Hellman *et al.*, 1979), it is pertinent to note that previous studies have shown that this high concentration of La^{3+} abolishes the islet uptake of ^{45}Ca in excess of the sucrose space (Hellman *et al.*, 1976a). The effect of La^{3+} on glucose-induced inhibition of ^{45}Ca efflux manifested during perfusion with a Ca^{2+} -deficient medium is shown in Fig. 4. It is evident that 2mM- La^{3+} also prevented the inhibitory action of glucose.

The observation that pretreatment with La^{3+} was associated with the subsequent stimulation of insulin release made it of interest to examine the effects of temporary exposure to La^{3+} on ^{45}Ca efflux. Fig. 5 illustrates the action of 0.8mM- La^{3+} on the radioactive efflux from islets loaded with ^{45}Ca in the presence of 3 or 20mM-glucose and perfused with medium containing 2.56mM- Ca^{2+} and 3mM-glucose. The inhibition observed during exposure to La^{3+} was not influenced by the glucose concen-

Fig. 3. Effects of La^{3+} on glucose stimulation of ^{45}Ca efflux

Experiments were performed in the parallel channels of a perfusion apparatus with islets loaded for 90min with 1.28mM- ^{45}Ca (780Ci/mol) in the presence of 20mM-glucose. The islets were perfused in medium containing 1.28mM- Ca^{2+} , with exposure to 20mM-glucose during the period from 60 to 100min as indicated by the horizontal open bar; 2mM- La^{3+} was also introduced in one of the channels, as indicated by the horizontal black bar. Filled symbols (●) signify islets exposed to La^{3+} and open symbols (○) refer to the corresponding control islets. The results are presented as the efflux of ^{45}Ca with the same specific radioactivity as in the loading medium, and are mean values $\pm$ S.E.M. for four different experiments.

Fig. 4. Effects of La^{3+} on glucose inhibition of ^{45}Ca efflux

Experimental details were as in the legend of Fig. 3, except that the islets were perfused in Ca^{2+} -deficient medium ($<50\text{ }\mu\text{M-Ca}^{2+}$).

tration of the loading medium. Removal of La^{3+} from the perfusion medium evoked a prompt increase in the rate of ^{45}Ca efflux. This effect was particularly pronounced with islets loaded in the presence of 20 mM-glucose, indicating that pre-treatment with La^{3+} leads to the preferential mobilization of ^{45}Ca incorporated in response to glucose.

Effects of La^{3+} on islet glucose oxidation

The oxidation of D-[U- ^{14}C]glucose was used as an index of the metabolism in islets exposed to La^{3+} . Since the removal of extracellular Ca^{2+} is in itself known to decrease the oxidation of glucose in *ob/ob*-mouse islets (Hellman *et al.*, 1974a), the effect of La^{3+} was examined at concentrations still permitting some entry of Ca^{2+} . As shown in Table 5, increasing the glucose concentration from 3 to 20 mM resulted in an approximate 4-fold elevation in the amount of glucose oxidized during 60 min. Even when included at concentration of 1 mM, La^{3+} was without effect on the oxidation rate at either glucose concentration.

Ultrastructure of β -cells exposed to La^{3+}

The electron-dense properties of the lanthanides

made it possible to observe directly the β -cell location of La^{3+} by conventional electron microscopy in both stained and unstained sections. The appearance of La^{3+} in islets incubated for 60 min with 2 mM- La^{3+} is shown in Plates 1 and 2. La^{3+} was confined exclusively to the plasma membranes. No La^{3+} deposits were seen in the interior of the β -cells, indicating the absence of intracellular penetration under standard incubation conditions. In addition to providing information about the site of La^{3+} action, electron microscopy allowed evaluation of possible toxic effects of the cation leading to alterations in the structure of the β -cells. As shown from the representative micrographs presented in Plates 1 and 2, the β -cells remained structurally unaffected after exposure to a high concentration of La^{3+} .

Discussion

Ca^{2+} is not only a prerequisite for insulin secretion, but also exerts direct stimulatory effects, as indicated by the observation that an increase in extracellular Ca^{2+} can actually initiate insulin release (Devis *et al.*, 1975; Hellman, 1976). However, it is evident from the dynamics of insulin release that temporary exposure of the islets to high con-

EXPLANATION OF PLATE 1

Low-power electron micrograph of β -cells exposed to La^{3+}

The islets were incubated for 60 min with 2 mM- La^{3+} in medium containing 3 mM-glucose and 2.56 mM- Ca^{2+} . The β -cells show well-preserved intracellular structures and clearly defined cell contours because of the exclusive localization of electron-dense La^{3+} deposits to the plasma membranes. Magnification $\times 6000$.

