CALCIUM AND PANCREATIC β-CELL FUNCTION

5. Mobilisation of a glucose-stimulated pool of intracellular $^{45}$Ca by metabolic inhibitors and the ionophore A-23187

By

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ABSTRACT

Glucose is believed to stimulate incorporation of calcium into the secretory granules of the pancreatic β-cells. The mechanism of the glucose-stimulated accumulation of calcium in the granule pool was evaluated by measuring fluxes of $^{45}$Ca in β-cell-rich pancreatic islets microdissected from ob/ob-mice. The incorporation of lanthanum-nondisplaceable $^{45}$Ca in response to glucose differed from both the basal uptake and that seen in response to phosphate in being suppressed by 10 μM antimycin A, 0.3 mM 2,4-dinitrophenol or 1 mM N-ethylmaleimide. Exposure to each of these metabolic inhibitors also resulted in a protracted efflux of the glucose-sensitive $^{45}$Ca under conditions when neither the $^{45}$Ca incorporated in the presence of 3 mM glucose nor in response to phosphate was significantly affected. The glucose-stimulated intracellular $^{45}$Ca existed in a state allowing it to be washed out with the ionophore A-23187. The results suggest that the glucose-stimulated incorporation of calcium into the secretory granules is mediated by transport against a concentration gradient into the granule sac.

Calcium ions play a unique and critical role in a wide variety of physiological processes including secretion of insulin. Evidence has been provided that Ca$^{2+}$ is not only a prerequisite for the secretory machinery in the pancreatic β-cells but also exerts a direct regulatory role in the insulin release process (Wollheim et al. 1975; Hellman 1978; Malaisse et al. 1978). Exposure of pancreatic islets to glucose, the major natural stimulator of insulin release, results in trapping of calcium in a slowly exchangeable intracellular pool (Hellman et al. 1976).
The available data suggest that the secretory granules comprise the predominant part of the glucose-sensitive calcium within the pancreatic β-cells (Bloom et al. 1977; Hellman 1978a).

The pancreatic β-cells are not exceptional in their capacity to accumulate calcium in the secretory granules upon exposure to an appropriate stimulus. A similar situation also exists for adrenal chromaffin cells (Borovitz 1969; Serck-Hanssen & Christiansen 1973) and neurosecretory fibres (Ishida & Yoneda 1974). The question therefore arises as to whether the accumulation of calcium in secretory granules is an integral part of the stimulus-secretion mechanism, being a prerequisite to the extrusion of granules by exocytosis. Evaluation of this hypothesis necessitates a more detailed knowledge of the behaviour of the calcium located to the secretory granules. The present study adds to such a characterization by reporting how various metabolic inhibitors and the ionophore A-23187 influence the glucose-stimulated pool of intracellular 45Ca in β-cell-rich pancreatic islets microdissected from ob/ob-mice. It will be shown that impairment of the energy metabolism results in the preferential mobilisation of the calcium believed to be located in the secretory granules. Despite its slow exchangeability the glucose-stimulated intracellular calcium could be washed out almost completely with the ionophore A-23187.

MATERIAL AND METHODS

Animals and isolation of islets

About 10 months old ob/ob-mice were taken from a non-inbred colony (Hellman 1965) and starved overnight. The animals were killed by decapitation and the pancreatic islets isolated by microdissection.

Chemicals

Reagents of analytical grade and deionized water were used. Sigma Chemical Company, St. Louis, Mo., USA supplied N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (Hepes), bovine serum albumin (fraction V), antimycin A, 2,4-dinitrophenol and N-ethylmaleimide. Ethyleneglycolbis-(aminoethylether)tetraacetic acid (EGTA), 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) and LaCl3 were obtained from British Drug Houses Ltd., Poole, England and dimethylsulfoxide was supplied by Mallinkrodt Chemical Works, St. Louis, Mo., USA. The ionophore A-23187 was a gift from Eli Lilly Int. Corp., Indianapolis, Ind., USA. 45Ca was purchased from NEN Chemical GmbH, Dreieich, W. Germany. Antimycin A and A-23187 were added from stock solutions of the drugs in dimethylsulfoxide, bringing the final dimethylsulfoxide concentrations of the test and control media to 0.1 %o.

