Journal of Supramolecular Structure 9:327–336 (1978)
Transmembrane Signaling 311–320

Cation-Dependent Phosphatase Activites in a Rat Pancreatic Islet Plasma Membrane Fraction Prepared by One-Step Gradient Centrifugation

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A plasma membrane-enriched fraction was prepared from homogenized rat pancreatic islets by a one-step sucrose gradient centrifugation. Using ^{125}I-wheat germ agglutinin as a plasma membrane probe, a fraction was obtained at a sucrose density of about 1.10 that was enriched in 5′-nucleotidase, Mg^{2+}-ATPase and alkaline phosphatase. The fraction contained little, if any, monoamino oxidase activity, insulin or DNA. Hydrolysis of 3-0-methyl-fluoresceinphosphate was stimulated by K^+ (10mM) at a pH optimum of pH 8.2. Hydrolysis of ATP-γ-^{32}P in the presence of $MgCl_2$ was of high specific activity and was optimum at pH 7.0 and 8.2. K^+ did not affect ATP-hydrolysis. At pH 8.2, a small fraction of the total Mg^{2+}-ATPase activity was inhibited by ouabain in the presence of Na^+ and K^+. Since K^+-stimulated phosphatase activity does not correlate with Mg^{2+}-ATPase, the two assay systems define separate enzymatic processes.

Key words: cation transport, Mg^{2+}-ATPase, insulin secretion

The process of insulin secretion seems to be dependent on the cation-composition of the extracellular medium. Insulin release in vitro is increased during conditions that would either increase the sodium permeability of the cells or inhibit a hypothetical sodium pump in the β-cell plasma membrane [1]. Ouabain or a K^+-free medium, modifications that both would inhibit a sodium pump, mimick the stimulatory effect of glucose on the electrical activity of β-cells impaled with microelectrodes [2]. It has also been found that glucose, but not non-insulin releasing sugars, inhibits efflux of radioactive potassium or rubidium from isolated islets [3, 4]. This suggests that the depolarization seen after glucose-stimulation may be mediated by a decrease in potassium permeability which, in turn, would require the presence of a monovalent cation-pump to establish a cation-gradient across the β-cell plasma membrane [3, 4].

Received April 3, 1978; accepted September 15, 1978.

The demonstration of an ouabain-sensitive uptake of radioactive potassium or rubidium in isolated islets [5, 6] support the hypothesis of a sodium pump in the β-cells. In many different cell types the ouabain-sensitive (Na^+ plus K^+) Mg^{2+}-ATPase is considered to be the enzymatic basis of the "sodium pump" activity. In homogenates or crude subcellular fractions of isolated islets this enzyme activity is low or hardly detectable [7–9]. Enzymes catalyzing cation-dependent cleavage of certain organophosphate indicators are, however, also implicated in cation transport in many cells.

Islet cell plasma membranes [8] prepared from ob/ob mouse islets were found to contain a ouabain-sensitive K^+-activated p-nitrophenyl phosphatase [10]. The K^+-activated phosphatase activity was markedly inhibited by ATP, but not other nucleotides. Since organophosphate indicators represent artificial substrates it was suggested that catalysis of monovalent cation flow in pancreatic β-cells may be coupled to a phosphoryl-transfer reaction with ATP as a natural regulator [10]. To test this hypothesis we have therefore studied the hydrolysis of ATP in the presence of potassium, sodium and ouabain, in a plasma membrane enriched fraction of isolated rat islets.

In the present study, we also describe a modification of our method of isolating plasma membranes from isolated islets [8]. Thus it will be shown that a fraction enriched in plasma membranes can be isolated from a few milligrams of total islet homogenate protein by a one-step sucrose gradient centrifugation procedure.

