Potassium Ion-Activated Hydrolysis of *p*-Nitrophenyl Phosphate in Pancreatic Islet-Cell Membranes

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Hydrolysis of *p*-nitrophenyl phosphate was measured in a fraction enriched in plasma membranes from pancreatic islets of non-inbred *ob/ob* mice. Hydrolysis was stimulated by K^+ (10 mM) in the pH range 5–10; a small peak of K^+ -induced activation was observed between pH 7.5 and 8. Both the K^+ -induced activation and the hydrolysis in the absence of K^+ were Mg^{2+} -dependent; maximum activation was obtained with 10 mM- K^+ plus 5 mM- Mg^{2+} . Rb^+ was as effective an activator as K^+ . Ouabain was inhibitory, the effect being inversely related to the K^+ concentration; 0.1–0.2 mM-ouabain caused about 50% inhibition in the presence of 1 mM- K^+ , but had no demonstrable effect in the presence of 4–5 mM- K^+ . The K^+ -stimulated activity was markedly inhibited by 0.1 mM-ATP, 35–140 mM- Na^+ , or 0.01 mM-*p*-chloromercuribenzenesulphonic acid. Similarities to Rb^+ accumulation suggest that catalysis of univalent cation flow in pancreatic β -cells may be coupled to a phosphoryl-transfer reaction with ATP as natural substrate or regulator.

Release of insulin from pancreatic β -cells may be regulated by transmembrane fluxes of univalent cations, as indicated by the influence of ions on secretory rates (Milner & Hales, 1970; Malaisse *et al.*, 1971; Henquin & Lambert, 1974; Hellman *et al.*, 1974; Malaisse, 1975) and the regulation of membrane electric potentials (Meissner & Schmelz, 1974; Atwater & Meissner, 1975; Dean *et al.*, 1975; Matthews & Sakamoto, 1975). Decreased permeability to K^+ was proposed as an electrodiffusional mechanism mediating the depolarizing and insulin-releasing actions of glucose (Sehlin & Täljedal, 1975). The hypothesis presupposes that β -cells contain a univalent-cation pump capable of establishing the necessary chemical gradients of ions, as does the explanation by Milner & Hales (1970) of insulin-secretory responses to ouabain or withdrawal of K^+ . In addition to providing the basis for electrodiffusional control, the univalent-cation pump may be directly electrogenic in transferring net charges across the β -cell plasma membrane (Atwater & Meissner, 1975; Matthews & Sakamoto, 1975).

Accumulation of Rb^+ in micro-dissected islets supports the assumption of a univalent cation pump in β -cells (Sehlin & Täljedal, 1974a). However, the vectorial function of the assumed pump has not been correlated with any scalar catalysis, such as a phosphoryl-transfer reaction; pancreatic islets have only barely detectable activities of (Na^+ + K^+)-dependent or ouabain-inhibited adenosine triphosphatases (Formby *et al.*, 1976; Idahl *et al.*, 1976). That early steps of stimulus-secretion coupling could involve some kind of phosphatase reaction is indirectly suggested by the prompt release of ortho-

phosphate from glucose-stimulated islets (Freinkel *et al.*, 1974; Bukowiecki & Freinkel, 1976).

In the present study, a subcellular fraction enriched in plasma membranes was isolated from *ob/ob*-mouse islets and assayed for K^+ -dependent hydrolysis of *p*-nitrophenyl phosphate. Although this substrate is artificial, enzymes catalysing its K^+ -dependent cleavage have been implicated in cation transport in other cells (Emmelot & Bos, 1966; Nagai *et al.*, 1966; Woodin & Wieneke, 1970a,b; Solyom & Trams, 1972; Dahl & Hokin, 1974; Hobbs & De Weer, 1976). The preliminary observation of K^+ -activated *p*-nitrophenyl phosphatase in pancreatic islets (Idahl *et al.*, 1976) is here extended to studies of the effects of pH, Mg^{2+} , Ca^{2+} , Na^+ , ouabain, ATP and some other agents of possible importance for the regulation of insulin release.

