Localization of Inorganic Phosphate in the Pancreatic B Cell and Its Loss on Glucose Stimulation

Abstract. *Perifusion experiments have shown that there is a discharge of inorganic phosphate into the medium when insulin secretion from isolated islets is stimulated by glucose. Histochemical and microprobe examination of resting pancreatic islets in the electron microscope shows a specific accumulation of inorganic phosphate adjacent to the plasmalemma and nucleolus of the B (beta) cells. This phosphate is lost from the cells during secretory stimulation of islets with high concentrations of glucose.*

When glucose stimulates the pancreatic B (beta) cell it triggers a complex series of chemical and physiological events finally resulting in the secretion of insulin. We have shown that a transitory increase in efflux of inorganic phosphate from the cell is one of the early responses to nutrient stimulants of insulin secretion (*1*). This phenomenon is highly specific. Thus D-glucose but not its enantiomorph evokes phosphate release (*1*); the α anomer of D-glucose rather than the β anomer is responsible (*2*); and absolute stereospecificity is shown by the stereoisomers of the nonmetabolizable analog of leucine, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (*3*). In this report we describe experiments in which we determined the subcellular location from which the substantial loss of inorganic phosphate occurs. With an electron microscope we examined pancreatic islets before and after exposing them to stimulating concentrations of glucose in which the phosphate ions had been fixed by histochemical methods. A slight modification of the lead acetate precipitation method for localizing inorganic phosphate by electron microscopy (*4*, *5*) was used.

Table 1. Characterization of lead phosphate precipitates in a standard and two samples of pancreatic islets by means of an electron microprobe analysis. The Araldite-embedded sections (0.1 μm) were picked up on copper grids and coated on both sides with carbon prior to examination. The probe was operated at 60 kV, 20 nA, ×40,000, 40 seconds count time, and 50 percent dead time. Phosphate counting at 200 KeV in 9 channels, 20 eV per channel. Lead counting at 2.38 KeV in 17 channels, 20 eV per channel. "Standard" denotes lead hydroxyapatite crystals $Pb_5(PO_4)_3OH$. "Sample" refers to precipitates in B cells perifused with glucose (0.5 mg/ml).

Specimen	X-ray emission counts		Ratio Pb/P counts
	Pb	P	
Standard			
Large crystal	20,972	1,705	12.3
Large crystal	17,620	1,717	10.3
Small crystal	4,665	474	9.8
Small crystal	10,616	848	12.5
Samples			
Experiment 1			
Plasmalemma	9,272	805	11.5
Plasmalemma	10,200	982	10.3
Plasmalemma	5,817	789	7.4
Plasmalemma	10,388	1,114	9.3
Nucleolus	4,474	777	5.7
Experiment 2			
Plasmalemma	9,431	1,205	7.8
Plasmalemma	8,647	1,231	7.0
Plasmalemma	6,125	897	6.8
Nucleolus	2,174	814	2.6
Nucleolus	1,698	1,019	1.7