METHODS

Islets of Langerhans were isolated by collagenase digestion of the pancreas from 8–12 rats as described [8]. After separating the islets from the pancreatic digest by Ficoll-gradient centrifugation they were individually chosen under a stereomicroscope and homogenized at 4°C in Mg-borate buffer (10 mM borate buffer, pH 8.0, containing 0.2 mM $MgCl_2$) supplemented with 3% (w/w) sucrose. The homogenate (1.8 ml) was layered on top of a sucrose gradient in Mg-borate buffer supplemented with 1 mM EGTA with the following composition from top to bottom: 4 ml 6-14% (w/w) linear sucrose gradient, 6 ml 23–43% (w/w) linear sucrose gradient, and 1 ml 50% (w/w) sucrose. The gradient was layered in 14 × 94 mm cellulose nitrate tubes and centrifuged at 4°C for 2 h at 25,000 rpm in a SW40 rotor with the Beckman L5-65 ultracentrifuge (Beckman Spinco, Palo Alto, California). The gradient was sampled from the top by a density gradient fractionator (Model 184, Instrumentation Specialities Company, Lincoln, Nebraska) into about 30 fractions. Sucrose densities were determined in an Abbe's refractometer (Erma Optical Works, Tokyo, Japan). Intact islets were labeled with ^{125}I-wheat germ agglutinin before homogenization and sucrose gradient centrifugations as described previously [8]. Determinations of 5′-nucleotidase, insulin, protein and DNA have also been given elsewhere [8]. Monoamino oxidase activity was determined by the method of Wurtman and Axelrod [11]. The conditions for the assays of 3-0-methylfluorescein phosphatase [12] and adenosine triphosphatase (ATPase) are given in Table I and in the legends to figures and tables. Sigma Chemical, St. Louis, Missouri supplied 3-0-methylfluorescein phosphate, 3-0-methylfluorescein, used as standard, as well as Na and Tris salts of ATP. Adenosine-5′-triphosphate, tetra (triethylammonium)-γ-^{32}P (25–35 Ci/mmol) was from New England Nuclear, Boston.

TABLE I. Conditions for Assays of 3-0-Methylfluoresceine Phosphatase and Adenosine Triphosphate Activities

Enzyme activity	Substrate	Buffer	Final volume	Product determination	References
3-0-methyl-fluoresceine phosphatase	0.8-1 μM 3-0-methyl fluoresceine phosphate	40mM Tris-acetate 5 mM $MgCl_2$	200 μl	3-0-methyl fluoresceine (fluorimetric)	[12, 13]
Adenosine-triphosphatase or	0.5-1 mM adenosine-5'-triphosphate-γ-^{32}P	40 mM Tris-acetate 5 mM $MgCl_2$	50 μl	Liberated $^{32}PO_4$	[14]
	0.25 mM adenosine-5'-triphosphate-γ-^{32}P	10 mM Imidazole-HCl 0.1 mM EDTA 0.2 mM $MgCl_2$	50 μl	as above	[14, 15]

All incubations were carried out in duplicate determinations at 37°C in the presence of 25-60 ng islet membrane protein. During these conditions hydrolysis of 3-0-methylfluoresceine phosphate and ATP-γ-^{32}P was linear for up to 60 min of incubation.

RESULTS

Fractionation of Islet Homogenate

A total homogenate of islets isolated from 8–12 rats usually yielded about 1.5–3 mg protein. Figure 1 shows the absorbance at 254 nm of an islet homogenate after centrifugation on the compound linear sucrose gradient. Four major peaks were found, two at sucrose concentrations lower than 10% (w/w) and two at concentrations higher than 25%. A similar absorbance pattern was obtained at 280 nm (not shown).

