Interaction between Insulin-Storage Granules and F-Actin *in vitro*

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Possible interactions between polymerized (F-) actin and insulin-storage granules from rat islets of Langerhans were examined *in vitro* by comparing the sedimentation of the granules in the presence of various actin concentrations. Actin in the concentration range 0.1–0.5 mg/ml produced a retardation in granule-sedimentation rates consistent with binding of the granules to the actin filaments. This interaction was increased by addition of ATP (2 mM), but was decreased by CaCl_2 (0.1 mM). Binding of granules to actin was unaffected by cyclic AMP or by preincubation of the granules with phospholipase C. Specificity of the interaction was confirmed by the use of depolymerized (G-) actin and of myosin to provide a solution of comparable viscosity; neither of these caused any alteration of granule sedimentation. Possible implications of this interaction of insulin-storage granules with actin for the mechanism of insulin secretion are briefly discussed.

Actin has been identified in many types of non-contractile cells, including the islets of Langerhans. Its presence has been demonstrated by immunofluorescence (Gabbiani *et al.*, 1974), and by identification of a protein band that co-migrates with muscle actin on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Pipeleers *et al.*, 1976). In addition, the microfilaments that are present in B cells are believed to be composed of actin, and it has been suggested that groups of these filaments form a 'cell web' beneath the plasma membrane, which impedes access of insulin-storage granules to the membrane (Orci *et al.*, 1972). An enhanced insulin-secretory response to glucose is obtainable after disruption of the microfilamentous web by the fungal metabolite cytochalasin B (Orci *et al.*, 1972).

As a first stage in an examination of the role of actin in the process of insulin secretion we have examined the effect of rabbit muscle actin on the sedimentation of isolated insulin-storage granules in a variety of conditions. These experiments give some indication of the interactions that might occur between actin and granules during stimulation of secretion in living B cells.

Methods

Preparation of storage-granule fractions

Islets of Langerhans were isolated from rat pancreas by collagenase digestion (Howell & Taylor, 1966). After preincubation for 30 min in a bicarbonate-buffered incubation medium (Gey &

Gey, 1936) containing 5.5 mM-glucose, the islets were homogenized in 0.3 M-sucrose/50 mM-Tris/HCl, pH 7.4. After centrifugation to remove unbroken cells, nuclei and debris, a crude mitochondria-storage granule-heavy microsomal fraction was prepared by differential centrifugation at 24000g for 20 min at 4°C in an MSE 18 centrifuge. After removal of the supernatant, the pellet was resuspended by gentle homogenization in sucrose/Tris/HCl, pH 7.4, and samples were used immediately for further sedimentation studies.

Preparation of muscle actin

Rabbit skeletal muscle was utilized in all the experiments described in this paper. It was extracted by homogenization in 0.1 M-KCl/0.05 M- NaHCO_3 at 4°C for 15 min, washing with 1 mM-EDTA and water and extraction with acetone. The resulting acetone-dried powder was stored at -20°C for up to 2 weeks. In each experiment the acetone-dried powder was further extracted with 2 mM-Tris/HCl/0.25 mM-ATP/1 mM-dithiothreitol, pH 7.4. Actin was polymerized from this soluble extract by addition of 0.1 M-KCl (Rees & Young, 1967). After incubation for 2 h the polymerized actin was precipitated by centrifugation at 105000g for 2 h in a Spinco L2 centrifuge at 4°C and the resultant pellet was resuspended by gentle homogenization in 0.1 M-KCl/2 mM-Tris/HCl/0.25 mM-ATP/1 mM-dithiothreitol, pH 7.4.

In some experiments further purification of the actin was attempted after depolymerization by dialysis against 2mM-Tris/HCl/0.25mM-ATP/1mM-dithiothreitol, pH7.4. The resulting dialysis residue was subjected to gel filtration on a column (60cm $\times$ 1cm) of Sephadex G-100 with elution in buffer of the same composition. The major peak of actin monitored by A_{280} was repolymerized by addition of KCl to a final concentration of 0.1M. After pelleting by centrifugation at 105000g for 2h, this pellet of purified actin was used in the same way as before.

Sedimentation studies

Polymerized actin was diluted with 0.15M-NaCl/10mM-Tris/HCl, pH7.4, to give a final concentration of 0.4–0.6mg/ml. Portions of the resuspended granule fraction were then added and the tubes were mixed vigorously before incubation at 37°C for 15min. After this period the tubes were centrifuged at 24000g for 10, 20 and 30min as shown in individual experiments. The supernatants remaining were removed and their protein and insulin contents were determined and compared with control tubes that had not been subjected to centrifugation. Results (mean $\pm$ S.E.M.) were expressed as the percentage of the total insulin and protein content of the system that remained in the supernatant after centrifugation.

