

SOLUBILIZING EFFECT OF NUCLEOTIDES ON ISOLATED INSULIN SECRETORY GRANULES

B. HELLMAN and I.-B. TÄLJEDAL. From the Department of Histology, University of Umeå,
Umeå, Sweden

INTRODUCTION

ATP has been reported to cause release of hormone from secretory granules isolated from the adrenal medulla (Poisner and Trifaró, 1967) and the posterior pituitary (Poisner and Douglas, 1968). This effect was suggested to be linked to enzymatic cleavage of the nucleotide, both the hormone release and the ATPase activity being inhibited by AMP. Using a partially purified fraction of insulin secretory granules from obese-hyperglycemic mice, we observed that ATP also has a solubilizing action on granule-bound insulin (Coore et al., 1969). The present study was undertaken to test whether the sensitivity of the β -granules to ATP can be explained by the same mechanisms that have been proposed for the other endocrine granules. A number of nucleotides were investigated for effects on the stability of isolated β -granules. In addition, the presence of ATP-splitting activity in this β -cell fraction was checked with regard to its sensitivity to AMP.

MATERIALS AND METHODS

Chemicals of analytical grade and water distilled two to three times were used.

In each experiment 20–30 fresh pancreatic islets were isolated from obese-hyperglycemic mice (gene symbol *obob*, Ingalls et al., 1950; Hellman, 1965) by means of microdissection (Hellerström, 1964). This tissue consists of more than 90% of β -cells, which respond normally to glucose (Lernmark and Hellman 1969). A subcellular fraction containing about 60% of the total and more than 95% of the particle-bound insulin in these cells was prepared by differential

centrifugation as previously described (Coore et al., 1969). After isolation, the material was suspended in a solution of 320 mM sucrose, adjusted to pH 6 by equilibration with ambient air or with HCl.

The stability of the isolated secretory granules in media of varying composition (see the Results section) was assessed as follows. Samples (50 μ l each) of the granule suspension were incubated with 250 μ l of test solution for 15 min at 37°C. After incubation and subsequent centrifugation of the test tubes at 6000 *g* for 20 min, the upper and lower halves (150 μ l each) of the tube contents were extracted with acid-ethanol and assayed for insulin by the radio-immunological method of Hales and Randle (1963). The relative amount of particle-bound insulin was calculated as the percentage excess of insulin in the lower fraction as compared with the upper one. For a more detailed description of the techniques, reference is made to Coore et al. (1969).

ATP-splitting activity in the granule fraction was assayed by the technique of Itaya and Ui (1966) as modified by Täljedal (1969). In these experiments cacodylate buffer (7 mM) was used. Protein was determined after Lowry et al. (1951), with human serum albumin as standard.

RESULTS

Effects of ATP and AMP in Phosphate Buffer of pH 6

Only about 50% of the insulin remained sedimentable after incubation of the granules in 11 mM phosphate buffer supplemented with 0.4 mM MgCl₂ (Table I). When added to this medium, 0.4 mM ATP did not increase the granule fragility.

Granule stability was not improved by AMP. On the contrary, 4.2 mM AMP brought about a further release of hormone.

Some cleavage of ATP occurred in the isolated granule fraction (Table I). AMP (4.2 mM) did not inhibit this ATP-splitting activity.

Effects of ATP and AMP in a High-K⁺/Low-Na⁺ Medium of pH 7

The stability of the granules was very low in a tris-buffered medium of pH 7, containing 133 mM KCl and 4.2 mM NaCl (Table I). ATP in the concentration of 0.4 mM did not affect the granule stability. A further release of insulin was also obtained in this medium by the addition of 4.2 mM AMP.

Effects of Various Nucleotides in Low-Electrolyte Media of pH 6

More than 90% of the insulin remained sedimentable after incubation of the granules in sucrose solutions devoid of electrolytes (Fig. 1; Table II). When ATP was added to this medium (Fig. 1), there was a tendency to decreased granule stability at 1.7 mM ATP. At an ATP concentration of 4.2 mM, the solubilizing effect was highly significant. The response to 4.2 mM ATP was not stimulated but was partially inhibited by MgCl₂. The latter compound alone caused only moderate release when added in a concentration of 4.2 mM (Fig. 1). It was checked that the low ATP-splitting activity associated with the granule fraction did not acidify the unbuffered medium to any measurable extent.