The distribution of various subcellular markers in relation to fraction number and sucrose concentration are shown in Figs. 2–6. When Figs. 1 and 2 are compared it can be seen that the distribution of protein generally followed that of the absorbance at 254 nm. There was a small but consistent absorbance peak at a sucrose density of about 1.03

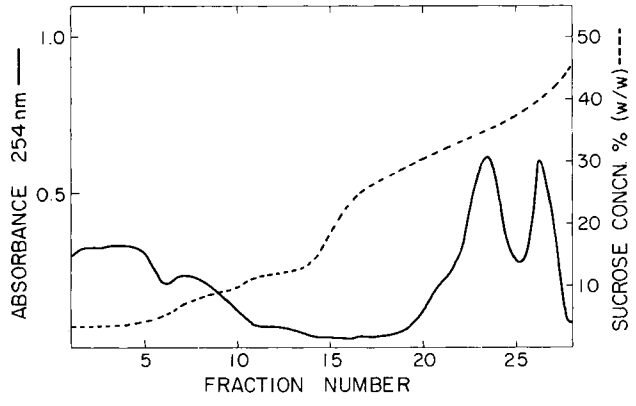

Fig. 1. Sedimentation of total rat islet cell homogenate on the compound sucrose gradient composed from bottom to top of 1 ml 50% (w/w) sucrose, 6 ml 23–43% (w/w) linear sucrose gradient and 4 ml 6–14% (w/w) linear sucrose gradient. The homogenate (1.8 ml) in 3% (w/w) sucrose was added to the gradient and centrifuged at 4°C for 2 hours at 25,000 rpm in a Beckman SW 40 rotor. Absorbance at 254 nm was measured when the gradient was sampled from above.

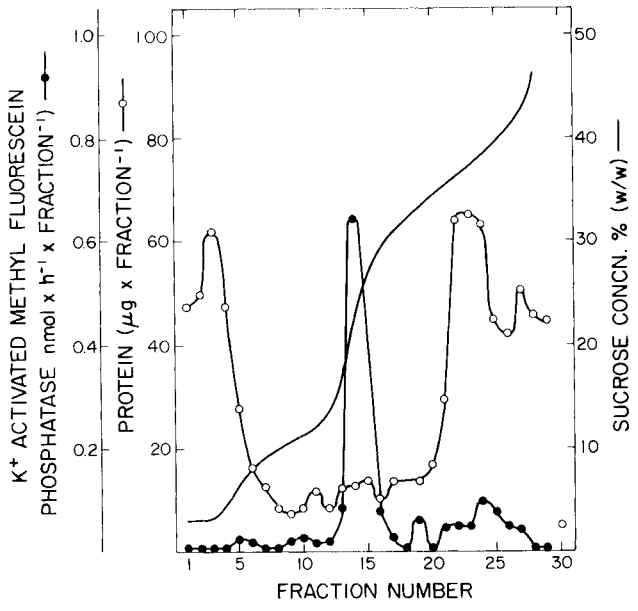

Fig. 2. Distribution of protein and K^+ activated methyl fluorescein phosphatase activity on the compound sucrose gradient after centrifugation of rat islet homogenates. There was an 80–85% recovery of protein added to the gradient. The methyl fluorescein phosphatase activated by 10 mM-K^+ was measured at pH 8.2 as described in Table I.

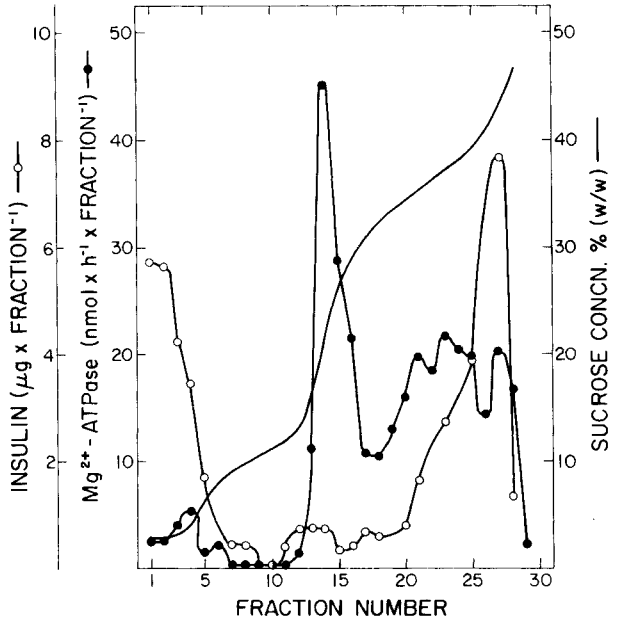

Fig. 3. Distribution of insulin and Mg^{2+}-ATPase activity on the compound sucrose gradient after centrifugation of rat islet cell homogenates. There was an 70-75% recovery of insulin added to the gradient. The Mg^{2+}-ATPase activity was measured at pH 7.0 in medium supplemented with 40 mM-Tris-acetate and 5 mM-$MgCl_2$ as described in Table I.

