Active calcium uptake was demonstrated in a subcellular fraction of islets which was enriched in endoplasmic reticulum. Calcium uptake was stimulated by ATP in a magnesium-dependent manner. The rate of calcium accumulation was sustained by oxalate (10 mM) and uptake was prevented or reversed by addition of the calcium ionophore A23187. This calcium uptake process was not affected by aziode or ruthenium red. Direct comparison of calcium uptake by endoplasmic reticulum-enriched and plasma membrane-enriched fractions indicated that the uptake was not due to contamination of the fraction with plasma membrane vesicles. These factors as well as the purity of the fraction indicate that the calcium uptake system resides in the endoplasmic reticulum. The properties of the endoplasmic reticulum calcium uptake system are similar to properties reported for endoplasmic reticulum derived from other cell types. These properties include stimulation by potassium and a $K_m$ for ionized calcium of 1.5 ± 0.3 μM. The islet-cell endoplasmic reticulum may play a critical role in cellular calcium homeostasis and contribute to the regulation of insulin secretion.

Calcium ions are a critical intracellular regulator of insulin secretion. Not only is calcium an absolute requirement for glucose-induced insulin secretion (1), but conditions which elevate cellular calcium stimulate insulin secretion. An increased intracellular calcium concentration probably initiates and modifies β-cell secretion by mediating events at the cell surface and intracellular matrix (2). Investigations using $^{45}$Ca$^{2+}$ have clearly demonstrated that glucose (3-5) and other insulinogenic agents (2, 6) stimulate calcium uptake by isolated islets. This net uptake of calcium is demonstrated even when superficial calcium is displaced by washing with lanthanum. It is generally assumed that the stimulus-induced cellular uptake of $^{45}$Ca$^{2+}$ into lanthanum-displaceable pools represents calcium accumulation into cellular organelles and that the handling of calcium by these organelles is dynamically involved in stimulus-secretion coupling (7-9).

While it is clear that cellular content and distribution of calcium ions are important determinants of β-cell function, the precise control of calcium homeostasis by β-cell organelles remains to be elucidated. ATP-stimulated calcium accumulation has been demonstrated in islet-cell homogenates (10) and in crude subcellular fractions (11, 12). However, these studies have centered on steady state accumulation of calcium and have not investigated the kinetics of calcium uptake.

Furthermore, these studies have not localized the calcium accumulation to a specific organelle. Recent evidence has indicated that endoplasmic reticulum of noncontractile cells (13, 14) has the similar ability to accumulate calcium as has been well characterized in sarcoplasmic reticulum of contractile cells (15). The present study describes and characterizes ATP-stimulated calcium uptake by islet-cell endoplasmic reticulum. This work is part of a series of investigations designed to determine the role of the endoplasmic reticulum in β-cell stimulus-secretion coupling.

EXPERIMENTAL PROCEDURES

Materials—Male Wistar rats (200 to 300 g) were purchased from Charles Rivers Laboratories (Wilmington, MA). Collagenase (CLS IV) was obtained from Worthington. $^{44}$CaCl$_2$ from New England Nuclear, and vanadate-free ATP and other chemicals from Sigma. Filters which were used in the calcium uptake assay were Millipore type GSWP, pore size 0.22 μm.

Islet Fractionation—Pancreatic islets were obtained under semisterile conditions using a modification of the standard collagenase digestion technique (16). Freed islets were collected on discontinuous Ficoll gradients at the interface of 20.5% and 11% Ficoll (17), rinsed in Hanks' solution, and individually selected under a dissecting microscope. Islets isolated in this way were maintained at 24 °C for 24 to 48 h in culture medium (CMRL 1066, Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 8 mM glucose at pH 7.4 (95% air, 5% CO$_2$).