ATP was not the only nucleotide to bring about a release of granule-bound insulin. ADP, AMP, ITP, NADH, NADP, and NADPH appeared to be equally effective when included in a concentration of 4.2 mM (Table III). In addition, EDTA exerted a solubilizing effect on the granules. Dibutyryl cyclic 3',5'-AMP, however, released little of the granule-bound insulin.

DISCUSSION

More than 90% of the insulin remained sedimentable after incubation in unbuffered sucrose solutions adjusted to pH 6, whereas only 50% was recovered by centrifugation after incubation in phosphate buffer with MgCl₂. This is in accordance with our previous conclusions that the stability of insulin secretory granules is best preserved in low-electrolyte media of pH 6 (Coore et al., 1969) and that simple physico-chemical mechanisms may account for the rapid extracellular dissolution of β -granules in vivo.

So far our results are in fair agreement with those of Howell et al. (1969), who used rat islets as the source of β -granules. In contrast, Lambert et al. (1970) found neither a stability optimum at pH 6 nor any effect of electrolytes in their study of insulin secretory granules from mice. The reasons for this discrepancy remain obscure. It is, however, obvious that the preparation and/or assay system used by the latter authors cannot be directly compared with ours, since Lambert et al. (1970) reported more than 50% of the granule-bound insulin to be released even during control incubations at pH 6 in sucrose alone. The exceptionally great stability observed in our control incubations

TABLE I
Effect of ATP and AMP on β -Granule Stability in the Presence of Electrolytes

Basic medium	Nucleotide added		
	None	0.4 mM ATP	0.4 mM ATP 4.2 mM AMP
11 mM phosphate buffer, pH 6 0.4 mM MgCl ₂	47.8 \pm 4.3 (26)	52.0 \pm 5.3 (26)	11.1 \pm 2.5 (14)
8.0 mM tris-HCl, pH 7 133.0 mM KCl 4.2 mM NaCl 0.4 mM MgCl ₂	20.8 \pm 8.7 (12)	19.2 \pm 7.1 (11)	5.3 \pm 3.9 (11)

The values denote percentage of sedimentable insulin (mean values \pm SEM) after incubation of β -granules in the indicated media. The numbers of observations are given in parentheses.

might be due to the fact that no collagenase was involved in the isolation procedure. It has recently been shown that commercially available preparations of collagenase also contain the membrane-active enzyme phospholipase C (Elsbach and Rizack, 1970).

There has been some controversy in the past concerning the effect of nucleotides on the β -granules. Neither Lambert et al. (1970) nor Howell et al. (1969) found that ATP released insulin from the β -granules under their experimental condi-

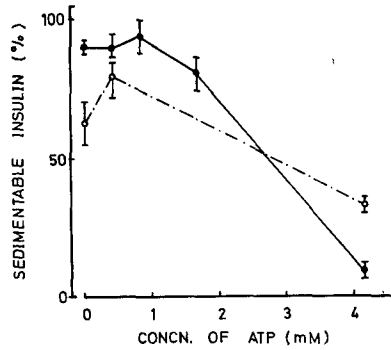


FIGURE 1 Effect of ATP on the stability of β -granules in 320 mM sucrose in the absence (●) and presence (○) of 4.2 mM $MgCl_2$. All media were unbuffered but carefully adjusted to pH 6.0 immediately before use. Each point represents the mean of six observations, for which the SEM is also indicated.

TABLE II
Effect of AMP on the Cleavage of ATP in the Isolated Granule Fraction

Experiment number	Rate of P_i liberation		Difference
	0.4 mM ATP	0.4 mM ATP 4.2 mM AMP	
I	0.69	0.91	0.22
II	1.40	1.33	-0.07
III	1.58	1.32	-0.26
Total	1.22	1.18	-0.04

Isolated β -granules were incubated for 15 min at 37°C in media containing 304 mM sucrose, 7 mM cacodylate buffer (pH 6) and the nucleotides listed in the Table. The results of three different experiments are given as moles of P_i liberated/kg protein per hr.