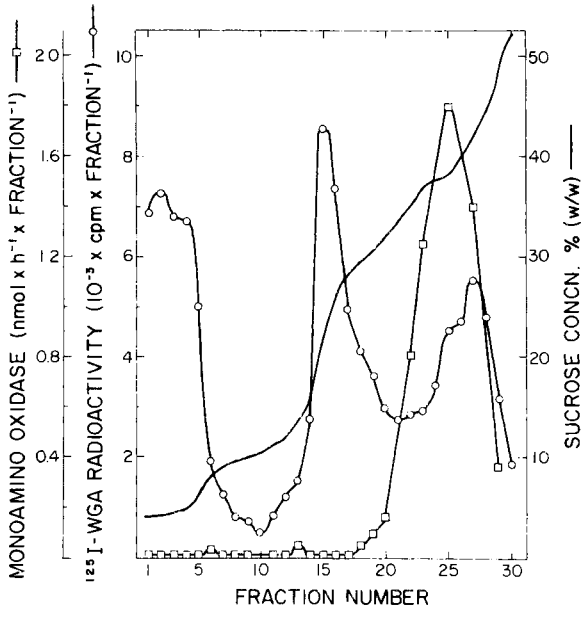

Fig. 4. Distribution of monoamino oxidase activity and ^{125}I-wheat germ agglutinin (^{125}I-WGA) radioactivity on the compound sucrose gradient after centrifugation of rat islet homogenates. In the experiments with ^{125}I-WGA islet homogenates were obtained from intact islets incubated with the radioactively labeled lectin in 130 mM borate buffer, pH 7.4, containing 0.5% (w/v) bovine serum albumin for 30 min at 4°C. The incubation was followed by washing in nonradioactive borate buffer before the islets were homogenized.

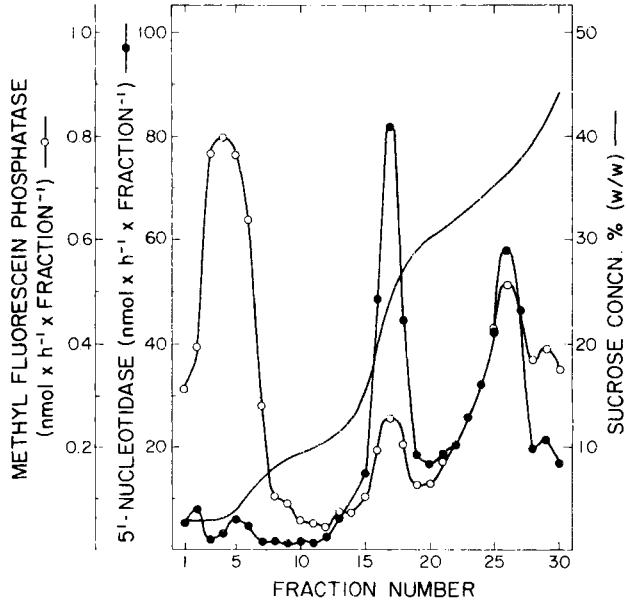

Fig. 5. Distribution of methyl fluorescein phosphatase and 5'-nucleotidase activities on the compound sucrose gradient after centrifugation of rat islet homogenates. There was a 70–80% recovery of activities added to the gradient. The methyl fluorescein phosphatase activity was measured at pH 7.8 as described in Table 1.