Materials and Methods

Adult non-inbred *ob/ob* mice from the Umeå colony were deprived of food overnight. Smooth membranes of pancreatic islets were prepared by using the principles described for rat islets (Lernmark *et al.*, 1976). In each experiment eight to twelve pancreatic glands were individually minced with scissors and digested by shaking for 20 min at 37°C with 2 mg of collagenase/ml of Hanks solution without Phenol Red (Paul, 1973). After the digests had been washed four times with approximately 10 vol. of Hanks solution, about 1500 islets were transferred to a buffer containing 10 mM-boric acid and 0.2 mM- $MgCl_2$, adjusted with NaOH to pH 8.0. The islets were washed three times with 400 μ l of

this borate buffer and homogenized by vigorous shaking in 300 μ l of the same buffer. In 16 separate experiments the homogenate contained 1.97 ± 0.82 mg of protein (mean $\pm$ s.d.). The islet homogenate was placed on top of a gradient of sucrose in the above borate buffer supplemented with 1 mM-EGTA; from bottom to top the gradient was composed as follows: 1 ml of 45% (w/w) sucrose, 0.5 ml of 31% sucrose, and 1.5 ml of a linear gradient of 14–3% sucrose. The gradient was centrifuged for 60 min at 25000 rev./min (70000 $g_{\max}$) in a Spinco L-50 ultracentrifuge equipped with rotor SW-39. To identify the plasma membranes, the homogenate in some experiments had been incubated with 125 I-labelled wheat-germ agglutinin before being placed on the gradient (Lernmark *et al.*, 1976). Peak radioactivity occurred in the region of 17.1 ± 2.3 – $33.7 \pm 2.7\%$ sucrose in 11 experiments (mean values $\pm$ s.d.). In each experiment 0.8 ml of this region was diluted with 0.6 ml of the borate/0.2 mM- Mg^{2+} -EGTA buffer and placed on a linear gradient of sucrose (23–45%, 4 ml) in the same buffer. After centrifugation as above for 90 min, fractions (250 μ l) were collected from the top and assayed for phosphatase activity.

Phosphatase activity was assayed in a final reaction volume of 100–125 μ l containing 25 μ l of the sucrose gradient, 5 mM-*p*-nitrophenyl phosphate (diTris salt), and unless otherwise stated, 40 mM-Tris base; pH was adjusted as required by the inclusion of acetic acid, and other reagents were included as described in the legends to Figures and Tables. Because of the buffer used in the sucrose gradient, the basal enzyme reaction medium contained 0.20–0.25 mM-EGTA, 0.04–0.05 mM- Mg^{2+} , and about 5 mM- Na^+ . The effect of bivalent cations on enzyme activity was tested by adding Mg^{2+} at concentrations above 1 mM, or by adding 0.01–9 mM- Ca^{2+} in the presence of 5 mM- Mg^{2+} ; the influence of EGTA on the effective concentration of ions must therefore be small and concentrations reported are those added to the medium without correction for ionic interaction with ligands. The pH of the reaction medium was controlled in the macro scale by mixing Tris/acetate and borate buffers as well as other ingredients in the same proportions as used during enzyme assay. After incubation for 60 min at 30°C, the reaction was stopped with 200 μ l of 0.5 M-NaOH, the test tubes were centrifuged (10000 g , 5 min), and the A_{410} was read in micro-cuvettes. One membrane preparation was first assayed (5 mM- Mg^{2+} and 10 mM- K^+) by the routine incubation for 60 min and found to split 2.4 nmol of substrate/h per μ g of protein. A portion of this preparation was stored for 2 days at -25°C ; on re-assay by incubation for 140 min its enzyme activity was 2.3 nmol/h per μ g of protein.

Fluorescamine (Udenfriend *et al.*, 1972) was used to assay protein as follows. Portions (10 μ l) of the sucrose gradient or whole-islet homogenate were

mixed with 300 μ l of 10% (w/v) trichloroacetic acid, left at 4°C for 30 min, and centrifuged at 4°C (10000 g , 5 min). The supernatant was discarded and the precipitate washed by being taken up and re-centrifuged in 300 μ l of ethanol/propan-2-ol (2:5, v/v). The supernatant was discarded and 100 μ l of 0.1% (v/v) Triton X-100 dissolved in 16 mM- Na_2CO_3 /34 mM- $NaHCO_3$ buffer, pH 9.6, was added to the pellet. After mixing followed by 40 min of incubation at 60°C, 30 μ g of fluorescamine in 100 μ l of acetone was added with vigorous shaking at room temperature (approx. 22°C). The fluorescence was read at 390 nm excitation and 475 nm emission wavelengths in an Aminco-Bowman spectrofluorophotometer. Human serum albumin was used as a reference. The method detects 0.1 μ g of protein.