Uptake of 45Ca

The medium used for the uptake studies was a salt-balanced Hepes buffer (Hellman 1975a) of pH 7.4 containing 1.28 mM Ca2+ and either 3 or 20 mM glucose. In some cases the basal medium was supplemented with phosphate in the form of NaH2PO4 and Na2HPO4 with a concomitant reduction of NaCl to maintain a constant Na+.
concentration (Borle 1972; Hellman & Andersson 1978). Preliminary incubation of batches of 3 islets for 60 min at 37°C was followed by loading for 20 min with 45Ca (specific radioactivity 15.6 Ci/mol). After incubation, the islets were washed for 60 min at 1°C in non-radioactive medium containing 2 mM LaCl₃ to remove extracellular radioactivity without the loss of 45Ca from the interior of the cells (Hellman 1978b). After freeze-drying and weighing of the islets, lanthanum-nondisplaceable radioactivity was measured by liquid scintillation counting.

**Efflux of 45Ca**

Batches of 8–10 islets were loaded with 45Ca (specific radioactivity 390–780 Ci/mol) in the presence of 3 or 20 mM glucose during 120 min of incubation at 37°C in 100 µl of either the above-mentioned Hepes medium (Hellman 1975a) or a salt-balanced Tris buffer with 2.56 mM Ca²⁺ (Hellman et al. 1976). After two washing periods of 5 min in non-radioactive Hepes medium supplemented with 1 mg/ml albumin, the islets were placed in 10 µl chambers of teflon tubing and perifused at 37°C with the same type of medium. The perifusate was delivered at a constant rate of about 40 µl/min by a

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![Image](image_url)

**Fig. 1.**

Modifications of the islet uptake of La³⁺-nondisplaceable 45Ca in response to glucose or phosphate by different metabolic inhibitors. The islets were pre-incubated for 60 min at 37°C with 1.28 mM Ca²⁺ and 3 mM glucose in media supplemented or not with 10 µM antimycin A, 0.3 mM 2,4-dinitrophenol or 1.0 mM N-ethylmaleimide (NEM). Subsequent loading with 45Ca was performed during 20 min of incubation in similarly composed media containing 3 or 20 mM glucose, or 3 mM glucose combined with 5 (NEM and dinitrophenol) or 10 (antimycin) mM phosphate. The bars indicate the incorporation of La³⁺-nondisplaceable 45Ca for each of the inhibitors in relation to the uptake obtained in response to glucose or phosphate respectively (± 100 %).

Mean values ± SEM for 8 experiments.
Effect of 2,4-dinitrophenol on $^{45}\text{Ca}$ efflux from islets loaded with $^{45}\text{Ca}$ in a phosphate-free Tris medium containing 3 (○) or 20 (●) mM glucose. The islets were placed in the parallel channels of a perifusion apparatus and exposed to a medium containing 3 mM glucose and 2.56 mM $\text{Ca}^{2+}$. After 60 min, the perifusion medium was supplemented with 0.3 mM 2,4-dinitrophenol as indicated from the horizontal black bar. The results are given as the efflux of $^{45}\text{Ca}$ with the same specific activity as in the loading medium.

Mean values ± SEM for 4 separate experiments.

Evaluation of results

The islet content of $^{45}\text{Ca}$ at a given moment during efflux was obtained by adding the radioactivity subsequently released to that remaining in the islets at the end of the experiment. By expressing the uptake and efflux data in terms of calcium with the same specific radioactivity as in the loading medium the same procedure was followed...
as previously employed in our laboratory (Hellman et al. 1976; Gylfe & Hellman 1978). This is a convenient way to correct for unavoidable variations in the labelling media within a series of experiments. It should be emphasized that isotopic equilibrium did not exist in the islets under study (Hellman et al. 1976; Gylfe et al. 1978). The data obtained can consequently be used neither for estimates of absolute changes in different calcium pools nor for direct comparison of calcium uptake and efflux. Statistical significance of effects was judged from the differences between paired test and control data using the two-tailed Student's distribution.