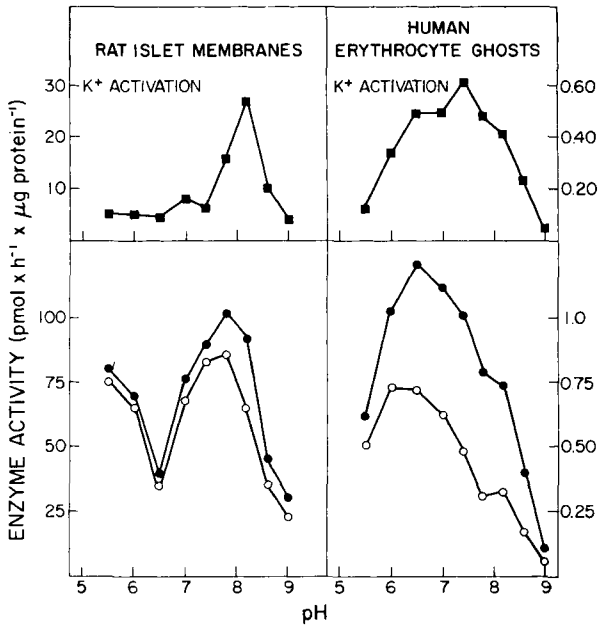

Fig. 6. Effect of pH on hydrolysis of methyl fluorescein phosphate catalyzed by the plasma membrane fraction of rat islets or human erythrocyte ghosts. Parallel incubations were performed in medium without (○——○) or with 10 mM-K^+ (●——●). The upper part of the figures shows the pH-dependency of the K^+-activated enzyme activity. The ordinates in the upper panels are expanded compared with the lower panels.

that did not seem to have a counterpart in any of the markers tested. Figure 2 demonstrates that about 30% of the total islet homogenate protein remained on top of the gradient, probably representing soluble proteins and material of low density. Some of these seem to represent immunoreactive insulin that is assumed to have leaked from damaged or broken secretion granules. Up to 40% of the total amount of the homogenate insulin remained at sucrose concentrations lower than 8% (Fig. 3).

Freshly isolated, intact islets were labeled with ^{125}I-wheat germ agglutinin (^{125}I-WGA) before being homogenized and fractionated by sucrose gradient centrifugation. Apart from the radioactivity of the free lectin remaining at the top of the gradient, Fig. 4 shows that the plasma membrane probe was recovered in a major peak corresponding to a sucrose density of about 1.10. For comparison, human erythrocyte ghosts were labeled with ^{125}I-WGA and centrifuged on the sucrose gradient. The labeled ghosts were recovered as a sharp labeled band at a sucrose density of about 1.15 (not shown). After centrifugation of islet homogenate the major peak of bound ^{125}I-WGA radioactivity was found in a region of the gradient which contained only 2–4% of the total homogenate protein. This peak of ^{125}I-WGA radioactivity coincided with several plasma membrane markers including Mg^{2+}-ATPase (Fig. 3), 5′-nucleotidase and alkaline phosphatase (Fig. 5), and the specific activities of these markers were 15–20-fold higher than in the initial whole islet homogenate. The relative enrichment of plasma membrane marker activities was comparable to that reported previously using a somewhat more cumbersome two-gradient system [8].

Figure 3 shows the distribution of insulin on gradients. The hormone (µg immunoreactive insulin) comprised about 4–8% of the total protein in the plasma membrane enriched fraction. However, as shown in Fig. 4 we were unable to detect monoamino oxidase activity, a mitochondrial outer membrane marker, in this fraction. Measurements of DNA indicated a major recovery of nuclear material at sucrose densities higher than 38% while less than 0.5% was found in the plasma membrane fraction (data not shown).