Our methods for isolating rat pancreatic islets by collagenase digestion, labeling them with ^{32}P, and then perifusing them in small plastic chambers have been described (*1–3*). Since it was anticipated that the sodium bicarbonate–containing medium used in the perifusion might be carried over on fixation of the islets and precipitate with the lead acetate, we tried, in initial experiments, to eliminate bicarbonate from the perifusion fluid. However, we found that the glucose-stimulated efflux of ^{32}P-labeled inorganic phosphate from the islet was critically dependent on the availability of bicarbonate. We therefore had to work out an exposure to bicarbonate that would permit phosphate efflux yet not allow sufficient carry-over to precipitate lead carbonate. The islets were labeled for 90 minutes in a modified Krebs-Ringer-bicarbonate medium at *p*H 7.4 containing glucose (1 mg/ml), calcium chloride (0.85 m*M*), sodium phosphate (0.5 m*M*), serum albumin (0.2 mg/ml), and [^{32}P]orthophosphate (150 μCi/ml). The islets were then washed three times with the same medium containing no phosphate and with the glucose reduced to 0.5 mg/ml. They were then transferred to perifusion chambers and perifused with the same medium as used for the washing except that the sodium bicarbonate had been replaced by 5 m*M* tris-HCl buffer, *p*H 7.4, and the NaCl concentration increased from 114 m*M* to 138 m*M*. After 20 minutes, the amount of glucose passing through one of the perifusion chambers was increased to 3 mg/ml to stimulate insulin secretion (Fig. 1). Under such conditions, a phosphate efflux was always obtained but its profile was obtunded compared with the release obtained in bicarbonate medium, in the sense that it occurred about 1 minute later and the peak was broader (Fig. 1). Stimulated insulin release was also delayed 1 to 2 minutes and slightly obtunded in confirmation of the experience of others (*6*). The glucose-stimulated and nonstimulated controls perifused in the tris-containing medium were fixed at 38 minutes, immediately after the heightened phosphate efflux had been completed (Fig. 1). The islets were transferred on the nylon mesh used in the perifusion chambers to fixation fluid containing sodium cacodylate (0.1*M*), glutaraldehyde (2 percent), and lead acetate (2 percent) at a *p*H of 7.0. The islets were then dehydrated and embedded in Araldite resin according to standard electron microscopic procedures. No further fixatives were used since osmium tetroxide and uranyl acetate removed all the lead precipitate present in the sections.

In the B cells in the "control" sam-

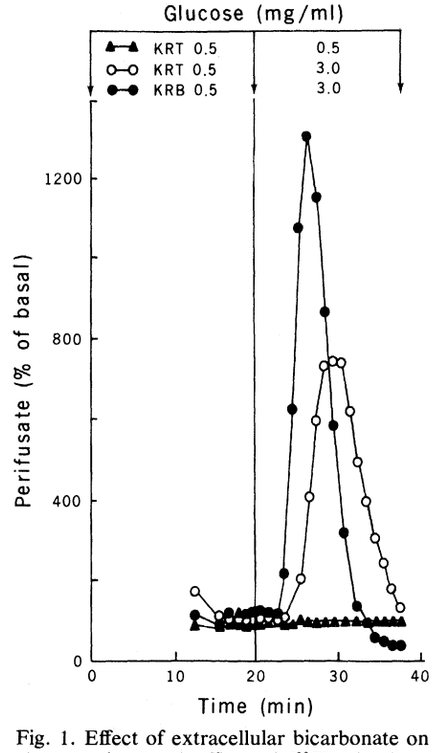

Fig. 1. Effect of extracellular bicarbonate on glucose-stimulated efflux of [^{32}P]orthophosphate. Rat pancreatic islets isolated with collagenase were labeled and then perifused at *p*H 7.4 with modified Krebs-Ringer-tris (*KRT*) or modified Krebs-Ringer-bicarbonate (*KRB*) as described in the text. Glucose in perifusates was maintained at 0.5 mg/ml (control) or increased to 3.0 mg/ml (stimulated) after 20 minutes. Islets from chambers perifused with Krebs-Ringer-tris were removed for histochemistry at 38 minutes. Results are from one of four similar experiments. The appearance of radioactivity in the effluent is expressed as a percentage of the average release of radioactivity (counts per minute per islet per minute) during the 15- to 20-minute interval.

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ples, precipitate was concentrated at or on the plasmalemma and over the nucleolus (Fig. 2A). There was a light scattering over the cytoplasm and nucleus. There was no significant amount of precipitate on or near the plasmalemmas of the A and D islet cells or on the plasmalemma of any of the exocrine cells remaining at the edges of the islets (Fig. 2, A and C). In the glucose-stimulated islets precipitate was not seen on the plasmalemma or nucleolus of the B cells (Fig. 2B). No precipitate was seen on any other type of cell present.