Chemicals

p-Nitrophenyl phosphate (diTris salt), ATP (Tris salt), cyclic AMP, phosphocreatine, 1-thio-D-glucose, adrenaline, 5-hydroxytryptamine, L-leucine, ouabain and *p*-chloromercuribenzenesulphonic acid were from Sigma Chemical Co., St. Louis, MO, U.S.A.; other nucleotides and glyceraldehyde 3-phosphate, 1,3-diphosphoglycerate, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate were from C. F. Boehringer Mannheim G.m.b.H., Mannheim, Germany; D-glucose, iodoacetamide and EGTA were from BDH Chemicals, Poole, Dorset, U.K.; 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; alloxan monohydrate was from Nutritional Biochemical Corp., Cleveland, OH, U.S.A.; *N*-bromoacetyl- β -D-glucosylamine (Hellman *et al.*, 1976) was given by Dr. E. W. Thomas, Department of Chemistry, University of Salford, U.K.; collagenase (CLS type IV) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; ultrapure sucrose (special enzyme grade) was from Schwarz/Mann, Orangeburg, NY, U.S.A.; wheat-germ agglutinin was from Miles Laboratories Inc., Elkhart, IN, U.S.A., and iodinated as previously described (Lernmark *et al.*, 1976); human serum albumin was from Kabi AB, Stockholm, Sweden; fluorescamine was from F. Hofmann-La Roche and Co. A.G., Basel, Switzerland. Inorganic salts were of analytical grade.

Results

Fig. 1 shows the distribution of phosphatase activities on the final sucrose gradient used for preparing islet-cell membranes. Enzyme activity in the absence of K^+ showed a fairly even distribution on the gradient. There was also a broad distribution of enzymes which became activated on addition of 10 mM- K^+ to the assay medium. However, the K^+ -induced activation consistently exhibited a peak in

Fig. 1. *p*-Nitrophenyl phosphatase activities and wheat-germ agglutinin in sucrose density gradient loaded with *ob/ob* mouse islet extract

Islet homogenates were incubated with ^{125}I -labelled wheat-germ agglutinin and fractionated on two consecutive sucrose density gradients (see the Materials and Methods section). Fractions of the second gradient were assayed for phosphatase activity in medium containing 5 mM *p*-nitrophenyl phosphate, 40 mM-Tris base, 5 mM- MgCl_2 and acetic acid to give a final pH of 7.8. Parallel incubations were also performed in medium supplemented with 10 mM- KCl . The activity in the absence of K^+ ($\circ$), the enhancement of activity due to 10 mM- K^+ ($\bullet$), and the content of the plasma-membrane marker, wheat-germ agglutinin ($\square$), are given for one experiment. Similar distribution of enzyme activities was observed in repeated experiments whether or not the homogenate had been labelled with wheat-germ agglutinin.

Fig. 2. Effect of pH on *p*-nitrophenyl phosphate hydrolysis catalysed by the plasma-membrane fraction of *ob/ob* mouse islets

Parallel incubations were performed in K^+ -free medium ($\circ$) and in medium containing 10 mM- K^+ ($\bullet$). All media contained 5 mM- Mg^{2+} , 5 mM-*p*-nitrophenyl phosphate and 40 mM-Tris base. The required pH values were obtained by including various amounts of acetic acid. Membranes sedimenting to 21–23% (w/w) sucrose were studied with and without prior labelling of the homogenate with ^{125}I -labelled wheat-germ agglutinin. Principally the same results were obtained with four different membrane preparations, irrespective of whether wheat-germ agglutinin was present or not. Results of one representative experiment without wheat-germ agglutinin are shown. The inset gives the pH-dependence of the K^+ -dependent enzyme activity, i.e. the difference between the curves in the main diagram. At pH 7.2, 8.2 and 8.7, both the K^+ -independent and the K^+ -stimulated enzyme activities were apparently uninfluenced by using HCl instead of acetic acid as the acidic species in the Tris buffer (results not shown).

fractions containing about 22% (w/w) sucrose. These fractions also contained peak amounts of the plasma-membrane label, ^{125}I -labelled wheat-germ agglutinin. Membranes sedimenting to the region of 21–23% (w/w) sucrose were used in the further enzyme studies. In 15 different sucrose gradients, the activity of this material at pH 7.7–7.8 and in the presence of 10 mM- K^+ and 5 mM- Mg^{2+} (30°C) was 4.9 ± 3.7 nmol of *p*-nitrophenol liberated/h per μg of protein (mean $\pm$ s.d.). Electron microscopy revealed that the material mainly consisted of smooth membranes forming vesicles of variable size (results not shown).