Insulin content of supernatants was determined after freeze-thawing of the solutions to ensure granule lysis, by an immunoassay procedure utilizing insulin-binding reagent (Wellcome Reagents, Beckenham, Kent, U.K.) and ^{125}I -labelled insulin prepared in our laboratory.

Sedimentation of actin was assessed by comparing the A_{280} of the solutions before and after centrifugation. The contribution of the islet homogenate to the overall A_{280} was less than 10% and was ignored.

Results

Characteristics of the system

Preliminary experiments were performed to determine the sedimentation properties of isolated insulin-storage granules in the presence of various concentrations of both polymerized (F-) and depolymerized (G-) actin. At actin concentrations of 0.01–0.1mg/ml, there was no significant difference between sedimentation of granules in polymerized or depolymerized actin. At concentrations greater than 0.1mg/ml, however, there was a statistically significant and concentration-dependent retardation of granule sedimentation in the presence of poly-

Fig. 1. Effect of actin concentration on sedimentation of insulin-storage granules

The percentage of insulin remaining in the supernatant after incubation of granules with depolymerized (G-) actin (○) or polymerized (F-) actin (■) for 15 min at 37°C followed by centrifugation at 24000g for 10 min are shown. Results shown are means $\pm$ S.E.M. (bars) for eight observations in each case.

merized, but not of depolymerized, actin (Fig. 1). Subsequent experiments therefore used polymerized actin concentrations in the range 0.4–0.6mg/ml.

Analysis of effects of duration of centrifugation on the retardation of granule sedimentation by actin were complicated by the slow dissolution of granules and release of their insulin content that occurs during their incubation *in vitro*: in these conditions this dissolution amounts to approx. 30%/h. Effects of increasing duration of centrifugation on granule sedimentation without (Fig. 2a) and with (Fig. 2b) correction for granule dissolution are shown, as is sedimentation of actin alone in the same experiments (Fig. 2c); the concentration of actin used in these experiments was 0.52mg/ml. In general it was found that the most consistent effect of actin on sedimentation of granules was demonstrable after 20min centrifugation, and this was used in all further experiments. Centrifugation of granules in the presence or absence of F-actin at 105000g for 60min to pellet all the remaining granules showed no significant difference in their sedimentation, suggesting that actin itself had no effect on granule stability.

Effects of myosin and phospholipase C

Addition of myosin in place of actin at similar concentrations had no effect on granule sedimenta-

Fig. 2. Effects of duration of centrifugation on granule and actin sedimentation

(a)–(c) show effects of 10, 20 or 30 min centrifugation at 24000g on sedimentation of insulin-storage granules in the presence of actin without (a) or with (b) correction for granule dissolution, and of actin alone (c). (a) shows that in the presence of actin progressive sedimentation of granules occurs during increasing periods of centrifugation. (b) The sedimentation curve of storage granules in the presence of actin after correction for their progressive dissolution during the period of centrifugation. (c) The sedimentation of actin alone during centrifugation in similar conditions. In (b), granule dissolution was determined simultaneously by estimating insulin concentration in supernatants after centrifugation of storage granules alone, the insulin appearing in the supernatant during this period being subtracted from the values shown in (a).

Table 1. Effects of myosin and phospholipase C pre-treatment on granule sedimentation

Sedimentation of storage granules was determined after incubation for 15 min at 37°C in the presence or absence of myosin (0.4 or 0.8 mg/ml), or after pre-treatment of the granules with phospholipase C (25 µg/ml) as described in the text.

(a) Effect of myosin

	Control	Myosin (0.4 mg/ml)	Myosin (0.8 mg/ml)
Insulin in supernatant (%)	53.5 ± 11.1	35.8 ± 6.7	46 ± 4.4
Myosin in supernatant (%)	—	70.5 ± 3.3	83.2 ± 1.8

(b) Effect of pre-treatment of granules with phospholipase C

	Control	+ Actin	Phospholipase-pretreated	
			Control	+ Actin
Insulin in supernatant (%)	51.3 ± 4.2	74.5 ± 7.5	42.4 ± 5.8	76.8 ± 9.3
Actin in supernatant (%)	—	68.0 ± 5.0	—	70.4 ± 4.3

tion, despite an apparently similar viscosity of the myosin-containing solutions (Table 1). Similarly, pre-treatment of the granules with phospholipase C (25 µg/ml) in the presence of 2 mM-CaCl₂ for 15 min at 37°C before centrifugation and resuspension as above did not alter the ability of actin to retard granule sedimentation.