TABLE III
Effects of Nucleotides and EDTA on β -Granule Stability in Low-Electrolyte Sucrose Media of pH 6

Test substance	Concentration	Sedimentable insulin	Number of observations
	mM	%	
None (control)		96.9 \pm 0.9	24
ITP (trisodium salt)	4.2	20.9 \pm 4.6	5
ADP (trisodium salt)	4.2	20.0 \pm 6.0	6
AMP (disodium salt)	4.2	39.5 \pm 12.8	6
Dibutyl cyclic 3'5'-AMP (monosodium salt)	4.2	85.0 \pm 2.9	6
NADP (disodium salt)	4.2	24.6 \pm 3.1	6
NADPH (tetrasodium salt)	4.2	34.3 \pm 5.9	6
NADH (disodium salt)	4.2	37.1 \pm 3.9	6
EDTA (disodium salt)	0.1	91.5 \pm 3.5	6
EDTA (disodium salt)	0.8	65.8 \pm 2.2	6
EDTA (disodium salt)	4.2	16.5 \pm 5.5	6

The values denote percentage of sedimentable insulin (mean values \pm SEM) after incubation of β -granules in 320 mM sucrose supplemented with the test substances listed. All media were unbuffered but carefully adjusted to pH 6.0 immediately before use. Nucleotides (inosine 5'-triphosphate, adenosine 5'-diphosphate, adenosine 5'-monophosphate, dibutyl adenosine 3',5'-monophosphate, nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate, and reduced nicotinamide dinucleotide) were obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany, and EDTA (ethylenediamine tetra-acetate) was from E. Merck AG, Darmstadt, Germany.

tions. The present results indicate that the concentrations of ATP used by Coore et al. (1969) as well as by Howell et al. (1969) are on the borderline of what is necessary for a significant effect. The action of ATP may furthermore appear erratic and difficult to demonstrate, if it is tested in media containing other electrolytes, which by themselves exert a solubilizing action. The present effect of 4.2 mM ATP in an unbuffered sucrose solution carefully adjusted to pH 6 was highly significant.

The solubilizing effect of ATP on the β -granules is not specific for this nucleotide. All nucleotides tested, except the dibutyryl derivative of cyclic 3',5'-AMP, released most of the granule-bound insulin. Since the improved assay conditions made it possible to show that EDTA was equally effective, solubilization of the granules is probably due to the chelation of heavy metal. It is well known that the insulin secretory granules contain a heavy metal (Coore et al., 1969), most probably zinc (Havu, 1969), and its chelation has previously been suggested as a mechanism of insulin release (Maske, 1957; Ninomiya et al., 1966). The idea that the effect of ATP on the isolated β -granules is due to its metal-chelating ability is consistent with the observation that the effect of 4.2 mM ATP was reduced in the presence of $MgCl_2$. Such a mechanism might also help to explain the difficulties in demonstrating the ATP-induced granule solubilization discussed above.

ATP has been reported to cause a release of hormone from secretory granules isolated from the adrenal medulla (Poisner and Trifaró, 1967) and from the posterior pituitary (Poisner and Douglas, 1968). This effect was suggested to be linked to enzymatic cleavage of ATP, both processes being inhibited by AMP (Poisner and Douglas, 1968). It is worthy of note that AMP did not stabilize the insulin secretory granules. Nor could AMP be shown to significantly inhibit the low ATP-splitting activity in the isolated β -granule fraction.

It is not known how regulation of insulin release is accomplished in the intact β -cell. There are even conflicting opinions as to whether the secretory granules are dissolved within the β -cell (Lever and Findlay, 1966) or ejected by emiocytosis (Lacy, 1968). The molecular events involved can at present only be the subject of speculation. It appears from the available data that not only pH and electrolyte changes but also the chelation of zinc may be involved in the physiological release mechanism. The nonspecific nature of these processes in

vitro does not exclude the participation of very specific regulator molecules in the intact β -cell. Nor can it be ruled out that the observed effects of nucleotides are due to some unknown metabolic interaction with the β -granules.

SUMMARY

A partially purified fraction of β -granules was prepared from microdissected islets of obese-hyperglycemic mice. More than 90% of the granule-bound insulin remained sedimentable by centrifugation after incubation for 15 min at 37°C and pH 6 in sucrose solutions containing a minimum of electrolytes. When ATP was added to this medium, a concentration-dependent release of the granule-bound insulin occurred. The threshold for a significant effect was about 2 mM ATP. With 4.2 mM ATP, the reduction of granule stability was highly significant. The solubilizing effect of ATP may be due to chelation of granule- Zn^{++} , since ADP, AMP, ITP, NADP, NADPH, NADH, and EDTA were also found to affect the stability of the granules in a similar manner. The β -cell granules differ from the secretory granules of the posterior pituitary in not being stabilized by AMP.