In whole homogenates of *ob/ob* mouse or rat islets (Hellerström *et al.*, 1964; Täljedal *et al.*, 1966) the acid phosphatase activity toward *p*-nitrophenyl phosphate is maximum at pH 5.3. In the isolated membrane fraction (Fig. 2), the phosphatase activity was also high at this pH and rapidly declined in the range pH 6–9. No peak of activity was detected around

pH 7 in K^+ -free medium. The enzyme activity was stimulated by K^+ over a wide pH range, and the activation by K^+ consistently displayed a small peak between pH 7.4 and 8 (four different experiments). In one experiment with more closely spaced pH values around pH 8, the relative enzyme activities in the presence of 5 mM- Mg^{2+} and 10 mM- K^+ were: pH 7.45, 87%; pH 7.80, 96%; pH 7.90, 100%; pH 8.15, 96%; pH 8.30, 91%; pH 9.25, 70%.

Fig. 3. Stimulation of the *p*-nitrophenyl phosphatase activity by K^+ at various Mg^{2+} concentrations. Fractions enriched in islet-cell membranes were incubated with 5 mM-*p*-nitrophenyl phosphate in 40 mM-Tris/acetate buffer at pH 6.1 (○) and pH 7.8 (●). The medium contained K^+ as indicated on the abscissa and Mg^{2+} as indicated by the numbers (mM) written near each curve in the graphs. (a) and (b) refer to separate experiments covering different ranges of K^+ concentration and performed with separate preparations of islet-cell membranes.

Fig. 4. Stimulation of the *p*-nitrophenyl phosphatase activity by Rb^+ or K^+ .

Fractions enriched in islet-cell membranes were incubated with 5 mM-*p*-nitrophenyl phosphate in 40 mM-Tris/acetate buffer, pH 7.8, containing 5 mM- Mg^{2+} . Rb^+ (■, ●) or K^+ (□, ○) was included in the medium at the concentrations indicated. Circles and squares refer to two separate experiments with different preparations of islet-cell membranes; parallel incubations with various concentrations of Rb^+ and K^+ were carried out within each experiment.

The K^+ -induced activation of the membrane-located phosphatase, as well as the activity in the absence of K^+ , was Mg^{2+} -dependent at both pH 6.1 and 7.8 (Fig. 3). In K^+ -free medium, 1–5 mM- Mg^{2+} caused a suboptimum enhancement of the hydrolysis of *p*-nitrophenyl phosphate; this effect was not increased by adding more Mg^{2+} . When the Mg^{2+} concentration was as low as 0.04 mM (originating from the sucrose gradient), 1–40 mM- K^+ had virtually no effect. In the presence of 1–10 mM- Mg^{2+} , K^+ was stimulatory to an extent depending on both the K^+ and the Mg^{2+} concentration. Maximum activation was obtained with a combination of 10 mM- K^+ and 5 mM- Mg^{2+} . Rb^+ was as good an activator as K^+ (Fig. 4).

Ca^{2+} could not replace Mg^{2+} as the permissive ion allowing K^+ to function as activator (Fig. 5). In medium containing no K^+ and only 0.04 mM- Mg^{2+} , 0.5–9 mM- Ca^{2+} had a very small, if any, stimulatory effect. Adding 10 mM- K^+ to media containing 0.5–9 mM- Ca^{2+} did not activate the hydrolysis of *p*-nitrophenyl phosphate. When the activity was fully stimulated by 10 mM- K^+ and 5 mM- Mg^{2+} , the addition of Ca^{2+} had inhibitory effects; the K^+ -dependent enzyme activity was almost completely blocked by 9 mM- Ca^{2+} and half-inhibited by about 0.2 mM- Ca^{2+} .