Measurements of 3-0-Methylfluorescein Phosphatase

3-0-methylfluorescein phosphate was used as a substrate in a fluorimetric assay to test the possibility that rat islet cell membranes contain a K^+-activated phosphatase. The hydrolysis of 3-0-methylfluorescein phosphate was high below pH 6 and at pH 8 in the isolated membrane fraction (Fig. 6). While the enzyme activity was stimulated by K^+ over a wide pH-range in human erythrocyte ghosts, there was a distinct peak of K^+-dependent activity at pH 8 in the rat islet membrane preparation. Ouabain (2 mM) inhibited the K^+-dependent enzyme activity in rat islet membranes and in human erythrocyte ghosts by about 25% and 60% respectively. Figure 2 indicates that the K^+-dependent phosphatase activity at pH 8.2 was mainly recovered in the plasma membrane fraction while hydrolysis of the phosphate indicator at pH 7.8 was apparent in low as well as high density regions of the sucrose gradient (Fig. 5).

Measurements of Mg^{2+}-ATPase

The hydrolysis of ATP- γ-^{32}P in relation to pH is shown in Fig. 7. The enzyme activity was highest at pH 7.0 or pH 8.2. The distribution on the compound sucrose gradient of Mg^{2+}-ATPase activity at either pH showed that the highest specific activity was present in the plasma membrane enriched fraction (Fig. 8). The hydrolysis of ATP-γ-^{32}P was studied in 2-3 combined fractions of the plasma membrane peak (Table II). Plasma membrane fractions were obtained from 14 different gradient centrifugations representing a specific activity of Mg^{2+}-ATPase between roughly 10–30 nmol $\times$ h^{-1} $\times$ μg $protein^{-1}$. Although K^+ (10 mM) stimulated hydrolysis of 3-0-methylfluorescein phosphate (Fig. 6), this cation (5–25 mM) did not affect the Mg^{2+}-ATPase activity at either pH 7.0 or 8.2. In the presence of both Na^+ and K^+ a small fraction of the total Mg^{2+}-ATPase activity was inhibited by ouabain (Table II, Experimental series 3). The specific activity at pH 8.2 of

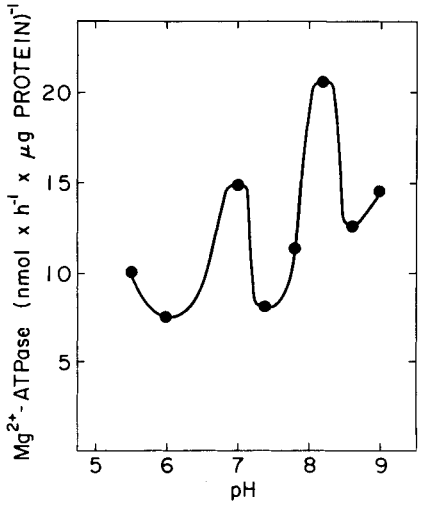

Fig. 7. Effect of pH on hydrolysis of ATP-γ-^{32}P catalysed by rat islet membranes. The hydrolysis of ATP was measured in medium supplemented with 40 mM-Tris-acetate and 5 mM-$MgCl_2$ as described in Table I.

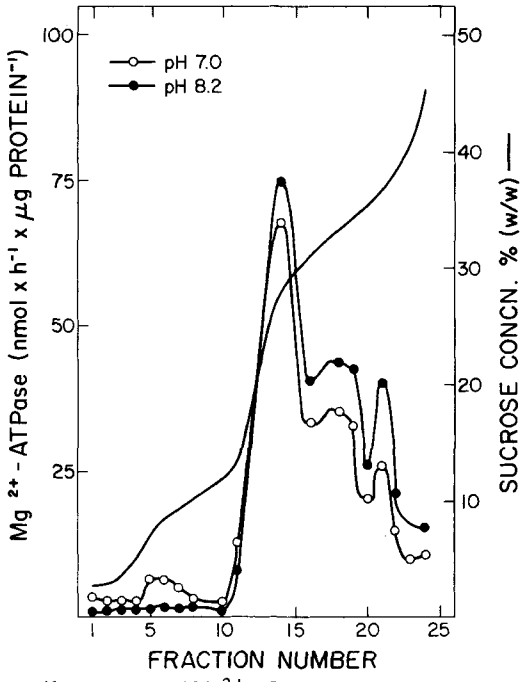

Fig. 8. Distribution of specific activities of Mg^{2+}-ATPase at either pH 7.0 or 8.2 on the compound sucrose gradient after centrifugation of rat islet cell homogenates.