RESULTS

The amount of La$^{3+}$-nondisplaceable $^{45}$Ca incorporated into the islets in the presence of 3 mm glucose was $3.46 \pm 0.18$ mmoles, kg$^{-1}$ dry weight (M ± SEM;

![Fig. 3.](image)

Effect of antimycin A on $^{45}$Ca efflux from microdissected islets. The left panel indicates the results obtained with islets loaded with $^{45}$Ca in the presence of 3 (○) or 20 (●) mm glucose in Tris medium containing 2.56 mm Ca$^{2+}$. The right panel indicates the results obtained with islets loaded with $^{45}$Ca in the presence of 20 mm glucose (●) or 3 mm glucose combined with 10 mm phosphate (○) respectively in Hepes-buffered medium containing 1.28 mm Ca$^{2+}$. The islets were placed in the parallel channels of a perifusion apparatus and exposed to a medium containing 3 mm glucose and 2.56 mm Ca$^{2+}$. After 60 min, the perifusion medium was supplemented with 10 μM antimycin A as indicated from the horizontal black bar. The results are given as the efflux of $^{45}$Ca with the same specific activity as in the loading medium. Mean values ± SEM for 4 separate experiments.

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Effect of N-ethylmaleimide (NEM) on $^{45}$Ca efflux from microdissected islets. The left panel indicates the results obtained with islets loaded with $^{45}$Ca in the presence of 3 (○) or 20 (●) mM glucose in Tris medium containing 2.56 mM Ca$^{2+}$. The right panel indicates the results obtained with islets loaded with $^{45}$Ca in the presence of 20 mM glucose (○) or 3 mM glucose combined with 5 mM phosphate (●) in Hepes-buffered medium containing 1.28 mM Ca$^{2+}$. The islets were placed in the parallel channels of a perifusion apparatus and exposed to medium containing 3 mM glucose and 2.56 mM Ca$^{2+}$. After 60 min the perifusate was supplemented with 0.1 or 1.0 mM MEM as indicated from the horizontal black bars. The results are given as the efflux of $^{45}$Ca with the same specific activity as in the loading medium. Mean values ± SEM for 4 separate experiments.

Elevation of the glucose concentration to 20 mM or the addition of 5 mM inorganic phosphate to the incubation medium increased the islet content of $^{45}$Ca by 2.19 ± 0.23 (n = 24) and 1.92 ± 0.49 (n = 16) mmoles, kg$^{-1}$ dry weight respectively. The basal uptake of $^{45}$Ca in the presence of 3 mM glucose was not significantly affected by 0.3 mM 2,4-dinitrophenol or 1.0 mM N-ethylmaleimide but increased by 43 ± 13% (n = 8) when the medium was supplemented with 10 μM antimycin A ($P < 0.01$). Fig. 1 indicates how the same amounts of these metabolic inhibitors affected the $^{45}$Ca taken up in response to glucose and phosphate. Whereas the glucose-stimulated uptake of $^{45}$Ca was effectively suppressed in the presence of antimycin A ($P < 0.005$), 2,4-dinitrophenol ($P < 0.025$) or N-ethylmaleimide ($P < 0.05$), these compounds did not affect (antimycin A and N-ethylmaleimide) or even tended to increase (2,4-
dinitrophenol; \( P \sim 0.05 \) the amounts of \(^{45}\)Ca taken up in response to phosphate.

Fig. 2 illustrates the influence of 2,4-dinitrophenol on the washout of \(^{45}\)Ca incorporated in response to glucose. Whereas the addition of 2,4-dinitrophenol to the perifusion medium did not result in marked modifications of the washout of radioactivity from islets loaded with \(^{45}\)Ca in the presence of 3 mM glucose, there was a clear effect on islets loaded in the presence of 20 mM glucose.

Fig. 3.

