THE EFFECTS OF CALCIUM AND DIVALENT IONOPHORES ON THE STABILITY OF ISOLATED INSULIN SECRETORY GRANULES

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1. Introduction

Insulin is stored in the pancreatic β-cells in the form of crystalline granules surrounded by membraneous sacs. The discharge of the hormone is generally recognized to occur by ejection of the granules by exocytosis [1–3]. Alternative or additional mechanisms, such as dissolution of the granules while still within the β-cells [4,5], have received less attention partly because the secretagogic action of glucose and other commonly employed stimuli fullfil the presumptions of exocytosis in being dependent both on the presence of extracellular Ca^{++} and production of ATP [6,7]. The fact that Ca^{++} and ATP are not prerequisites for all stimulation of insulin release was evident from recent experiments in our laboratory with compound X-537A, an antibiotic facilitating the transmembrane flux of divalent cations [8]. This ionophore was found to stimulate insulin release at low glucose concentrations, an effect which was considerably potentiated after excluding Ca^{++} from the incubation medium or exposing the β-cells to metabolic inhibitors [9]. The present study lends further support to the view that the insulin-releasing effect of X-537A can be accounted for by mechanisms other than exocytosis. It will be shown that X-537A increases the fragility of isolated β-granules and that this effect resembles the secretagogic action on the intact β-cell in being inhibited by Ca^{++}.

2. Experimental

Chemicals of analytical grade and deionized water were used. Adult female *ob/ob* mice were taken from a non-inbred strain [10] and starved overnight. Thirty islets were microdissected from each animal and homogenized at 4°C in 300 μl of a solution consisting of 304 mM sucrose adjusted to pH 6.0 with 8 mM Tris–HCl. A crude granule fraction was prepared by the technique of Coore et al. [11] with the modification that the material taken for analyses was allowed to sedimentate for 10 min at 14 000 *g*. This sediment was suspended in 450 μl of the sucrose solution at pH 6.0. The stability of the isolated granules was tested by incubating 50 μl of the crude granule suspension for 15 min at 37°C with 250 μl of a solution consisting of 304 mM sucrose, 8 mM Tris–HCl and the various test substances. The ionophores were added from stock solutions of the drugs in dimethylsulfoxide (DMSO), bringing the final concentration of DMSO in test and control tubes to 0.1%. It was established from separate experiments that this low concentration of DMSO neither influences granule stability at pH 6.0 nor 7.4. After incubation and subsequent centrifugation for 10 min at 14 000 *g*, the upper and lower halves (150 μl each) of the tube contents were extracted with acid–ethanol and radioimmunologically assayed for insulin. 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 [11].

3. Results

As shown from the control values in table 1, twice as much insulin was sedimentable at pH 6.0 as at pH 7.4. Furthermore, the amounts of insulin sedimented tended to be reduced when 1 mg/ml albumin was intro-

Table 1
Effects of divalent ionophores on granule stability in media of different pH and albumin content

Test substance	pH	Albumin (mg/ml)	Control (a)	Test (b)	(b) – (a)
X-537A	6.0	1.0	83.8 ± 2.8	58.3 ± 4.5	−25.5 ± 5.3[a]
X-537A	7.4	0	51.3 ± 6.1	22.6 ± 2.1	−28.7 ± 6.9[a]
X-537A	7.4	1.0	42.1 ± 3.7	14.6 ± 1.4 (7)	−27.9 ± 4.5[b] (7)
A-23187	7.4	1.0	34.5 ± 2.2	38.1 ± 3.4	+ 3.5 ± 2.8

The values denote percentage sedimentable insulin (mean values ± SEM) after incubating a crude fraction of β-granules for 15 min at 37°C with and without 25 μg/ml of the ionophores X-537A or A-23187 in media containing 304 mM sucrose, 0.1 mM $CaCl_2$, 1 μl/ml DMSO and 8 mM Tris–HCl.The albumin content (bovine serum albumin, fraction V, Sigma) and the pH in the various media are listed. Eight separate experiments were performed when not otherwise stated within parenthesis. In addition to the results obtained with each medium the differences between parallel test and control incubations are given.
[a] $P < 0.01$; [b] $P < 0.001$.

duced into the incubation media. Exposure of the granules to X-537A caused a significant release of the bound insulin which was not affected by the presence or absence of albumin or by the change of pH from 6.0 to 7.4. The integrity of the β-granules was unaffected by the addition of A-23187 to an albumin-containing medium of pH 7.4.