Islets (6,000 to 10,000) obtained from 33 to 36 rats were pooled and fractionated by a modification of the technique of Naber et al. (18). In the present studies, initial homogenization of the islets was performed at pH 7.2 rather than at pH 6.0. Homogenization at pH 7.2 resulted in retention of a greater percentage of endoplasmic reticulum marker enzymes (NADH-cytochrome c reductase and glucose-6-phosphatase) in the post-mitochondrial supernatant than under the latter conditions. The islets were homogenized in 1 ml of fractionation buffer (50 mM MES, 250 mM sucrose, 1 mM EDTA, pH 7.2 at 4 °C). The homogenate was centrifuged at 600 × g for 5 min, resuspended in 1 ml of fractionation buffer, and recentrifuged at 600 × g for 5 min to remove nuclear material and debris. The supernatant was further pelleted at 20,000 × g for 20 min and the resulting (post-mitochondrial) supernatant was then centrifuged at 150,000 × g for 90 min to yield an endoplasmic reticulum-enriched pellet. This pellet was resuspended in 600 to 900 μl of fractionation buffer minus EDTA, rehomogenized, and utilized immediately in calcium uptake assays. In some cases, the endoplasmic reticulum-enriched fraction was stored at -70 °C for up to 3 days with an average loss of 23% of the original calcium uptake activity. Biochemical assessment of endoplasmic reticulum enrichment and relative purity of the fraction employed marker enzyme assays as described previously (18), except in the case of glucose-6-phosphatase which was assayed by the method of Kitcher et al. (19). Total protein was determined by a fluorescamine method (20) using bovine serum albumin as standard.

In three separate experiments, calcium uptake was also assessed in a fraction enriched in plasma membrane. The plasma membrane-

*This work was supported in part by AM03373, AM25897, and a grant from the Juvenile Diabetes Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid.
enriched fraction was obtained from the 20,000 × g pellet following rehomogenization in 10 mM MES at pH 6.0 and separation on discontinuous sucrose gradients after centrifugation at 150,000 × g for 90 min. The plasma membrane fraction was collected at the surface of 1.14 sucrose density (18), pelleted at 150,000 × g for 60 min, and rehomogenized in fractionation buffer minus EDTA prior to assay.

Assay of Calcium Uptake—Calcium uptake was assayed by a filtration technique using tracer 45CaCl2. Standard incubations were that system for ionized calcium, calcium concentrations were buffered with oxalate/[Mg"]/[Mg"]/[oxalate] which are given by atomic absorption spectrophotometry.

In other experiments designed to determine the Ka of the uptake system for ionized calcium, calcium concentrations were buffered with 0.2 mM EGTA, and free calcium levels at molar ratios of 0.5 to 0.95 [calcium]/[EGTA] were estimated assuming an association constant (pH = 6.8) for [Ca.EGTA]/[Ca2'][EGTA] (23) and association constants for calcium and magnesium oxalate which are given above.

The calcium uptake assay was initiated by the addition of 2 to 5 μg of protein to a final volume of 100 μl in 1.5-ml capacity polypropylene tubes. The uptake assay was stopped by filtration through 0.22-μ Millipore filters (presoaked in 0.25 mM NaCl at room temperature. The total washing time was 25 s/sample. The filters were air-dried, dissolved in 0.5 ml of ethylene glycol monomethyl ether, and counted by standard liquid scintillation procedures. Assays were performed in duplicate or triplicate. N refers to the number of separate tissue preparations which were used for a particular experiment.

**RESULTS**

**Characterization of the Endoplasmic Reticulum-enriched Fraction**—The endoplasmic reticulum-enriched fraction from islet cells contained a 2- to 3-fold enhancement of endoplasmic reticulum marker enzymes (NADH-cytochrome c reductase and glucose-6-phosphatase) relative to the cell homogenate. By contrast there was little or no biochemical evidence (Table I) for contamination of this fraction with mitochondria (succinic cytochrome c reductase) or insulin secretory granules (extractable immunoreactive insulin). The endoplasmic reticulum-enriched fraction contained some contamination of plasma membrane marker (5'-nucleotidase) but a much greater enhancement of 5'-nucleotidase occurred in other subcellular fractions (Table I).