Effect of the ionophore A-23187 on \(^{45}\)Ca efflux from microdissected islets loaded with \(^{45}\)Ca in phosphate-free Tris medium containing 3 (○) or 20 (●) mM glucose. The islets were placed in the parallel channels of a perifusion apparatus and perifused in the presence of 3 mM glucose with medium deficient in Ca\(^{2+}\) (left panel) or containing 2.56 mM Ca\(^{2+}\) (right panel). After 40 min of perifusion the islets were exposed for a subsequent period of 40 min to 10 \( \mu \)g/ml A-23187 as indicated from the horizontal black bars. The results are given as the efflux of \(^{45}\)Ca with the same specific activity as in the loading medium. Mean values \( \pm \) SEM for 4 separate experiments.
Effect of the ionophore A-23187 on $^{45}$Ca content in microdissected islets initially loaded with $^{45}$Ca in the presence of 3 (○) or 20 (●) mM glucose and later exposed to 10 μg/ml of the ionophore during perfusion with calcium-deficient medium. The islets are identical to those presented in the left panel of Fig. 5. The results are given as the islet content of $^{45}$Ca with the same specific activity as in the loading medium.

In the latter case, the introduction of 2,4-dinitrophenol resulted in an initial transitory stimulation followed by a more permanent increase in the efflux rate. The second phase was reversible as indicated from experiments where 2,4-dinitrophenol was omitted from the perfusion medium after 40 min exposure to the drug (not shown). Antimycin A also caused selective mobilisation of the $^{45}$Ca incorporated in response to glucose; a similar biphasic pattern was observed for the washout of radioactivity from islets loaded with $^{45}$Ca in the presence of 20 mM glucose (left panel, Fig. 3). Islets loaded with $^{45}$Ca in the presence of phosphate reacted in a different way, exhibiting only the transient first peak of stimulated efflux rate after exposure to antimycin A (right panel, Fig. 3). Fig. 4 illustrates the influence of N-ethylmaleimide on the washout of $^{45}$Ca taken up in response to glucose and phosphate respectively. Introduction of a high concentration (1.0 mM) of this sulphhydryl reagent resulted in a transient initial peak even when the islets had been loaded with $^{45}$Ca in the presence of 3 mM glucose. However, the most impressive result of exposure to
N-ethylmaleimide was the late and protracted phase of stimulated $^{45}$Ca efflux from the islets loaded with $^{45}$Ca in the presence of 20 mM glucose. The fact that N-ethylmaleimide induced selective mobilisation of $^{45}$Ca incorporated in response to glucose was also evident from the washout of radioactivity from islets loaded with $^{45}$Ca in the presence of phosphate. In the latter case exposure to 0.1 mM N-ethylmaleimide resulted in neither a transient nor permanent increase in the rate of $^{45}$Ca efflux as was found with islets loaded in the presence of 20 mM glucose.

The effect of 10 $\mu$g/ml of A-23187 on $^{45}$Ca washout is shown in Fig. 5. Addition of the ionophore to medium containing 2.56 mM Ca$^{2+}$ resulted in a rapid but transient stimulation of efflux with a peak rate of about 40 $\mu$moles, kg$^{-1}$ dry islet, min$^{-1}$. The initial phase of stimulation was followed by a prolonged increase of $^{45}$Ca washout from islets loaded with $^{45}$Ca in the presence of 20 mM glucose. The stimulatory effect of A-23187 became particularly impressive during perifusion with medium deficient in Ca$^{2+}$. In the latter case, the initial response of islets loaded in the presence of 20 mM glucose was a peak efflux rate of no less than 140 $\mu$moles, kg$^{-1}$ dry islet, min$^{-1}$. The combination of such a marked initial effect with the absence of a secondary phase of stimulation suggests that the addition of A-23187 resulted in complete depletion of the glucose-stimulated pool of $^{45}$Ca when the perifusion medium was deficient in Ca$^{2+}$. Fig. 6 shows that this is actually the case. It is evident that insignificant amounts of $^{45}$Ca remained in the islets at the end of the incubation irrespective of whether loading with $^{45}$Ca was made in medium containing 3 or 20 mM glucose.