The electron microprobe microanalysis was carried out on an EMI EMMA microscope with a probe measuring 20 nm in diameter. An energy dispersive investigation in which we used an EDAX readout was sufficient to distinguish the peaks of phosphate and lead (see Table 1). Microprobe analysis showed that the precipitate on the nucleolus and at the plasmalemma of the B cell contained only lead and phosphate in significant amount. No sodium, potassium, manganese, or calcium was detected. The mean ratio of lead to phosphate x-ray emission counts from the granules near to the plasmalemma (8.6) was just significantly lower ($P < .05$) to that found for standard lead phosphate crystals (mean: 11.2) embedded in a similar Araldite section; $t = 2.51$, Student's t-test (Table 1). This means that there was a slightly higher P signal than we would have expected if the emissions had been entirely derived from lead hydroxyapatite, $Pb_5(PO_4)_3OH$, which, according to Tandler and Solari (*5*) is the chemical form of the precipitate formed by lead acetate–containing fixative. This small difference could be due to a background P signal being derived from glutaraldehyde-cross-linked amino phospholipids in the plasmalemma which are not removed by the solvent dehydration procedures and which are included with the signal coming from the Pb-containing granules. If the precipitate in the B cells contained significant lead carbonate it would be expected, in contrast to the present results, that the Pb/P ratio would be higher than the standard. The results therefore indicate that the precipitate adjacent to the plasmalemma is substantially lead phosphate and that contamination with lead carbonate is absent or minimal. The apparent low ratio of Pb/P emission counts for the nucleolus (Table 1) could result from the nucleic acid contained within it contributing to the signal.

The exact location of the precipitate with respect to the plasmalemma of the B cell is very difficult to determine. This is because any attempt to increase the membrane contrast of the islets with osmium tetroxide or uranyl acetate completely removed the precipitate of lead phosphate. With the resolution available, nearly all the particles of precipitate appeared to be on the internal surface of the plasmalemma (Fig. 2D). That the phosphate is not in the extracellular space in the nonstimulated state is supported by the observation that it cannot be easily washed from the islet. There are no cytoplasmic vesicles or any organelles consistently found on the intracellular side of the plasmalemma; and the precipitate does not seem to be related in any way to the B cell granules. It seems more likely that the phosphate is bound to the plasmalemma in some way.

In other histochemical characterizations of ions in pancreatic islets, a precipitate produced by potassium pyroantimonate was observed along the plasmalemma of B cells (*7–9*), confined principally to the inner leaflet, by some (*7, 8*) but not all (*9*) workers. The precipitate contained a variety of ions, but mainly antimonate with potassium and calcium. The ions producing the pyroantimonate precipitate seemed to remain in position after fixation in buffered glutaraldehyde with no other additions, and could be precipitated by subsequent treatment with osmium tetroxide–potassium pyroantimonate solution (*7, 9*). This contrasts sharply with our experiments in which, if islets were fixed in glutaraldehyde without lead acetate, no precipitate was found however much soaking in lead acetate was subsequently