**Table 1**

|                     | Total protein | NADH-cytochrome c | 5'-Nucleotidase | Succinic cytochrome c reductase | Insulin |
|---------------------|--------------|-------------------|----------------|-------------------------------|---------|
|                     | μg           | % rec             | % rec          | % rec                         | % rec   |
| Endoplasmic reticulum-enriched fraction | 181 ± 34 | 4.42 ± 0.87 | 7.06 ± 0.85 | 2.35 ± 0.38 | 7.38 ± 0.58 | 1.84 ± 0.35 | 0 | 0 | 0.68 ± 0.02 | 0.18 ± 0.03 |
| Plasma membrane-enriched fraction | 73 ± 15 | 1.75 ± 0.25 | 2.20 ± 0.12 | 1.40 ± 0.17 | 9.58 ± 0.59 | 5.71 ± 0.54 | 0.23 ± 0.14 | 0.15 ± 0.09 | 0.45 ± 0.13 | 0.25 ± 0.03 |

Electron microscopic examination of the endoplasmic reticulum fraction documented that this fraction was composed primarily of small membrane vesicles with few secretory granules (Fig. 1). A single mitochondrion was located in one preparation.

**Time Course of Calcium Uptake**—Initial studies indicated that a small amount of calcium was associated with the endoplasmic reticulum in the absence of added ATP and that this amount of calcium was approximately doubled by addition of ATP. Fig. 2 demonstrates that the small amount of calcium associated with the membranes in the absence of ATP was not affected by the addition of 10 mM oxalate which
was added to trap intravesicular calcium by precipitation (24). By contrast, in the presence of ATP (1.25 mM), 10 mM oxalate stimulated calcium uptake for at least 30 min and stabilized the rate of calcium uptake for at least 30 min. Thus, under these conditions in the presence of oxalate it was possible to measure the rate of ATP-stimulated calcium uptake. In 23 experiments performed under conditions similar to Fig. 2, calcium accumulation by the islet endoplasmic reticulum fraction was 6.85 ± 0.54 nmol of calcium/mg of protein/15 min in the absence of ATP and was increased to 28.78 ± 2.45 nmol/mg of protein/15 min during a 15-min incubation by the addition of 1.25 mM ATP.

Effect of Calcium Ionophore A23187—The probability that ATP-stimulated calcium accumulation by the endoplasmic reticulum fraction represented vesicular uptake of calcium against a concentration gradient was investigated by the addition of 6 mM ionophore A23187 to the calcium uptake system after 15 min incubation. The ionophore depleted vesicular calcium within 5 min. Calcium remaining in the vesicles after the ionophore treatment is presumably precipitated within the vesicles as calcium oxalate. Addition of the ionophore prior to initiation of the assay prevented ATP-stimulated uptake of calcium (Fig. 3).

ATP and Magnesium Dependency of the Calcium Uptake Process—Variation of the concentration of ATP (magnesium held constant at 5 mM) demonstrated that addition of as little as 200 μM ATP produced maximum activation of calcium uptake. The rate of ATP-stimulated calcium uptake was not appreciably changed as ATP was increased from 0.2 to 5 mM (N = 3, data not shown).

In contrast to the saturation of the uptake process at low levels of ATP, there was a marked dependence on higher concentrations of magnesium. When the concentration of ATP was held constant (1.25 mM), the rate of ATP-stimulated calcium uptake increased with the magnesium concentration (Fig. 4). Although saturation of the MgATP requirement (200 μM MgATP, see above) occurred at 350 μM MgCl₂, maximum stimulation of calcium uptake did not occur until MgCl₂ attained 2 to 5 mM or 200 to 1000 μM ionized Mg²⁺ (assuming stability constants given for MgATP and Mg-oxalate stated under "Experimental Procedures"). This result would imply a requirement for Mg²⁺ in excess of MgATP. ATP-stimulated calcium uptake was absolutely dependent on Mg²⁺ since in the absence of added magnesium the rate of calcium accumulation was unaffected by the addition of ATP.
The possibility that calcium uptake by the endoplasmic reticulum fraction may be due in part to contamination by inside-out vesicles of plasma membrane was evaluated by simultaneous determination of calcium uptake in the plasma membrane-enriched islet-cell fraction (Table I) and in the endoplasmic reticulum-enriched fraction. Although the amount of calcium associated with the membranes in the absence of ATP was similar for both subcellular fractions, the rate of calcium uptake in the presence of 1.25 mM ATP was much higher in the endoplasmic reticulum-enriched fraction, and ATP-dependent uptake (corrected for accumulation in the absence of ATP) by the plasma-membrane-enriched fraction was only 11% of that observed in the endoplasmic reticulum-enriched fraction (Fig. 6). Additionally, ATP-dependent calcium uptake by the plasma-membrane-enriched fraction was similar in the presence or absence of 100 mM KCl (0.21 ± 0.13 versus 0.21 ± 0.08 nmol/mg of protein/min).