This study is part of a project supported by the United States Public Health Service (AM-12535), the Swedish Medical Research Council (12x-562, 12x-2288), the Swedish Diabetes Association, and Nordisk Insulinfond.

The albumin used in the insulin assays was kindly donated by AB Kabi, Stockholm. The skillful technical assistance of Miss Karin Janze is gratefully acknowledged.

Received for publication 2 March 1970, and in revised form 29 April 1970.

REFERENCES

- COORE, H. G., B. HELLMAN, E. PIHL, and I.-B. TÄLJEDAL. 1969. Physicochemical characteristics of insulin secretion granules. *Biochem. J.* 111:107.
- ELSBACH, P., and M. A. RIZACK. 1970. The effect of collagenase preparations contaminated with phospholipase C activity on adipose tissue lecithin. *Biochim. Biophys. Acta.* 198:82.
- HALES, C. N., and P. J. RANDLE. 1963. Immunoassay of insulin with insulin-antibody precipitate. *Biochem. J.* 88:137.
- HAVU, N. 1969. Sulfhydryl inhibitors and pancreatic islet tissue. *Acta endocrinol. (Copenhagen) Suppl.* 139.
- HELLERSTRÖM, C. 1964. A method for the micro-

- dissection of pancreatic islets in mammals. *Acta endocrinol. (Copenhagen)*. **45**:122.
- HELLMAN, B. 1965. Studies in obese-hyperglycemic mice. *Ann. N. Y. Acad. Sci.* **131**:541.
- HOWELL, S. L., D. A. YOUNG, and P. E. LACY. 1969. Isolation and properties of secretory granules from rat islets of Langerhans. III. Studies of the stability of the isolated beta granules. *J. Cell Biol.* **41**:167.
- INGALLS, A. M., M. M. DICKIE, and G. D. SNELL. 1950. Obese, a new mutation in the mouse. *J. Hered.* **41**:317.
- ITAYA, K., and M. UI. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta.* **14**:361.
- LACY, P. E. 1968. Ultrastructural changes of the β -cells during insulin secretion. *Acta diab. latina.* **5**(Suppl. 1):436.
- LAMBERT, A. E., Y. KANAZAWA, L. ORCI, and G. M. GRODSKY. 1970. Properties of isolated β -granules in suspension. In *The Structure and Metabolism of the Pancreatic Islets*. S. Falkmer, B. Hellman, and I.-B. Täljedal, editors. Pergamon Press Ltd., Oxford. 397.
- LERNMARK, Å., and B. HELLMAN. 1969. The β -cell capacity for insulin secretion in microdissected pancreatic islets from obese-hyperglycemic mice. *Life Sci.* **8**:53.
- LEVER, J. D., and J. A. FINDLAY. 1966. Similar structural bases for the storage and release of secretory material in adreno-medullary and β pancreatic cells. *Z. Zellforsch. Mikrosk. Anat.* **74**:317.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem.* **193**:265.
- MASKE, H. 1957. Interaction between insulin and zinc in the islets of Langerhans. *Diabetes.* **6**:335.
- NINOMIYA, R., Y. MIURA, K. KOSAKA, K. NAKAO, and S. OKINAKA. 1966. Stimulatory effect of chelating agents and mesoxalate on the in vivo release of insulin in the pancreas of the dog. The possible role of amino acids. *Diabetes.* **15**:44.
- POISNER, A. M., and W. W. DOUGLAS. 1968. A possible mechanism of release of posterior pituitary hormones involving adenosine triphosphate and an adenosine triphosphatase in the neurosecretory granules. *Mol. Pharmacol.* **4**:531.
- POISNER, A. M., and J. M. TRIFARÓ. 1967. The role of ATP and ATPase in the release of catecholamines from the adrenal medulla: I. ATP-evoked release of catecholamines, ATP and protein from isolated chromaffin granules. *Mol. Pharmacol.* **3**:561.
- TÄLJEDAL, I.-B. 1969. Presence, induction and possible role of glucose 6-phosphatase in mammalian pancreatic islets. *Biochem. J.* **114**:387.