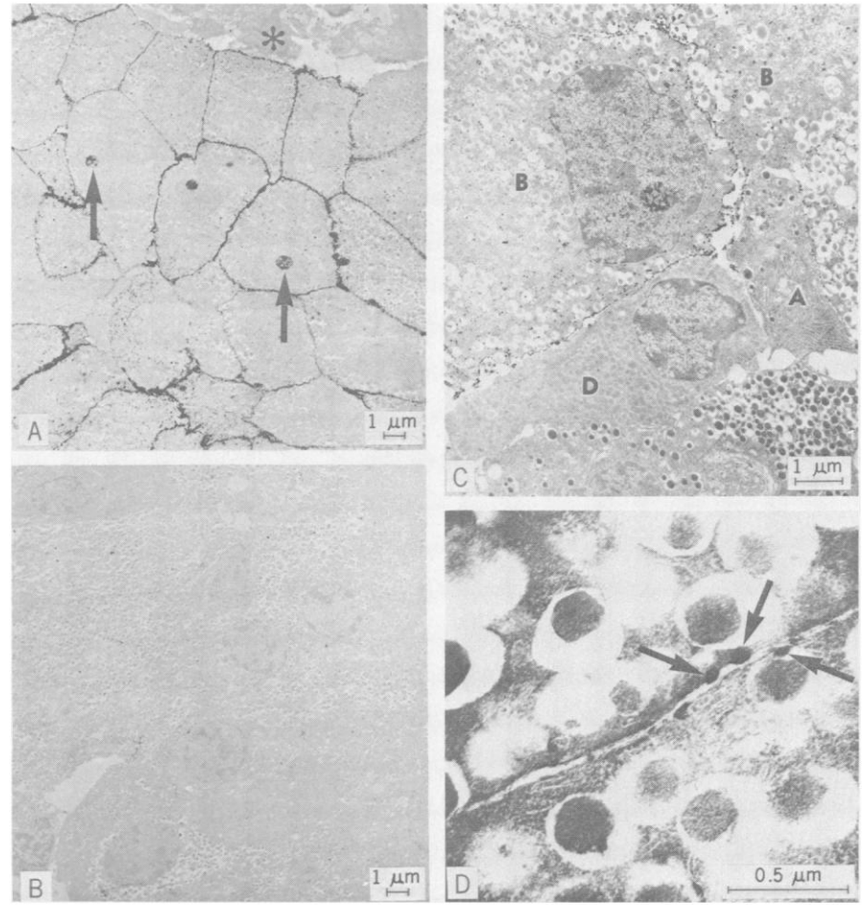

Fig. 2. Electron micrographs of isolated perifused pancreatic islets fixed in 2 percent glutaraldehyde in 0.1*M* cacodylate buffer, *p*H 7.0, containing 2 percent lead acetate for 3 hours at room temperature, then left in 4 percent lead acetate in 35 percent acetic acid at 4°C overnight. Fixed islets were dehydrated and embedded in Araldite without further fixation. (A) Perifusion with glucose (0.5 mg/ml). The section is unstained and shows a precipitate around the periphery of each B cell, and in the nucleoli (arrows). Cells at the edge of the islet (A cells and connective tissue cells) show no precipitate (asterisks) (×3,200). (B) Perifusion with glucose (3.0 mg/ml) for 18 minutes then fixed and processed as in (A); no precipitate can be seen (×3,200). (C) and (D) Perifusion with glucose (0.5 mg/ml) and fixed and processed as in (A), but stained with uranyl acetate (saturated solution in 50 percent alcohol) and lead citrate. In (C) the precipitate is on the plasmalemma of the B cells but not on the A or D cells (×8,300); in (D) the plasmalemmas of two B cells are shown with precipitates restricted to the cytoplasmic side (arrows) (×50,500).

allowed. This demonstrates that the Pb-containing precipitates near to the plasmalemma are formed from soluble inorganic phosphate which is lost into the medium if a glutaraldehyde prefixation is used. The similarity in location of the two precipitates thus seems fortuitous, which is borne out by the fact that the amount of pyroantimonate precipitate was increased by an increase in glucose concentration (*7–9*), whereas the lead phosphate precipitate disappeared.

As far as we are aware, this is the first demonstration by any electron microscope technique of a specific localization of phosphate at the plasmalemma. The localization of orthophosphate in the nucleolus was previously described in maize root-tip cells (*4, 5*). The early autoradiographic experiments of Causey and Harris (*10*) suggested that ^{32}P injected into the frog was selectively concentrated near to the cell surface of the muscle cells, but there was no proof that this existed as inorganic P. These results on muscle can also be contrasted with more recent experiments which show no preferential localization of ^{32}P near the plasmalemma of the hepatocyte both by light (*11*) or electron microscope (*12*) autoradiography. The fact that the plasmalemma precipitate is found only on the B cells and not on A, D, or exocrine cells under the present experimental conditions, coupled with the disappearance of the precipitate under conditions which are known to deplete the B cell phosphate and cause insulin secretion (*13*), complement the evidence already obtained that the two processes are connected in some way. Although the form in which the phosphate is kept near to the plasmalemma is not apparent, it is obvious that it would be strategically placed to support rapid extracellular migration and passage to the perfusion fluid on stimulation, as is observed in practice.

