Actomyosin interactions with insulin-storage granules *in vitro*

Simon L. HOWELL and Margaret TYHURST

Department of Physiology, Queen Elizabeth College, University of London, Campden Hill Road, London W8 7AH, U.K.

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Interactions between actomyosin and insulin-storage granules isolated from rat islets of Langerhans have been examined in a simple system *in vitro*, which allows comparison of the sedimentation of the granules in the presence and absence of actomyosin in various conditions. Actomyosin altered granule-sedimentation rates in a manner consistent with the binding of the granules to actomyosin filaments. This interaction was enhanced by addition of ATP (1.5 mM) but unaltered by addition of CaCl_2 , by calmodulin or by calmodulin in the presence of $10\text{ }\mu\text{M}$ - CaCl_2 . Addition of EGTA (0.1 mM), cyclic AMP (10 μM) or cytochalasin B (10 $\mu\text{g}/\text{ml}$) were also without effect in these conditions. Pre-incubation of granules with phospholipase C did not affect granule-actomyosin interaction. Ultrastructural studies showed close contacts between the membranes of the granules and actomyosin filaments. The results indicate the possibility that actomyosin might provide the motile force for granule translocation during the insulin secretory process.

Actin and myosin have both been identified as constituent proteins of catfish and rat islets of Langerhans (Gabbiani *et al.*, 1974; Ostlund *et al.*, 1978). Attention has been paid recently to the possible interactions between exogenous actin filaments and isolated insulin-storage granules (Howell & Tyhurst, 1979) and also the regulation of globular=filamentous actin interconversion in B-cells during stimulation of secretion (Howell & Tyhurst, 1980; Swanson-Flatt *et al.*, 1980). However, there have been few studies of the possible role of myosin in the insulin-secretory process, and in particular of putative interactions between actomyosin and isolated insulin-storage granules. We have therefore examined these aspects by the use of a simple technique *in vitro* similar to that used for our previous studies on actin-granule interaction.

Experimental

Preparation of storage-granule fractions

Islets of Langerhans were isolated from rat pancreas by collagenase digestion (Howell & Taylor, 1966). After pre-incubation for 30 min in a bicarbonate-buffered incubation medium (Gey & Gey, 1936) containing 5 mM-glucose, the islets were homogenized in 0.3 M-sucrose/50 mM-Tris/HCl,

pH 7.4. After centrifugation ($600\text{ g}\times 5\text{ min}$) to remove unbroken cells, nuclei and debris, a crude mitochondria/storage-granule/heavy-microsomal fraction was prepared by differential centrifugation at 24000 g 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, in a Kontes all-glass homogenizer, and samples were used immediately for further sedimentation studies.

Myosin, actin and actomyosin

These were extracted from rabbit muscle and were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Their purity was confirmed by polyacrylamide-slab-gel electrophoresis, which also showed the presence of light and heavy chains in the myosin preparations. Actin depolymerization was effected by dialysis against 2 mM-Tris/HCl/0.25 mM-ATP/1 mM-dithiothreitol, pH 7.4. Repolymerization was achieved by addition of KCl to a final concentration of 0.2 M.

Sedimentation studies

Myosin, actin or actomyosin were diluted with 0.15 M-NaCl/10 mM-Tris/HCl, pH 7.4, to give final

concentrations of 0.02–0.6 mg/ml (Howell & Tyhurst, 1979). Portions of the resuspended granule fraction were then added and the tubes were mixed vigorously before incubation at 37°C for 15 min. After this period the tubes were centrifuged at 24 000 *g* for 20 min. 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 (means $\pm$ S.E.M.) were expressed as percentages of the total insulin and protein content of the system that remained in the supernatant after centrifugation, and were compared by use of Student's *t* test.