Affinity of the Uptake Mechanism for Calcium—ATP-stimulated calcium uptake increased as the total calcium concentration was increased from 1 to 80 μM under standard assay conditions. The apparent $K_m$ of the uptake process for total calcium (calculated from double reciprocal analysis of seven experiments) was 19.7 ± 1.9 μM total calcium. This corresponds to an apparent $K_m$ for ionized calcium of 7.2 ± 0.7 μM.

The affinity of the calcium uptake system for calcium was also evaluated by varying free calcium levels with calcium/EGTA ratios (0.5 to 0.95). Calculation of free calcium is described under "Experimental Procedures." The apparent $K_m$ for free Ca$^{2+}$ (five experiments) was 1.5 ± 0.3 μM. Fig. 7 shows a representative experiment where both total calcium and EGTA-buffered free calcium levels were compared in the same assay.

**DISCUSSION**

ATP-stimulated calcium uptake was demonstrated in an endoplasmic reticulum-enriched fraction obtained from pancreatic islet cells. The vesicular nature of the membranes, potentiation of ATP-stimulated calcium uptake by oxalate, and effects of pre- and post-treatment of the vesicles with ionophore A23187 indicated that the ATP-stimulated calcium accumulation represents uptake of calcium into membrane vesicles against a concentration gradient.

The active calcium uptake process studied in this fraction was localized to the endoplasmic reticulum by biochemical and morphological characterization of the fraction. Consistent with these results, neither sodium azide nor ruthenium red had an effect on calcium uptake by the endoplasmic reticulum-enriched fraction. A similar dose of ruthenium red has been reported to reduce calcium uptake by islet-cell mitochondria to 5% of control values (8). As there was some contamination of the endoplasmic reticulum-enriched fraction with 5'-nucleotidase, calcium uptake by this fraction could be due in part to the presence of inside-out vesicles of plasma membrane. Although there was a 3-fold greater enhancement of 5'-nucleotidase activity in the plasma membrane-enriched fraction as compared to the endoplasmic reticulum-enriched fraction, the specific activity of calcium uptake was more than 9 times greater in the endoplasmic reticulum-enriched fraction. The data indicated, therefore, that the majority of the active calcium uptake reported in these studies can be attributed to the endoplasmic reticulum.
The properties of calcium uptake by the islet-cell endoplasmic reticulum were similar to the properties of calcium uptake described for endoplasmic reticulum in other cell types. Similar requirements for ATP, dependence on magnesium, specific stimulation by potassium, have been demonstrated for endoplasmic reticulum derived from tissues as diverse as liver, fat, platelets, and brain. The kinetic properties of the calcium uptake system of pancreatic islet-cell endoplasmic reticulum with regard to calcium are similar to those reported for endoplasmic reticulum of other cells (13, 14). As a considerable portion of the total calcium is chelated by anions present in the assay medium, the actual affinity for free calcium is much higher. Under the experimental conditions employed here, approximately 36.4% of the total calcium (at 40 to 80 μM total calcium) remains free. Hence, an apparent Kₘ of 19.7 μM total calcium would correspond to an apparent Kₘ for free calcium of 7.2 μM. A second independent estimation of the affinity of the uptake mechanism for free calcium employed EGTA-buffered free calcium and yielded an apparent Kₘ of 1.5 ± 0.2 μM free calcium. Although these estimates are higher than the submicromolar levels of free calcium which are assumed to persist in the cytosol, higher calcium levels may be attained at this subcellular site. Furthermore, B-cell calcium content may fluctuate with the secretory state of the cell.