EXPLANATION OF PLATE 2

Ultrastructural details of β -cells exposed to La^{3+}

The islets were incubated for 60 min with 2 mM- La^{3+} in medium containing 3 mM-glucose and 2.56 mM- Ca^{2+} . (a) Peripheral region of two β -cells, showing lack of association of La^{3+} precipitates with secretory granules or other intracellular structures. Magnification $\times 21\,000$. (b) Detailed appearance of β -cell plasma membranes showing an abundant amount of La^{3+} precipitates. Magnification $\times 51\,000$.

Fig. 5. Effects of La^{3+} on the efflux of ^{45}Ca incorporated in response to glucose

Experiments were performed in the parallel channels of a perfusion apparatus with islets loaded for 120 min with 2.56 mM-Ca^{2+} (390 Ci/mol) in the presence of either (O) 3 mM- or (●) 20 mM- glucose. The islets were perfused with medium containing 3 mM-glucose , 2.56 mM-Ca^{2+} and albumin (1 mg/ml), with exposure to 0.8 mM-La^{3+} during the period from 40 to 80 min as indicated by the black horizontal bar. The results are presented as the efflux of ^{45}Ca with the same specific radioactivity as in the loading medium, and are mean values $\pm \text{S.E.M.}$ for four different experiments.

Table 5. Effects of La^{3+} on glucose oxidation

After preincubation in medium containing 2.56 mM-Ca^{2+} , the islets were incubated for 60 min in the absence and presence of La^{3+} in the same type of medium supplemented with 3 or $20 \text{ mM-D-[U-}^{14}\text{C]glucose}$ (5.67 and 0.85 Ci/mol). The amounts of $^{14}\text{CO}_2$ produced are expressed as mmol of glucose equivalents oxidized/h. Results are given as mean values $\pm \text{S.E.M.}$ for n experiments. No statistically significant effects of La^{3+} were found, as judged from the differences between paired test and control incubations.

Concn. of La^{3+} (mM)	Rate of oxidation (mmol/h per kg dry wt. of islets)			
	3 mM-glucose ($n = 7$)		20 mM-glucose ($n = 8$)	
	Primary data	Effects of La^{3+}	Primary data	Effects of La^{3+}
0	6.0 ± 0.6	—	27.3 ± 2.9	—
0.1	6.2 ± 0.6	$+0.2 \pm 1.1$	25.7 ± 3.9	-1.6 ± 4.5
0.3	5.7 ± 0.6	-0.3 ± 0.4	27.4 ± 3.0	$+0.1 \pm 2.7$
1.0	6.8 ± 0.7	$+0.8 \pm 0.8$	25.2 ± 2.0	-2.1 ± 3.0

centrations of Ca^{2+} is associated with both stimulatory and inhibitory effects (Hellman *et al.*, 1979). Accordingly, after an initial stimulatory phase, raising the extracellular Ca^{2+} concentration resulted in a progressive decline in the secretory rate until restoration of the original Ca^{2+} concentration

prompted a new phase of stimulated release. These observations, together with the existence of an optimal concentration of extracellular Ca^{2+} for glucose-induced insulin release (Milner & Hales, 1967; Hellman, 1975), have led to the concept that the regulation of insulin release is ultimately depend-

ent on the interaction between stimulatory and inhibitory pools of β -cell Ca^{2+} (Hellman *et al.*, 1979, 1980a,b).

In accordance with previous studies (Hellman *et al.*, 1974b), the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine not only potentiated the secretory response to glucose but also stimulated the basal insulin release. It has been observed that the optimal concentration of extracellular Ca^{2+} for insulin release is decreased after the addition of glucose and phosphodiesterase inhibitors (Hellman *et al.*, 1979). Consistent with the sensitization of the secretory machinery to the stimulatory effects of extracellular Ca^{2+} , the optimal secretory response to glucose even persisted after lowering of the Ca^{2+} concentration to 0.26 mM in the presence of 3-isobutyl-1-methylxanthine. In the present study, 0.5 mM- La^{3+} inhibited insulin release irrespective of the concentrations in the medium of Ca^{2+} , glucose and 3-isobutyl-1-methylxanthine. Whereas the addition of 3-isobutyl-1-methylxanthine was associated with a marked enhancement of the extent of inhibition, the relative effectiveness of La^{3+} was uninfluenced by raising the glucose concentration. It has been reported that 0.2 mM- La^{3+} evokes similar percentage decreases in insulin release at different glucose concentrations (Hellman, 1975). The observation that basal and glucose-stimulated insulin release were inhibited to a similar extent suggests that the inhibitory action reflects general stabilization of the β -cell plasma membrane. However, it seems probable that La^{3+} also exerts more specific effects, since inhibition could even be demonstrated after the addition of as little as 0.03 mM- La^{3+} to an albumin-containing medium.