It is evident from table 2 that the solubilizing effect of X-537A was counteracted by $CaCl_2$. Nearly all granules were dissolved in the absence of $CaCl_2$, whereas the stability was not significantly affected when the granules were exposed to the ionophore in the presence of 2 mM $CaCl_2$. Addition of $CaCl_2$, itself, tended to increase granule stability as shown from the control values in table 2; when estimated from the mean difference between 7 paired test and control incubations the increase in the percentage of insulin was 14.3 ± 5.1 ($P < 0.05$). There was a marked

Table 2
Effects of X-537A and $LaCl_3$ on granule stability in media of different Ca^{++} concentrations

Test substance	$CaCl_2$ (mM)	Control (a)	Test (b)	(b) – (a)
X-537A	–	31.0 ± 3.3	1.7 ± 2.2	−29.3 ± 5.0[a]
X-537A	0.1	34.5 ± 2.2	10.7 ± 1.5	−23.8 ± 2.4[a]
X-537A	2.0	45.4 ± 2.3 (7)	38.9 ± 3.0	− 6.7 ± 2.8 (7)
$LaCl_3$	–	31.0 ± 3.3	57.8 ± 3.2	+26.8 ± 3.2[a]

The values denote percentage sedimentable insulin (mean values ± SEM) after incubating a crude fraction of β-granules for 15 min at 37°C with and without 25 μg/ml of X-537A or 0.1 mM $LaCl_2$ in media of pH 7.4 containing 304 mM sucrose, 1 mg/ml albumin, 1 μg/ml DMSO and 8 mM Tris–HCl. The amounts of $CaCl_2$ in the various media are listed. Eight separate experiments were performed when not otherwise stated within parentheses. In addition to the results obtained with each medium the differences between parallel test and control incubations are given.
[a] $P < 0.001$.

stabilization of the granules after adding 0.1 mM $LaCl_3$; the amounts of sedimentable insulin being nearly twice as high as in the control incubations.

4. Discussion

No technique is available for the preparation of fractions of β-granules free of microsomal and lysosomal contamination, despite extensive efforts in several laboratories [11–14]. We therefore refrained from attempts to more extensive purification with the accompanying hazards of damaging the granules and adhered to a technique for the isolation of a crude granule fraction by differential centrifugation. The major modification of the technique described by Coore et al. [11] was the use of a higher *g*-value to include more small secretion granules in the analyses. It should be noted that the granule fraction obtained displayed the same increase in fragility when the pH was raised from 6.0 to 7.4 as found in other studies [11,13,15]. There was only a slight tendency toward reduction in the amounts of sedimentable insulin in the presence of 1 mg/ml albumin. This reinforces previous arguments [11] that neither adsorption of insulin to the tube wall nor to the subcellular particles greatly influences the results obtained in the test system employed.

The stability of the isolated β-granules appeared to be increased in the presence of $CaCl_2$. It is tempting to attribute the previous failure to demonstrate a stabilizing effect of Ca^{++} to the fact that these experiments were performed in the presence of competitive cations including a considerably lower pH [11,13,15]. The subcellular fraction obtained after sedimentating the homogenates of the present types of islets for 30 min at 110 000 *g* has, for example, been found to have its optimal binding capacity for Ca^{++} at a pH as high as 6.6–7.4 [16]. The idea that the solubilization of insulin is prevented by increased binding of Ca^{++} to the membraneous sacs or their granule contents is supported by the considerable increase of sedimentable insulin seen in the presence of $LaCl_3$. The lanthanum ion has been recognized as an effective and specific blocker of the binding sites for Ca^{++}; it has a high charge density and a hydrated size which is only slightly larger than Ca^{++} [17,18]. It is evident from recent studies with particle electrophoresis that there are numerous binding sites for Ca^{++} on the surface of the islet granules [19]. Histochemical studies at the ultrastructure level have revealed deposits of Ca^{++} associated with the secretory granules [20,21]. Therefore, it seems likely that Ca^{++} contributes to the stability of the granules also intracellularly.

The divalent ionophores X-537A and A-23187 are useful tools for exploring secretory mechanisms in view of their ability to enable Ca^{++} to cross biological membranes [8]. When the β-cell-rich pancreatic islets of *ob/ob* mice were exposed to these ionophores at 3 mM glucose only X-537A was shown to stimulate insulin release [9]. The potentiation of the secretagogic effect by metabolic inhibitors or withdrawal of extracellular Ca^{++} led to the suggestion that X-537A acts by increasing the permeability of cellular membranes with solubilization of the granule-stored insulin rather than by increasing the ejection of the β-granules by exocytosis. The present data support this hypothesis in showing that X-537A increases the fragility of the isolated β-granules under conditions when A-23187 lacks such an effect. Furthermore, in analogy to the observed influence of Ca^{++} on the stimulation of insulin release from intact islets by X-537A, the solubilization of the isolated granules diminished with increasing Ca^{++} concentration.

The mechanisms by which exposure to X-537A leads to dissolution of isolated β-granules remain obscure. One important function of the membraneous sac surrounding the β-granule is probably to secure optimal physico-chemical conditions for retaining insulin in its stored form. Provided that X-537A induces labilization of cellular membranes, the dissolution of the granules may result from a generally increased permeability of the granule sac. The greater stability of the granules at lower pH rises the question of whether the ionophore effect can be specifically attributed to elimination of a pH gradient between the interior of the granule sac and the surrounding medium. The present data provide no support for this, because X-537A was as effective in dissolving the granules at pH 6.0 as at pH 7.4. Since X-537A can both complex zinc [22] and facilitate the transport of divalent cations across phospholipid membranes, it is also worthwhile to consider whether this ionophore directly solubilizes the stored zinc–insulin complex within the granules.

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