Effects of ATP, cyclic AMP and Ca²⁺

ATP (2 mM) had no significant effect on granule stability in these conditions. However, addition of ATP to suspensions of granules in actin-containing media significantly decreased the extent of their sedimentation, so that less insulin was present in the pellet (Table 2). There was no comparable effect on actin sedimentation. Cyclic AMP (0.1 mM) had

no effect on sedimentation of granules or of actin alone, nor on sedimentation of granules in the presence of actin (Table 2). Finally, Ca²⁺ at concentrations of 1 mM (but not 0.1 mM) increased the degree of sedimentation of granules in actin-containing media so that it was not significantly different from that observed in control (non-actin-containing) solutions (Table 2). Ca²⁺ alone had no effect on granule stability or on actin sedimentation.

Discussion

The specificity of the observed interaction of F-actin with insulin-storage granules was investigated by use of experiments with depolymerized (G-) actin, and with myosin solutions with viscosity

Table 2. *Effects of ATP, cyclic AMP and Ca²⁺ on granule-actin interaction*

Storage-granule sedimentation after centrifugation at 24000g for 20min was assessed after incubation of the granules with or without actin under the conditions shown in each experiment. Results shown are means $\pm$ S.E.M. of 12 observations. *Indicates significant difference ($P < 0.05$) from sedimentation observed in the presence of actin alone (t test). Differences between sedimentation of granules alone and in the presence of actin are significant in every case.

(a) Effect of ATP				
	Control	+ Actin	+ Actin + 0.2mM-ATP	+ Actin + 2mM-ATP
Insulin in supernatant (%)	47.4 $\pm$ 4.0	62.1 $\pm$ 1.5	65.3 $\pm$ 3.5	75.2 $\pm$ 4.2*
Actin in supernatant (A ₂₈₀) (%)	—	72.4 $\pm$ 2.6	71.6 $\pm$ 2.9	73.5 $\pm$ 2.1
(b) Effect of 0.1mM-cyclic AMP				
			+ Actin + 0.1mM- cyclic AMP	
Insulin in supernatant (%)	51.1 $\pm$ 5.9	70.1 $\pm$ 3.8	77.8 $\pm$ 3.8	
Actin in supernatant (%)	—	76.4 $\pm$ 3.75	70.3 $\pm$ 1.2	
(c) Effect of Ca ²⁺				
			+ Actin + 0.1mM- CaCl ₂	+ Actin + 1mM- CaCl ₂
Insulin in supernatant (%)	54.2 $\pm$ 4.9	69.8 $\pm$ 2.9	65.7 $\pm$ 4.2	58.4 $\pm$ 4.4*
Actin in supernatant (%)	—	69.4 $\pm$ 2.4	66.3 $\pm$ 2.4	70.0 $\pm$ 1.8

comparable with that of F-actin (Ostlund *et al.*, 1977); none of these agents showed effects similar to those of F-actin (Table 1). Furthermore, phospholipase C pre-treatment of granules before estimation of their interaction with actin did not alter their sedimentation. In this, as in other aspects of their specificity, the results reported in the present paper are similar to those described by Ostlund *et al.* (1977) in a study of pituitary granule-actin interactions. These authors suggested that lipid-protein but not phospholipid-protein or protein-protein interaction may be important for pituitary granule-actin binding.

It has been proposed that microtubules and microfilaments exist in close association in the B-cell cytoplasm, thereby constituting a system for the directed transport of storage granules from the cytoplasmic pool to the plasma membrane (Lacy *et al.*, 1968). There is to date little evidence for an association of actin filaments with the microtubules, so that it may not be helpful to speculate about the possible role of granule-actin interactions in this process. However, it has been shown that actin-containing microfilaments are present in the B cell as a 'cell web' running parallel to the plasma membrane and that reorganization of the cell web during stimulation of secretion or by cytochalasin B might facilitate the fusion of granule and plasma membranes in exocytosis (Orci *et al.*, 1972). The existence of an

interaction of actin and granule membranes implies that granules that contact filaments of the cell web will tend to stay in contact with it, and therefore remain close to the site of secretion.

The effect of ATP in promoting granule-actin interaction is of some interest, since it occurs in the presence of ATP concentrations that may be found in intact cells, suggesting that such interactions might similarly be facilitated by ATP *in vivo*. However, overall B-cell ATP concentrations do not alter in proportion to the degree of stimulation of insulin secretion (Ashcroft *et al.*, 1973), so that a regulatory role of ATP in secretion by promoting granule-actin adhesion seems unlikely. On the other hand, stimulation of secretion may be effected by an increase in cytosolic Ca²⁺ concentrations (Malaisse, 1973), so it is of some interest that addition of Ca²⁺ to the system results in a lower affinity of the granules for the actin filaments. *In vivo* this would presumably render them more mobile, and more readily accessible to the plasma membrane. Decreasing the surface charge on granule and/or plasma membranes by increased Ca²⁺ concentrations may in turn facilitate their fusion in exocytosis (Dean, 1975; Dean & Matthews, 1975; Howell & Tyhurst, 1977).

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