Investigations of calcium movements in whole islets has left little doubt that dynamic handling of calcium by the islet is intimately involved in the regulation of the insulin secretory process. Glucose (3–5), other secretogogues, as well as elevation of extracellular potassium (28) increase calcium uptake by the islets. Although the initial effect of insulin secretogogues may be to increase calcium entry at the plasma membrane by affecting voltage-dependent channels (29) and/or affecting the calcium extrusion mechanism (30, 31), the resulting increase of calcium accumulation into lanthanum-non-displaceable pools is generally assumed to indicate accumulation of calcium by intracellular organelles. Thus, cellular organelles may participate in the regulation of calcium concentration in the cytosol or at specific subcellular loci. In this way, for example, factors such as starvation may increase calcium accumulation by the islets but reduce the subsequent secretory response by lowering the concentration of calcium at a critical regulatory site(s) (32). Hellman and co-workers (33) have preloaded islets which were preloaded with "Ca²⁺" in an attempt to locate the intracellular sites of calcium sequestration. These studies demonstrated that although much of the accumulated radiocalcium was confined to the fraction containing insulin secretory granules, the mitochondrial and microsomal fractions were the only metabolically labile calcium stores.

Because of the difficulty of obtaining large quantities of islet cells for subcellular fractionation, few studies have focused on the direct properties of the islet organelles with regard to calcium uptake. Sugden and Ashcroft (8) have measured calcium uptake by islet-cell mitochondria, and Howell (10, 11) and Sehlin (12, 33) have reported ATP-stimulated calcium accumulation in islet-cell microsomal fractions. Sehlin's recent studies (33) have demonstrated that the steady state calcium accumulation at 1 h by such a fraction is dependent on magnesium and potassium. However, because of the limitation of the quantity of islets, these studies have not utilized well defined subcellular fractions and the calcium accumulation observed cannot be ascribed conclusively to a particular organelle. Neither have these studies elucidated the kinetics of a calcium uptake process.

By utilizing large numbers of islets (6,000 to 10,000) and refinement of our subcellular fractionation techniques, we have been able to characterize an islet subcellular fraction which is enriched in endoplasmic reticulum. Furthermore, we have defined an active calcium uptake system in this fraction and localized this active process to the endoplasmic reticulum. This calcium uptake process has properties which are similar to those reported for endoplasmic reticulum isolated from other cell types. The affinity of this uptake system for calcium is at least 5 times greater than that reported for the calcium uptake by islet-cell mitochondria (8). It seems likely, therefore, that the islet-cell endoplasmic reticulum which mediates ATP-dependent calcium accumulation may be involved in controlling intracellular calcium homeostasis and play an important role in the regulation of insulin secretion. The definition of this calcium uptake system will allow direct investigation of these possibilities.

Acknowledgments—We acknowledge the assistance of Dr. Barry Siegfried, Jewish Hospital of St. Louis, and Dr. Marilyn Ackerman, Washington University Department of Chemistry, in the consideration of ionized levels of Ca²⁺ and Mg²⁺.