Insulin content of supernatants was determined by an immunoassay procedure after freeze–thawing of the solutions to ensure granule lysis. Anti-insulin serum was a gift from Dr. W. Montague, University of Leicester, Leicester, U.K., and ^{125}I -labelled insulin was prepared in our laboratory by a chloramine-T procedure. Cytochalasin B, adenosine 3':5'-cyclic monophosphate (cyclic AMP) and phosphodiesterase 3':5'-cyclic nucleotide activator (calmodulin) were obtained from Sigma Chemical Co.

Electron microscopy

Pellets containing actomyosin, which were sedimented at 24 000 *g* for 20 min after incubation in the presence of the isolated granule fractions for 15 min at 37°C, were fixed with 3% glutaraldehyde in 0.1 M-phosphate buffer immediately after sedimentation and post-fixed with 2% OsO_4 in similar buffer. After dehydration in ethanol and embedding in a standard epoxy resin, thin sections were stained with a saturated solution of uranyl acetate in 50% ethanol and examined in an AEI EM 6B electron microscope.

Results and discussion

The present study seeks to complement our previous work on interactions of actin with isolated insulin-storage granules from rat islets of Langerhans in which it was shown that such interaction significantly retards the sedimentation of the granules (Howell & Tyhurst, 1979). It is clear from comparison with these previous experiments (see also Fig. 1) that actomyosin is also effective in altering granule sedimentation, at a concentration far lower than that required for granule–F actin interaction, whereas myosin itself has no such interactive effects (Fig. 1). This was shown not to be a viscosity effect since similar results were not seen with Ficoll or soluble collagen, solutions that are of comparable viscosity (Howell & Tyhurst, 1979). Ultrastructural studies of the sedimented material show the existence of direct contacts between

Fig. 1. Effect of F-actin, G-actin, myosin and actomyosin concentration on sedimentation of insulin-storage granules

The percentage of insulin remaining in the supernatant after incubation of granules with globular actin (G-actin) filamentous actin (F-actin), myosin or actomyosin for 15 min at 37°C followed by re-centrifugation at 24 000 *g* for 20 min is shown. Results are means $\pm$ S.E.M. (bars) from six observations derived from 3 separate experiments in each case. * indicates a significant difference from control (no additions) at $P < 0.05$.

actomyosin filaments and isolated β -granules and leave no doubt that the interactions are directly between actomyosin filaments and granule membranes (Fig. 2), and not merely the result of a mesh effect that might also serve to alter their sedimentation characteristics. Similar images were obtained after incubation in the presence or absence of ATP, and occasionally with other organelles in the subcellular fractions.

The interaction between granules and actomyosin that was observed was enhanced by addition of ATP at a concentration (1.5 mM) similar to that known to exist within the B-cells, but to be unaffected by addition of calcium, even at relatively high concentrations (Table 1). However, it is not certain whether these reflect the true Ca^{2+} concentrations in the system since several components (mitochondria, storage granules) each contain Ca^{2+} , which is potentially releasable into the medium during incubations of this type. Removal of all free Ca^{2+} from the medium by addition of 0.1 mM-EGTA did not alter granule sedimentation (Table 1), while addition of exogenous calmodulin (0.1 or 0.4 $\mu\text{g/ml}$) in the presence or absence of 10 μM - Ca^{2+} again did not significantly affect the interaction between granules and actomyosin that was observed (Table 1). None of these additions altered the sedimentation characteristics of actomyosin alone, where higher speed centrifugation (30 000 *g* for 60 min) served to demon-

Fig. 2. Electron micrographs of actomyosin-insulin-storage-granule interactions

The actomyosin and storage-granule fractions were incubated in the presence of 1.5 mM-ATP for 15 min at 37°C, centrifuged for 20 min at 24000g, fixed and prepared for electron microscopy as described in the text. The filamentous nature of the actomyosin is evident, as are close contacts with the storage-granule membranes (arrows). G indicates the electron-opaque granule core. Staining was with uranyl acetate in 50% ethanol. Magnification: (a), approx. 55 000; (b) approx. $\times 84\,000$.