ouabain-sensitive hydrolysis of ATP-γ-^{32}P was 580 ± 170 pmol/h per μg protein (mean value ± SE for 6 determinations). K^+-activated 3-0-methylfluorescein phosphatase activity measured under similar conditions in other membrane preparations amounted to 14 ± 4 pmol/h per μg protein (mean value ± SE for 8 determinations). For comparison, the activity of K^+-dependent 3-0-methylfluorescein phosphatase in human erythrocytes was 0.75 ± 0.09 pmol/h per μg protein (12 determinations). The activity of (Na^+ plus K^+) Mg^{2+}-ATPase, measured at pH 7.0, was 125 ± 23 pmol/h per μg protein (3 determinations). In the presence of 0.1 mM ouabain this activity was reduced by 34%.

DISCUSSION

A wide variety of observations suggest that the β-cell plasma membrane participates in many ways in the regulation of insulin release. The plasma membrane is likely to harbor recognition sites for modifiers of secretion such as glucose and other sugars, amino acids and hormones. It may also regulate the movements of cations important for stimulus-secretion coupling and the exocytosis process for insulin. A major obstacle to the study of these and related problems is the difficulty of obtaining sufficient amounts of islet material for subcellular fractionation and biochemical analyses. Relatively large scale isolation of islets, as employed here, yields only about 2–3 mg of total islet protein from 8–12 rats. In the present study we describe an important modification of an earlier technique [8] demonstrating that the plasma membrane-enriched fraction can be obtained by a one-step sucrose gradient centrifugation. By external labeling of the cell surface with ^{125}I-wheat germ agglutinin before homogenization a membrane fraction can be identified and used for further experiments within three hours after isolating the pancreatic islets.

The major peak of ^{125}I-wheat germ agglutinin radioactivity had a density in sucrose of about 1.10 g/cm^3, as found earlier [8] and also reported for a plasma membrane

TABLE II. Mg^{2+}-ATPase Activities in the Rat Islet Cell Membrane Fraction

Experimental series	Agents tested	pH	n	Mg^{2+}-ATPase (nmol × h^{-1} × μg $protein^{-1}$)
I	None	7.0	7	33.9 ± 5.4
	5-20 mM-KCl	7.0	7	33.7 ± 5.2
	60-100 mM-NaCl	7.0	7	30.9 ± 4.5
	KCl plus NaCl	7.0	7	30.8 ± 4.6
II	None	8.2	5	21.8 ± 6.5
	5-25 mM-KCl	8.2	5	21.2 ± 6.8
III	10-25 mM-KCl and 60 mM-NaCl	8.2	6	9.8 ± 0.8
	KCl and NaCl plus 0.1 mM ouabain	8.2	6	9.2 ± 0.8[a]

[a] Effect of ouabain $P < 0.02$. Comparisons are made only within each experimental series which represents 2–3 gradient fractions of the plasma membrane region from several gradient centrifugations.

enriched fraction of ob/ob mouse islets [10]. This value probably represents an accurate estimate of the buoyant density of islet cell membranes since human erythrocyte ghosts were recovered as a sharp band at the expected density of 1.15 [16]. The peak activity of ^{125}I-wheat germ agglutinin on rat islet gradients coincided with that of other plasma membrane markers such as 5′-nucleotidase, Mg^{2+}-ATPase and alkaline phosphatase. These activities as well as that of ^{125}I-WGA radioactivity also appeared at higher sucrose densities. This could be due to, eg particle aggregation or membrane particles with other physical properties. However, this observation underlines the necessity to utilize a number of appropriate markers. For example, there was little contamination of the membrane fraction by insulin and DNA. The fact that monoamino oxidase activity was not detected in the plasma membrane fraction indicates that contamination by smooth membranes derived from mitochondria is negligible.