Evaluation of the dynamics of insulin release from islets temporarily exposed to La^{3+} revealed a rapid onset of inhibition, which persisted until the lanthanide was omitted from the perfusion medium. Glucose-stimulated insulin release has been reported to be inhibited even after removal of 1 mM- La^{3+} (Lorenz *et al.*, 1979). It is therefore noteworthy that the present study revealed an impressive and prolonged enhancement of the secretory rate over the initial value on exclusion of the lanthanide from the perfusate. This stimulatory effect was subsequently inhibited by a second exposure to La^{3+} . More important, however, it was abolished by exposure of La^{3+} -pretreated islets to L-adrenaline, a substance which antagonizes the stimulatory action of glucose and other commonly used insulin secretagogues (Milner & Hales, 1969). Further support for the idea that the stimulation of insulin release after pretreatment with La^{3+} is related to a physiological mechanism was provided by the observation of a considerable potentiation with 3-isobutyl-1-methylxanthine. It has been suggested that changes in the transmembrane ionic milieu result in increased

activity of adenylate cyclase in the β -cell (Gagerman *et al.*, 1979). However, measurements of cyclic AMP provided no support for the idea that raised concentrations of this nucleotide were involved in the enhancement of insulin release after pretreatment with La^{3+} . It was not surprising to observe stimulation of insulin release in association with exposure to La^{3+} . In fact, La^{3+} has been shown to exert stimulatory effects in a number of secretory systems, including the release of adrenal catecholamines (Borowitz, 1972; Borowitz & Noller, 1977), histamine (Foreman & Mongar, 1972), neurohypophysial hormones (Matthews *et al.*, 1973) and amylase (Chandler & Williams, 1974). However, it was not possible to demonstrate a direct stimulatory effect of La^{3+} on insulin secretion, inhibition being exclusively found under a wide variety of experimental conditions. The fact that stimulation only occurred after a previous exposure to La^{3+} does not necessarily preclude the existence of a direct stimulatory mechanism, since this may well be masked by the prominent inhibitory action of the lanthanide.

In order to understand the mode of action of La^{3+} , it was essential to establish the cellular localization of the cation and examine its effects on both the structure and metabolism of the β -cells. Ultrastructural studies conclusively demonstrated that La^{3+} deposits were entirely restricted to the plasma membrane. Furthermore, there was no evidence of disruption of cell structures or other lesions after exposure to a high concentration of La^{3+} . Consistent with this observation, there was no increase in the sucrose space of islets exposed to La^{3+} . It therefore seems probable that the enhancement of insulin release seen after pretreatment with La^{3+} is due to neither leakage of insulin through damaged plasma membranes nor penetration of the lanthanide into the β -cells. Since insulin release is an energy-dependent process (Hellerström & Brodin, 1975), it is possible that La^{3+} -induced inhibition could reflect adverse effects of the cation on the metabolism of the β -cell. However, there was no significant suppression of glucose oxidation or prolonged decrease in cyclic AMP in islets exposed to La^{3+} . Furthermore, the relative magnitude of glucose stimulation of insulin release and ^{45}Ca uptake remained unaffected. Accordingly, it seems likely that the binding of La^{3+} to the plasma membrane is responsible for both the stimulatory and inhibitory effects of this cation on insulin secretion.

Evidence that La^{3+} both competes with Ca^{2+} at superficially located membrane binding sites and suppresses the intracellular uptake of ^{45}Ca was obtained by studying ^{45}Ca uptake in the presence of 0.5 mM of the lanthanide. It has been reported that 2 mM- La^{3+} , the concentration employed in the wash

technique, completely blocks the intracellular uptake of ^{45}Ca into islets from *ob/ob* mice (Hellman *et al.*, 1976a). However, as indicated from previous studies (Hellman, 1978; Herchuelz & Malaisse, 1978) and confirmed by the present data, 2 mM- La^{3+} markedly inhibits ^{45}Ca efflux, but fails to abolish it completely. In view of the persistent efflux of small amounts of ^{45}Ca in the presence of high concentrations of La^{3+} , it is advisable to perform the La^{3+} -wash technique at lower temperature in order to obtain a better estimate of the intracellular calcium content (Hellman, 1978). The demonstration that inhibition of ^{45}Ca efflux is often preceded by a transient stimulatory phase probably indicates a rapid displacement of ^{45}Ca bound to the exterior of the cells. Since proportionately more of the escaping ^{45}Ca can be supposed to be trapped at the cell surface at low Ca^{2+} concentrations, it was not surprising to observe a more pronounced stimulation when the islets were perfused with a Ca^{2+} -deficient medium.