**DISCUSSION**

In the present study, inhibitors were selected with different primary actions on metabolism and with established effects on the $\beta$-cell function in the concentrations employed. Both antimycin A as a blocker of respiration and 2,4-dinitrophenol as an uncoupler of the oxidative phosphorylation inhibit the glucose-stimulated insulin release (Coore & Randle 1964; George et al. 1971). A permeability increase of the plasma membrane in terms of sucrose space has been observed after exposing microdissected islets from ob/ob-mice to 10 $\mu$m antimycin A (Hellman et al. 1973). N-ethylmaleimide was taken as representing a rapidly penetrating sulphydryl reagent. When the present type of islets are exposed to 0.1 mM N-ethylmaleimide both the oxidation of glucose and the glucose-stimulated insulin release are markedly suppressed (Hellman et al., unpublished observations).

Outward transport of Ca$^{2+}$ across the cell membrane is known to occur against a concentration gradient. Lowering the temperature has been found to
result in a rapid reduction of $^{45}$Ca efflux from islets microdissected from ob/ob-mice, suggesting a relationship between the metabolism of the pancreatic $\beta$-cells and their ability to extrude Ca$^{2+}$ (Hellman 1978b). It is likely that inhibition of efflux accounts for the increased islet uptake of $^{45}$Ca produced by antimycin A under basal conditions and by 2,4-dinitrophenol in the presence of phosphate. Metabolic inhibitors have also been reported to increase the net uptake of $^{45}$Ca in smooth muscle (Weiss & Goodman 1976). A depression of the active extrusion of Ca$^{2+}$ from the pancreatic $\beta$-cells might mask a concomitant mobilisation of calcium from intracellular organelles. It is for example well established that mitochondria incorporate Ca$^{2+}$ against a concentration gradient by an active transport process (Lehninger 1970; Mela 1977). Nevertheless there was no obligatory stimulation of the $^{45}$Ca washout when the pancreatic islets were exposed to the metabolic inhibitors.

Previous studies have indicated that both glucose and phosphate stimulate the intracellular uptake of $^{45}$Ca into $\beta$-cell-rich pancreatic islets microdissected from ob/ob-mice (Hellman & Andersson 1978). The $^{45}$Ca incorporated in response to glucose differed from that taken up in the presence of phosphate in being selectively mobilised during glucose stimulation of insulin release. The difference in behaviour between $^{45}$Ca incorporated in response to glucose and phosphate can be understood in terms of the location of the respective calcium pools within the pancreatic $\beta$-cells. Whereas both electron microscopic observations (Herman et al. 1973; Schäfer & Klöppel 1974) and subcellular fractionation analyses of $^{45}$Ca loaded islets (Bloom et al. 1977) indicate precipitation of glucose-sensitive calcium in the secretory granules, it seems likely that the presence of phosphate leads to deposition of calcium in the mitochondria (Borle 1972; Hellman & Andersson 1978).

The present study further contributes to the characterization of the calcium sequestered in the secretory granules by demonstrating that the $^{45}$Ca incorporated in response to glucose is particularly sensitive to agents interfering with the metabolism of the $\beta$-cells. Under conditions when neither the $^{45}$Ca taken up in the presence of 3 mM glucose nor in response to phosphate was significantly affected except for a peak of transient stimulation, exposure to either antimycin A, 2,4-dinitrophenol or N-ethylmaleimide resulted in an enhanced and protracted efflux of the glucose-sensitive intracellular $^{45}$Ca. It is likely that the biphasic pattern of this mobilisation is accounted for by concomitant depression of the outward transport across the plasma membrane as discussed above. It has been suggested that sulphydryl groups in the $\beta$-cell membrane participate in the control of insulin secretion (Hellman et al. 1974). This draws attention to the possibility that stimulated exocytosis of the secretory granules accounts for the selective mobilisation of the glucose-sensitive calcium in the presence of N-ethylmaleimide. However, the pronounced increase of $^{45}$Ca washout seen in the presence of 0.1 mM N-ethylmaleimide de-
finitely contrasts with the previous observation of a transient and insignificant stimulation of the insulin release process (Hellman et al., unpubl. observations).