Alkaline phosphatase activity as well as K^+-activated p-nitrophenyl phosphatase are enriched in several plasma membrane preparations of various tissues [14]. Using methyl-umbelliferonephosphate as the substrate we found an eightfold enrichment of the phosphatase activity when measured at pH 8 in rat islet membranes [8]. Hydrolysis of p-nitrophenyl phosphate in a fraction enriched in plasma membranes from ob/ob mouse islets was stimulated by K^+ in the pH-range of 5–10 with a small peak of K^+-induced activation between pH 7.5 and 8.0 [10]. Using a highly sensitive fluorimetric assay [12] we now demonstrate that a K^+-activated phosphatase activity with a pH optimum around 8 is also present in plasma membrane enriched fraction of rat islets. Furthermore, in these membranes as well as in the human erythrocyte ghosts we found that the enzyme activity was sensitive to inhibition by ouabain. However, when measured during similar conditions the specific activity of the islet membrane enzyme appears to be at least 20-fold that of the erythrocyte ghosts (Fig. 6). Despite this and the fact that ATP was a powerful inhibitor of K^+-activated phosphatase in other islet membranes [10] we were unable to detect cation-activated ATP hydrolysis in the rat membranes. Furthermore, hydrolysis of Mg^{2+}-ATP in the presence of Na^+ and K^+ was only slightly inhibited by ouabain. Thus, as demonstrated earlier [8] ouabain-sensitive (Na^+ plus K^+) Mg^{2+}-ATPase activity represents only a minor fraction of the total islet membrane Mg^{2+}-ATPase activity. With the present degree of purification the specific activity of the plasma membrane peak was in the same order of magnitude as

reported for highly purified membrane preparations of other rat tissues [14].

The relationship between K^+-stimulated phosphatase activity and (Na^+ plus K^+) Mg^{2+}-ATPase is unclear [17, 18]. In human erythrocyte ghosts it was suggested (13) that the K^+-activated 3-0-methylfluorescein phosphatase activity was a partial reaction catalyzed by the (Na^+ plus K^+) Mg^{2+}-ATPase since a) K^+-phosphatase activities seemed to copurify with that of (Na^+ plus K^+) Mg^{2+}-ATPase, and b) that the relative inhibition of the two enzyme activities by ouabain was similar. However, when hydrolysis of 3-0-methylfluorescein phosphate and ATP by rat islet membranes was assayed under the conditions used for 3-0-methylfluoresceine phosphatase (Table I) we were unable to detect such relationship. Thus the rat islet membrane preparation resembles that [8] prepared from ob/ob mouse islets which contain a readily demonstrable ouabain sensitive K^+-activated phosphatase but not (Na^+ plus K^+) Mg^{2+}-ATPase activity [10]. It is generally assumed that (Na^+ plus K^+) Mg^{2+}-ATPase is a plasma membrane constituent in most cells. However, ATP hydrolysis by rabbit leucocyte membranes was found to be unaffected by sodium, potassium or ouabain [19]. These membranes contain, on the other hand, a potassium-sensitive, ouabain-sensitive p-nitrophenol phosphatase which was suggested to be part of an electrogenic potassium pump in the leucocyte [19, 20]. The present plasma membrane preparation, readily isolated from rat pancreatic islets by one-step centrifugation, will allow a more detailed characterization of K^+-activated 3-0-methylfluorescein phosphatase to test the possibility that it may be the enzymic counterpart for a monovalent cation pump in the islet cells. However, the relatively high specific activity of Mg^{2+}-ATPase in the plasma membrane fraction remains to be explained in relation to functional characteristics of the insulin-producing β-cells.

ACKNOWLEDGMENTS

Portions of this work were supported by grants from the USPHS (AM-17046, AM-13914), the University of Chicago Diabetes Research and Training Center (AM-20595) and the Swedish Medical Research Council. We are indebted to Dr. T.L. Steck for supply of human erythrocyte ghosts and to Ms Sally Parks for preparing the manuscript.

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