Table 1. Actomyosin-insulin-storage-granule interactions in the presence of various additions

Storage-granule sedimentation after centrifugation at 24000g for 20 min was assessed after incubation of the granules with or without actomyosin under the conditions shown in each experiment. Each test result represents the pooled mean $\pm$ s.e.m. of 16 observations from four separate experiments. The variation of results between experiments was of the same order as that seen within an experiment. * indicates a significant difference from control containing no actomyosin at $P < 0.05$; ** indicates a significant difference ($P < 0.05$) from control containing actomyosin alone. Abbreviation used: AM, actomyosin.

Additions	Insulin in supernatant (%)
None	53.6 $\pm$ 3.8
AM (62.5 μ g/ml)	69.0 $\pm$ 5.5*
AM + 0.75 mM-ATP	71.1 $\pm$ 4.6
AM + 1.5 mM-ATP	78.5 $\pm$ 3.9**
AM + 10 μ M-Ca ²⁺	65.2 $\pm$ 2.4
AM + 100 μ M-Ca ²⁺	67.9 $\pm$ 3.1
AM + calmodulin (0.1 μ g/ml)	65.7 $\pm$ 4.5
AM + calmodulin (0.4 μ g/ml)	64.9 $\pm$ 2.6
AM + calmodulin (0.1 μ g/ml) + 10 μ M-Ca ²⁺	64.4 $\pm$ 5.2
AM + calmodulin (0.4 μ g/ml) + 10 μ M-Ca ²⁺	62.7 $\pm$ 4.1
AM + 0.1 mM-EGTA	66.1 $\pm$ 4.3
AM + 0.10 μ M-cyclic AMP	72.1 $\pm$ 4.8
AM + cytochalasin B (10 μ g/ml)	73.5 $\pm$ 5.6

strate that the granules had not become unstable in the conditions (results not shown).

Cyclic AMP at a concentration of 10 μ M did not alter sedimentation rates, nor did the addition of cytochalasin B, a fungal metabolite that is known to interfere with function of microfilaments, probably by preventing their polymerization (Lin *et al.*, 1981). Finally, pre-treatment of the isolated granule fraction with phospholipase C in the presence of 1 mM-CaCl₂ did not significantly affect their interaction with actomyosin, which was subsequently observed, the percentage sedimentation sites being $62 \pm 3\%$ in the absence and $73 \pm 5\%$ (mean $\pm$ s.e.m.; $n = 12$) in the presence of actomyosin. The observed effects of ATP, and lack of effect of the other agents tested broadly parallel results that were obtained previously in studies of granule F-actin interactions (Howell & Tyhurst, 1979).

One crucial element missing from the hypothesis that actomyosin might be involved in the intracellular transport of the insulin-storage granules before their secretion, possibly along a predetermined course that is delineated by the microtubules, is a demonstration of actomyosin in contact with granules or with microtubules or with both in the intact

cells. There has been a recent immunocytochemical illustration of the existence of actin in the membranes of insulin-storage granules as well as at other sites within the cell (Benadayan, 1981), but little is known of myosin distribution within B-cells or indeed within other non-muscle cell types. A role of actomyosin in granule movement in B-cells, as indicated in the present experiments, suggests the possibility of a mechanism for a direct linkage between elevation of intracellular Ca^{2+} concentrations and the stimulation of insulin secretion. Thus many of the effects of Ca^{2+} are believed to be mediated via calmodulin, which is itself a subunit of myosin light-chain kinase (Adelstein & Eisenberg, 1980). By analogy with other systems, activation of this kinase in B-cells would elicit myosin phosphorylation, which in turn induces actin-myosin interaction and contraction (Adelstein & Eisenberg, 1980) and in this case might initiate granule movement. Myosin kinase has been identified in rat islets, and in a transplantable insulin-producing tumour (Penn *et al.*, 1982), but there appears so far to be little direct evidence that endogenous myosin is a major substrate for Ca^{2+} -dependent phosphorylation in islets of Langerhans (Harrison & Ashcroft, 1982).

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