N. FREINKEL
K. C. PEDLEY
Center for Endocrinology, Metabolism and Nutrition, Northwestern University Medical School, Chicago, Illinois 60611
P. WOODING
R. M. C. DAWSON
Institute of Animal Physiology, Babraham, Cambridge, United Kingdom

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Mitochondrial Thyroid Hormone Receptor: Localization and Physiological Significance

Abstract. *Binding studies of thyroid hormone to submitochondrial fractions from rat liver suggest that the component responsible for high-affinity, low-capacity (saturable) binding of hormones arises from the inner mitochondrial membrane. The partially purified component, approximately 150,000 daltons, appears to be half protein and half lipid, largely phospholipids, tentatively identified as lecithin, phosphatidyl ethanolamine, and cardiolipin. A similar hormone-binding macromolecule was found in mitochondria from rabbit kidney, from human liver and kidney, and from rat kidney, myocardium, skeletal muscle, intestinal mucosa, whole small intestine, adipose tissue, and lung. It was absent from mitochondria of adult rat brain, spleen, and testis, organs calorigenically unresponsive to thyroid hormones injected in vivo, but was present in mitochondria from brains of rats 12 days old and younger. The organ distribution of the hormone-binding protein and its presence in neonatal brain mitochondria supports the biological relevance of the mitochondrial component as a thyroid hormone receptor.*

We have described a membrane component of rat liver mitochondria (*1, 2*) which binds triiodothyronine (T_3) with an extremely high association constant ($K_A > 10^{11} M^{-1}$). This binding exhibits high-affinity, low-capacity (saturable) characteristics; we infer that binding by mitochondria may be of importance in thyroid hormone action. This view is supported by the demonstration of binding of thyroid hormone analogs in proportion to the potency of the analog (*2, 3*).

In view of data indicating a direct effect of T_3 on mitochondrial oxidative phosphorylation (*3*), we investigated the intramitochondrial localization of the presumed receptor. We now report that the receptor appears to be part of the inner mitochondrial membrane and provide data on its nature and organ distribution.

Materials and methods. Euthyroid male Sprague-Dawley rats (Charles River, North Wilmington, Massachusetts), 400 to 700 g, were used after an overnight fast. Pregnant female rats were purchased from Camm Research, Wayne, New Jersey. Mitochondria were prepared as described (*1, 3*). A highly purified preparation of phospholipase A_2 from cobra venom was provided by M. Rapport, Columbia University.

Two methods of mitochondrial fractionation were used (Fig. 1).

1) Digitonin fractionation was performed as described by Greenawalt (*4*). A mitochondrial suspension (100 mg of mitochondrial protein per milliliter) was treated with digitonin (0.12 mg per milligram of mitochondrial protein) by adding an equal volume of digitonin solution (12 mg/ml) and stirring for 15 minutes at 0°C. After centrifugation twice at 9250 rev/min (1000*g*), the resultant "mitoplast" fraction was resuspended in medium at a protein concentration of 30 mg/ml. Lubrol WX (19 mg/ml) was then added (final concentration, 0.16 mg per milligram of mitoplast protein). After 15 minutes the suspension, diluted 1 : 2 with isolation medium, was centrifuged at 144,000*g* for 60 minutes. The sediment (inner mitochondrial membrane) was resuspended in tris buffer, *p*H 7.2, containing 0.01*M* EDTA. The supernatant from this spin represents matrix protein solution (*4*). Outer mitochondrial membranes were obtained by collecting the sediment after centrifugation (144,000*g*) of the combined supernatants from the mitoplast preparation. The supernatant from this spin is intermembrane protein solution (*4*).

2) Hypotonic lysis was done by the method of Caplan and Greenawalt (*5*). Rat liver mitochondria were prepared in 0.25*M* sucrose and washed twice. The pellet was then treated for 10 minutes with distilled water at 4°C (1 ml per gram of liver equivalent). The suspension was centrifuged at 105,000*g* for 30 minutes

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