Although 2 mM- La^{3+} did not completely prevent the efflux of ^{45}Ca , it abolished the glucose stimulation of ^{45}Ca washout normally obtained in the presence of physiological concentrations of Ca^{2+} . In view of the effectiveness of 2 mM- La^{3+} to prevent the islet uptake of ^{45}Ca (Hellman *et al.*, 1976a), these observations add further support to the view that glucose stimulation of ^{45}Ca efflux represents the increased turnover of ^{45}Ca incorporated in response to glucose (Hellman *et al.*, 1979). The mechanism by which glucose suppresses the efflux of ^{45}Ca during perfusion with a Ca^{2+} -deficient medium remains unclear. The inhibitory action might be a consequence of a lowered cytoplasmic concentration of Ca^{2+} caused by trapping of the cation by intracellular organelles, or alternatively a reflection of the competitive inhibition of ^{45}Ca efflux at the level of the plasma membrane (Malaisse *et al.*, 1979; Hellman *et al.*, 1980a,b). The present study indicates that 2 mM- La^{3+} completely abolishes the glucose-induced inhibition of ^{45}Ca efflux recorded in a Ca^{2+} -deficient medium. It is therefore tempting to attribute the glucose inhibition to an interaction with Ca^{2+} transport in the plasma membrane. However, possible indirect actions make it difficult to attribute the glucose effect to any specific mechanism. The intracellular Ca^{2+} distribution is, for example, dependent on the intricate nature of the ionic milieu within the cell (Rasmussen, 1977). In view of this relationship, it is pertinent to note that La^{3+} has also been shown to interfere with univalent-ion fluxes, leading to a decrease in the cellular content of cations such as Na^+ (Brading & Widdicombe, 1977).

In view of the essential role of Ca^{2+} in insulin secretion, it is possible to attribute the inhibitory action of La^{3+} to alterations in glucose-dependent

Ca^{2+} fluxes. Support for the involvement of Ca^{2+} in the stimulatory phase of insulin release after temporary exposure to La^{3+} was obtained with the demonstration of a preferential mobilization of the ^{45}Ca incorporated in response to glucose. Such a pattern of mobilization has previously been attributed to the increased entry of Ca^{2+} , which is subsequently exchanged for the radioactivity in the glucose-sensitive pools of intracellular Ca^{2+} (Hellman *et al.*, 1979). Consequently, the differences between ^{45}Ca efflux from islets loaded in the presence and absence of high concentrations of glucose suggest that there is an increase in the permeability to Ca^{2+} after pretreatment with La^{3+} . Such an idea is consistent with electrophysiological data indicating an increase of spike activity in the β -cell during this phase (Meissner *et al.*, 1980). Although the stimulated net uptake of Ca^{2+} could account for increased amounts of this cation in a stimulatory cytoplasmic pool after exposure to La^{3+} , it seems probable that additional mechanisms must exist. This is indicated by the fact that the stimulatory phase of insulin secretion persisted in a Ca^{2+} -deficient medium supplemented with 3-isobutyl-1-methylxanthine. An elevation of the cytoplasmic Ca^{2+} concentration can also be expected to result from the decrease in the efflux of Ca^{2+} and from modifications in the buffering capacity of organelles. In addition to probable changes in Ca^{2+} distribution following from alterations of physiologically important univalent cations within the cell (Brading & Widdicombe, 1977), the localization of La^{3+} makes it tempting to consider inward displacement of Ca^{2+} from the inner surface of the plasma membrane. Such a mechanism has previously been implicated for the La^{3+} -induced stimulation of oxytocin and neurophysin secretion (Matthews *et al.*, 1973). The possibility that La^{3+} might affect the inner structure of the plasma membrane through alterations in protein-lipid interactions has also been suggested from freeze-fracture studies of heart muscle (Weihe *et al.*, 1977).

The observation that insulin release was inhibited in the presence of La^{3+} does not exclude the possibility that Ca^{2+} actually accumulates in the stimulatory cytoplasmic pool of the β -cell during this phase. In fact, the effects of La^{3+} on insulin release can be best understood if the action of the stimulatory cytoplasmic Ca^{2+} pool is over-ridden by the binding of La^{3+} to inhibitory sites in the plasma membrane. In view of the competition between La^{3+} and Ca^{2+} for the superficially located membrane binding sites of the β -cell, insulin secretion may well be regulated by the interaction between stimulatory and inhibitory Ca^{2+} pools. Indeed, it is well established that extracellular Ca^{2+} actually exerts inhibitory as well as stimulatory effects on the secretory activity of the β -cell (Milner & Hales,

1967; Hellman, 1975, 1976; Wollheim *et al.*, 1978; Hellman *et al.*, 1979).

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