At K^+ concentrations of 4–5 mM or more, 1.5 mM-ouabain inhibited the K^+ -dependent phosphatase activity by 20–30%; 0.1–0.5 mM-ouabain had virtu-

Fig. 5. Effect of Ca^{2+} on the *p*-nitrophenyl phosphatase activity in islet-cell membranes

Fractions enriched in islet-cell membranes were incubated with 5mM-*p*-nitrophenyl phosphate in 40mM-Tris/acetate buffer, pH7.8, containing CaCl_2 at the concentrations indicated. The main Figure shows enzyme activities recorded when samples of one membrane preparation were incubated in the absence of both Mg^{2+} and K^+ ($\circ$), in the presence of 10mM- K^+ ($\bullet$), and in the presence of both 5mM- Mg^{2+} and 10mM- K^+ ($\blacksquare$). The inset shows the averages of experiments with two or three separate preparations of islet-cell membranes and shows how Ca^{2+} affected the K^+ -stimulated portion of enzyme activity; 100% is the activity in the presence of 10mM- K^+ and 5mM- Mg^{2+} minus the activity in the presence of 5mM- Mg^{2+} alone.

ally no effect (Table 1). The relative effect of ouabain was inversely related to the K^+ concentration, since the enzyme activity response to 1mM- K^+ was half inhibited by 0.1–0.2mM-ouabain and nearly completely blocked by 1.5mM-ouabain.

Table 2 summarizes the effects of some other inhibitors of the K^+ -dependent phosphatase. Inhibition by 35–140mM- Na^+ is not attributable to ionic strength, since 140mM- Li^+ or 140mM-choline ions were less inhibitory than equimolar Na^+ . Moreover, the enzyme activity was unaffected by varying the concentration of Tris in the buffer within the range of 40–80mM (results not shown).

ATP at a concentration of 0.1mM or more was strongly inhibitory, the inhibition being nearly complete with 0.25mM-ATP. ADP also inhibited the phosphatase, but less effectively than equimolar ATP. *p*-Chloromercuribenzenesulphonic acid (0.01mM) abolished the K^+ -dependent enzyme activity. The following compounds had no obvious effect when tested on two different membrane

Table 1. Effect of ouabain on the K^+ -stimulated *p*-nitrophenyl phosphatase activity in ob/ob-mouse islet membranes

Fractions enriched in islet-cell membranes were incubated with 5mM-*p*-nitrophenyl phosphate and 5mM- Mg^{2+} in 40mM-Tris/acetate buffer, pH7.8. Parallel incubations were performed in media containing no K^+ or ouabain and in media containing K^+ and ouabain at the concentrations indicated. Results are mean values for experiments, with the numbers of different membrane preparations in parentheses; for each K^+ concentration, 100% is the activity in the presence of K^+ and Mg^{2+} minus the activity in the presence of Mg^{2+} alone.

Concn. of K^+ (mM)	Concn. of ouabain (mM)	Enzyme activity (% of K^+ -stimulated activity in absence of ouabain)
1	0.1	52 (1)
1	0.2	51 (2)
1	0.5	42 (2)
1	1.0	22 (2)
1	1.5	10 (3)
4–5	0.1	102 (1)
4–5	0.2	93 (2)
4–5	0.5	96 (2)
4–5	1.0	83 (2)
4–5	1.5	67 (2)
10	1.5	75 (5)
40	1.5	84 (2)

Table 2. Effect of various agents on the K^+ -stimulated *p*-nitrophenyl phosphatase in ob/ob-mouse islet membranes

Fractions enriched in islet-cell membranes were incubated with 5mM-*p*-nitrophenyl phosphate and 5mM- Mg^{2+} in 40mM-Tris/acetate buffer, pH7.8. Parallel incubations were performed in media containing no K^+ and in media containing 10mM- K^+ and an agent to be tested as indicated. Results are mean values of experiments with two or three different membrane preparations as indicated in parentheses; 100% is the activity in the presence of K^+ and Mg^{2+} minus the activity in the presence of Mg^{2+} alone.