In view of their ability to facilitate the transmembrane fluxes of divalent cations, carboxylic acid ionophores have been widely employed for studying the role of Ca\(^{2+}\) in secretory processes. In the present study the ionophore A-23187 was preferred to the related compound X-537A, since it lacks pronounced effects on the energy metabolism of the \(\beta\)-cells (Tamarit-Rodriguez et al. 1977). Irrespective of the extracellular calcium concentration, A-23187 exerts only moderate stimulatory effects on insulin release from islets microdissected from ob/ob-mice (Hellman 1975b). Although it is generally accepted that A-23187 acts at the level of the plasma membrane by increasing its permeability to Ca\(^{2+}\), the ionophore might also mobilise calcium from intracellular stores (Chandler & Williams 1977; Borle & Studer 1978). The latter mechanism is compatible with the observation that A-23187 stimulates the unidirectional \(^{45}\)Ca efflux from preloaded islets isolated from rats (Ashby & Speake 1975) and ob/ob-mice (Tamarit-Rodriguez 1978). Despite the low mobility of the glucose-stimulated intracellular \(^{45}\)Ca, the present study indicates that it exists in a state allowing complete washout in the presence of the ionophore A-23187. Since divalent cations suppress the cellular uptake of A-23187 (Chandler & Williams 1977), it was not surprising to notice that extensive depletion of the islet radiactivity was only achieved in a calcium-deficient medium.

The present study provides support for the view that Ca\(^{2+}\) transport across the granule membrane is an energy-dependent process by demonstrating that metabolic inhibitors both counteract the glucose-stimulated accumulation of \(^{45}\)Ca and promote the mobilisation of \(^{45}\)Ca already taken up in response to glucose. It can be anticipated that the energy is provided by ATP hydrolysis. ATP has been found to be a component of a secretory granule fraction isolated from rat islets (Leitner et al. 1975). Furthermore, Ca\(^{2+}\)-stimulated ATPase has been demonstrated in a subcellular fraction of secretory granules obtained by differential centrifugation of mouse islet homogenates (Formby et al. 1976). A dependence of the Ca\(^{2+}\) uptake of secretory granules on ATP hydrolysis does not necessarily imply involvement of an ATPase which is directly stimulated by Ca\(^{2+}\). The present data can also be understood if granule uptake of Ca\(^{2+}\) occurs via exchange for protons. The inhibitory effect of 2,4-dinitrophenol can for example be attributed not only to uncoupling of the oxidative phosphorylation in the mitochondria but also to short-circuiting of a granule proton pump due to leakage of protons across the granule membrane. The adrenal chromaffin cells behave in the same way as the \(\beta\)-cells by incorporating calcium in the secretory granules when exposed to a secretory stimulus (Borovitz 1969; Serck-Hanssen & Christiansen 1973). It is therefore of considerable interest that the chromaffin granules contain an ATPase serving as an inwardly directed electrogenic proton pump (Njus & Radda 1978; Phillips & Allison 1978).

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The fact that the glucose-stimulated accumulation of $^{45}$Ca in the $\beta$-granules appeared to be particularly sensitive to the inhibitors tested might be related to the properties of the ATPase involved. It was observed in studies of adrenal chromaffin cells that N-ethylmaleimide inhibits granule ATPase at concentrations lower than that required to inhibit ATPase from other subcellular fractions (Kirshner et al. 1966). It also seems likely that, under conditions of suppressed metabolism, the processes of active $Ca^{2+}$ transport most affected are those distant to the sites of energy production. The latter idea is consistent with data suggesting that the adenine nucleotide content of the $\beta$-granules does not readily exchange with those adenine nucleotide pools present in either the cytoplasm, plasma membrane or mitochondria (Sussman & Leitner 1977).

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