REFERENCES

1. Grodsky, G. M., and Bennett, L. L. (1966) Diabetes 15, 910-913.
2. Malaisse, W. J., Hirschuezl, A., Devis, G., Somers, G., Boscherio, A. C., Hutton, J. C., Kawazu, S., Sener, A., Atwater, I. J., Duncan, G., Ribalet, B., and Rojas, E. (1978) An. N. Y. Acad. Sci. 307, 562-582.
3. Malaisse-Lagae, F., and Malaisse, W. J. (1971) Endocrinology 88, 72-80.
4. Hellman, B., Sehlin, J., and Taljedal, I.-B. (1976) J. Physiol. 254, 659-656.
5. Naber, S. P., McDaniel, M. L., and Lacy, P. E. (1977) Endocrinology 101, 686-693.
6. Hellman, B., Lenzen, S., Sehlin, J., and Taljedal, I.-B. (1977) Diabetologia 13, 49-53.
7. Rizzarelli, M., Malaisse-Lagae, F., Ambert, M., Perrelet, A., Malaisse, W., and Orli, C. (1976) J. Cell Sci. 27, 107-117.
8. Sugden, M. C., and Ashcroft, S. J. H. (1978) Diabetologia 15, 173-180.
9. Hellman, B., Sehlin, J., and Taljedal, I.-B. (1971) Am. J. Physiol. 221, 1795-1801.
10. Howell, S. L., and Montague, W. (1975) FEBS Lett. 52, 48-52.
11. Howell, S. L., Montague, W., and Tryhurst, M. (1975) J. Cell Sci. 19, 395-409.
12. Sehlin, J. (1976) Biochem J. 156, 66-69.
13. Moore, L., Chen, T., Knapp, H. R., Jr., and Landon, E. J. (1975) J. Biol. Chem. 251, 6252-6258.
14. Bruns, D. E., McDonald, J. M., and Jarett, L. (1976) J. Biol. Chem. 251, 7191-7197.
15. Erbashi, S., and Endo, M. (1968) Prog. Biophys. Mol. Biol. 18, 183-183.
16. Lacy, P. E., and Kostianovskiy, M. (1967) Diabetes 16, 35-39.
17. Shibata, A., Ludvigsen, C. E., and Lacy, P. E. (1977) Diabetes 26, 701-709.
18. Naber, S. P., McDonald, J. M., Jarett, L., McDaniel, M. L., and Lacy, P. E. (1976) Diabetes 25, 667-672.
19. Sehlin, J. (1976) Diabetes 15, 439-444.
20. Kitcher, S. A., Siddle, K., and Luzio, J. P. (1978) Anat. Rec. 188, 29-36.
21. Orr, H. T., Cohen, A. I., and Lowry, O. H. (1976) J. Neurochem. 26, 609-611.
22. Katz, A. M., Repke, D. L., Upshaw, J. E., and Polascik, M. A. (1970) Biochim. Biophys. Acta 208, 473-490.
23. Perrin, D. D. (1979) Stability Constants of Metal-Zon Complexes, Part B Organic Ligands, IUPSC Chemical Data Series No. 2, Pergamon Press, New York.
24. Schatzman, J. H. (1973) J. Physiol. 235, 551-559.
25. Solano, R. J., and Briggs, F. N. (1973) Circ. Res. 31, 531-539.
26. Black, B. L., Jarett, L., and McDonald, J. M. (1980) Biochim. Biophys. Acta 596, 359-371.
27. de Meis, L., Rubin-Altschul, B. M., and Machado, R. D. (1970) J. Biol. Chem. 245, 1883-1889.
28. Roberts, L. S., Sheiner, D., and Belamarich, F. A. (1973) J. Gen. Physiol. 61, 461-461.
Calcium Uptake by Islet-Cell Endoplasmic Reticulum

28. Hellman, B., Sehlin, J., and Taljedal, I-B. (1978) Pflügers Arch. Eur. J. Physiol. 378, 93–97
29. Meissner, H. P. (1976) J. Physiol. (Paris) 72, 757–767
30. Pershadsingh, H. A., McDaniel, M. L., Landt, M., Bry, C. G., and Lacy, P. E. (1980) Nature 288, 492–495
31. Siegel, E. G., Wollheim, C. B., Renold, A. E., and Sharp, G. W. G. (1980) J. Clin. Invest. 66, 996–1003
32. Hahn, H-J, Gylfe, E., and Hellman, B. (1980) Biochim. Biophys. Acta 630, 425–432
33. Sehlin, J. (1981) Am. J. Physiol. 240, C35–38
Active calcium uptake by islet-cell endoplasmic reticulum.

J R Colca, J M McDonald, N Kotagal, C Patke, C J Fink, M H Greider, P E Lacy and M L McDaniel

_J. Biol. Chem. 1982, 257:7223-7228._

Access the most updated version of this article at [http://www.jbc.org/content/257/12/7223](http://www.jbc.org/content/257/12/7223)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/257/12/7223.full.html#ref-list-1](http://www.jbc.org/content/257/12/7223.full.html#ref-list-1)