Agent tested	Enzyme activity (% of K^+ -stimulated activity in absence of agent tested)
35mM- Na^+	67 (2)
70mM- Na^+	40 (2)
105mM- Na^+	14 (2)
140mM- Na^+	3 (2)
140mM- Li^+	57 (2)
140mM-Choline ions	62 (2)
0.001mM-ATP	108 (2)
0.01mM-ATP	92 (3)
0.1mM-ATP	38 (2)
0.25mM-ATP	8 (3)
1.0mM-ATP	0 (2)
0.001mM-ADP	114 (2)
0.01mM-ADP	100 (2)
0.1mM-ADP	83 (2)
0.25mM-ADP	79 (2)
0.01mM- <i>p</i> -Chloromercuribenzenesulphonic acid	0 (2)

preparations (average effect less than $\pm 15\%$): 0.25 mM-AMP, 0.01–0.25 mM-GTP, 0.01–0.25 mM-GDP, 0.25 mM-phosphocreatine, 0.05 mM-glyceraldehyde 3-phosphate, 0.05 mM-1,3-diphosphoglycerate, 20 mM-D-glucose, 5 mM-1-thio-D-glucose, 5 mM-N-bromoacetyl- β -D-glucosylamine, 2 mM-alloxan, 0.1 mM-iodoacetamide, 10 mM-leucine, 1 μ M-adrenaline, 10 μ M-5-hydroxytryptamine, 0.1 mM-3-isobutyl-1-methylxanthine or 0.1 mM-cyclic AMP; or when tested on one membrane preparation: 0.005–0.25 mM-glucose 6-phosphate, 0.1 mM-fructose 6-phosphate or 0.1 mM-fructose 1,6-bisphosphate.

Discussion

K⁺-dependent *p*-nitrophenyl phosphatase activity distributed broadly on the sucrose gradient; perhaps islets contain several enzymes of this type. At least one is likely to occur in plasma membranes, since a peak of activity coincided with the maximum binding of wheat-germ agglutinin. That the agglutinin labels plasma membranes was shown by studying enzyme markers and ultrastructure after fractionating rat (Lernmark *et al.*, 1976) and *ob/ob*-mouse (Idahl *et al.*, 1976) islets. Because islets of *ob/ob* mice contain more than 90% β -cells, we believe that the isolated fraction was enriched in β -cell plasma membranes. These membranes possibly contain more than one K⁺-activated enzyme, as the pH-activity curve was wide and multiphasic. Although finer fractionation could perhaps resolve a complex of catalysts with different sensitivities to activators and inhibitors, already the properties of the present membrane preparation appear relevant to the theory of insulin secretion.

To explain stimulatory effects of K⁺ withdrawal or ouabain on insulin release, Milner & Hales (1970) postulated a univalent-cation pump in the β -cells. The postulate is supported by measurements of electric potentials (Matthews & Sakamoto, 1975; Atwater & Meissner, 1975) and fluxes of ²²Na⁺ and ⁸⁶Rb⁺ (Sehlin & Täljedal, 1974a,b). However, the biochemical nature of the pump is unclear. Although they can accumulate Rb⁺ 40-fold (Sehlin & Täljedal, 1974a), β -cells fail to keep the intracellular Na⁺ lower than 95 mM (Sehlin & Täljedal, 1974b); a high Na⁺ permeability or a slow Na⁺ pump must be assumed. Moreover, the vectorial ion transfer is not known to be coupled to any scalar catalytic function in the β -cells. A specific adenosine triphosphatase was undetectable in homogenates of *ob/ob*-mouse islets (I.-B. Täljedal, unpublished work), was barely detectable in islet-cell plasma membranes from rats (Lernmark *et al.*, 1976) or *ob/ob* mice (Idahl *et al.*, 1977), and in microsomal fraction of normal mouse islets represented less than 1% of the activity in brain microsomal fraction (Formby *et al.*, 1976). β -Cells appear to resemble polymorpho-

nuclear leucocytes, which secrete proteins, are defective in Na⁺ extrusion, accumulate K⁺, lack a specific adenosine triphosphatase, and possess a membrane-located K⁺-activated and ouabain-inhibitable *p*-nitrophenyl phosphatase of assumed importance for K⁺ transport (Woodin & Wieneke, 1970a,b).

Because studies of ion pumping in β -cells have used Rb⁺ as a functional analogue of K⁺ (Sehlin & Täljedal, 1974a), it is noteworthy that Rb⁺ replaced K⁺ satisfactorily as activator of the *p*-nitrophenyl phosphatase. That the enzyme may be involved in transporting Rb⁺ and K⁺ is suggested by the ouabain experiments. When mouse islets are incubated with about 5 mM-K⁺, 1 mM-ouabain, but not 0.1 mM-ouabain, inhibits ⁸⁶Rb⁺ accumulation (Sehlin & Täljedal, 1974a). Membrane depolarization of mouse β -cells was induced by 1 mM-ouabain, but not by 0.1 mM-ouabain, in one study (Matthews & Sakamoto, 1975), and by 0.1 mM-ouabain, but not 0.05 mM-ouabain, in another (Atwater & Meissner, 1975). These dose-response relationships conform to the weak enzyme inhibition caused by less than 1.5 mM-ouabain in the presence of 4–5 mM-K⁺. The greater enzyme inhibition at 1 mM-K⁺ agrees with less ouabain being required to depolarize mouse β -cells the lower the K⁺ concentration (Matthews & Sakamoto, 1975). Species differences in the β -cell sensitivity to cardiac glycosides may exist, since 0.1 mM-ouabain inhibited ⁴²K⁺ uptake by rat islets (Howell & Taylor, 1968), and as little as 0.001 mM-ouabain stimulated the release of insulin from pieces of rabbit pancreas (Hales & Milner, 1968).

p-Chloromercuribenzenesulphonic acid (0.1 mM) inhibits Rb⁺ accumulation in islets, and this effect is partly due to increased permeability to Rb⁺ (Sehlin & Täljedal, 1974a). The marked decrease in K⁺-activated phosphatase activity suggests that the mercurial may also inhibit active Rb⁺ influx. Sensitivity to Ca²⁺ is another instance of at least qualitative similarity between the K⁺-dependent phosphatase and the univalent-cation pump. Rb⁺ uptake is enhanced in Ca²⁺-depleted islets (Sehlin & Täljedal, 1974a); Ca²⁺ inhibited the K⁺-activated hydrolysis of *p*-nitrophenyl phosphate. Ca²⁺ was inhibitory at concentrations below that in the extracellular fluid or the basal buffer used for studying Rb⁺ accumulation (2.56 mM). The intracellular concentration of dissociated Ca²⁺ may be much lower, and we do not know the Ca²⁺ concentration to which the enzyme is exposed in living β -cells.

Replacing extracellular Na⁺ by choline ions inhibits Rb⁺ accumulation as effectively as does 1 mM-ouabain (Sehlin & Täljedal, 1974a). For perfect correspondence between the K⁺-activated phosphatase and the cation pump, one would thus expect the enzyme to be Na⁺-activated. However, the K⁺-dependent phosphatase was more active at low (5 mM) than at high (70–140 mM) concentrations of Na⁺.

Although this finding is in agreement with the inhibitory effect of Na⁺ on K⁺-stimulated *p*-nitrophenyl phosphatase activity in other systems (Nagai *et al.*, 1966; Dahl & Hokin, 1974), it complicates the assumption of an identity between the enzyme and the univalent-cation pump. *Ad hoc* explanations could be that the enzyme is barred from Na⁺ in living β -cells, that the *p*-nitrophenyl phosphatase activity reflects only part of the catalytic mechanisms involved in Rb⁺ transport, or that the Na⁺ sensitivity is an artifact of the preparation and assay conditions.

p-Nitrophenyl phosphate is an artificial substrate. The relationship between K⁺-stimulated *p*-nitrophenyl phosphatase and (Na⁺+K⁺)-activated adenosine triphosphatase in other organs has been much discussed, although the nature of any mechanistic relationship is unclear (Nagai *et al.*, 1966; Dahl & Hokin, 1974; Hobbs & De Weer, 1976). Because (Na⁺+K⁺)-activated adenosine triphosphatase cannot readily be measured in mouse islet membranes, we screened some natural phosphate esters for inhibitory effects on the *p*-nitrophenyl phosphate hydrolysis. The efficiency of ATP as inhibitor suggests that this molecule has a strong affinity for the enzyme. More than 50% inhibition was obtained with 0.1 mM-ATP in the presence of 5 mM-Mg²⁺; occasionally, as little as 5–10 μ M-ATP appeared inhibitory. The effect of ATP therefore cannot be due to chelation of Mg²⁺. It seems reasonable to envisage ATP as a physiological substrate or as a regulator of the activity toward an as yet unidentified substrate. It should be noted that the phosphoryl cleavage was measured in the presence of about 55 M-H₂O. Since the unperturbed environment of the membrane-located enzyme may be less aqueous, the physiological reaction could well be transphosphorylation to some substrate other than water.

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