CALCIUM EXCHANGEABILITY IN SUBCELLULAR FRACTIONS OF PANCREATIC ISLET CELLS

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SUMMARY
The distribution and exchangeability of calcium within subcellular compartments of rat pancreatic islet cells was determined in pulse-chase experiments following isotopic labelling with $^{45}$Ca$^{2+}$. In unstimulated islets the mitochondrial fraction showed the greatest, and the insulin secretory granule fraction the least, uptake of $^{45}$Ca$^{2+}$; subsequently the rate of loss from each compartment during 120 min was in the order mitochondrial $>$ microsomal $>$ secretory granule fraction. On exposure to D-glucose, 16.8 mM, the $^{45}$Ca labelling of all 3 subcellular compartments was increased after 5 and 30 min stimulation. In contrast, theophylline 10 mM caused a significant decrease in mitochondrial $^{45}$Ca content with little effect on other compartments. These results are discussed in relation to the importance of calcium disposition, exchangeability, and mobility for the initiation and control of insulin secretion.

INTRODUCTION
In the β-cells of the pancreas electrophysiological studies indicate that concentrations of exogenous D-glucose which elicit insulin release cause a rapid influx of Ca$^{2+}$ (Dean & Matthews, 1970; Matthews, 1977; Matthews & Sakamoto, 1975a, b). This is reflected in an enhanced rate of extracellular $^{45}$Ca uptake into islet cells stimulated by D-glucose (Hellman, Sehlin & Täljedal, 1971, 1976; Malaisse-Lagae & Malaisse, 1971). These rapid kinetic events are evidently associated with the more immediate changes in cell membrane permeability which form an integral part of the stimulus-secretion coupling process for insulin release (Matthews, 1975, 1977). Mechanisms must exist also for the subsequent termination of calcium action by sequestration or extrusion, especially if, in the β-cell, as in many other cells, the prevailing concentration of free cytoplasmic Ca$^{2+}$ is to be maintained at a low level, i.e. in the nM to μM range (Baker, 1976; Carafoli & Crompton, 1976). Furthermore, an intracellular redistribution of calcium in response to various stimulants of insulin secretion has been demonstrated both histochemically (Schäfer & Klöppel, 1974a) and ultracytochemically (Herman, Sato & Hales, 1973; Klöppel & Schäfer, 1976; Ravazolla et al. 1976; Schafer & Klöppel, 1974b). Thus a precise knowledge of the location, exchangeability, capacity, and mobility of subcellular calcium stores is...
essential for a full understanding of both the physiological activity and pharmacological response of the β-cell.

This study therefore describes pulse-chase experiments designed to elucidate the uptake, distribution and exchangeability of calcium in defined subcellular compartments of the pancreatic islet cell following isotopic labelling with $^{46}\text{Ca}^{2+}$ and stimulation with D-glucose. Comparative experiments were also carried out to assess the extent to which mobilization of endogenous calcium might account for the action of theophylline in initiating insulin release (Brisson, Malaisse-Lagae & Malaisse, 1972).

**MATERIALS AND METHODS**

**Tissue preparation**

Islets of Langerhans were isolated by collagenase digestion (Lacy & Kostianovsky, 1967) of pancreatic tissue from fed male rats (150–200 g). After isolation the islets were incubated 3 times for 5-min periods at 37 °C in oxygenated (95 % O$_2$; 5 % CO$_2$) Krebs-Ringer bicarbonate (KRB) solution containing (mM): NaCl, 116; NaHCO$_3$, 25; KCl, 4.7; MgCl$_2$, 1.13; CaCl$_2$, 2.56; Na$_2$HPO$_4$, 1.42; D-glucose, 2.8 mM and supplemented with 0.5 % bovine serum albumin (Fr. V, Sigma). At the end of each 5-min period the medium was removed and fresh KRB solution added. During isolation islets were distributed into 2 equal groups, one for use as a control and the other for subsequent drug treatment.

**Islet incubation**

Islets were incubated with approximately 10 $\mu$Ci of $^{46}\text{Ca}^{2+}$ (2 mCi/ml; 20 mCi/mg; 100 $\mu$g Ca$^{2+}$/ml, obtained from the Radiochemical Centre, Amersham, Bucks) in 1 ml of oxygenated KRB solution with 0.5 % bovine albumin for 1 h at 37 °C. The islets were collected by centrifugation and rapidly washed by resuspending twice in 46Ca-free KRB solution (1 ml). The tissue was then incubated at 37 °C in oxygenated KRB solution for 5, 30, 60 or 120 min. Control experiments (no additions) were carried out simultaneously with those in which the D-glucose concentration was increased (to 16.8 mM) or theophylline added (10 mM). After incubation, islets were removed from the medium by centrifugation and washed by resuspension once with KRB solution free of albumin.

In additional control experiments islets were incubated for 30 min, one group with, and the other without, an increase in D-glucose concentration to 16.8 mM. In these experiments 46Ca was added only after homogenization as a check for redistribution and adsorption during subcellular fractionation.

**Isolation of subcellular fractions**

Immediately following removal of albumin at the end of the incubation period the islets were homogenized at 4 °C in 0.3 M sucrose, containing 5 mM phosphate buffer pH 6, using a ground glass homogenizer of small capacity (1 ml). Subcellular fractions were then isolated by differential rate and sucrose gradient centrifugation according to the method of Howell, Fink & Lacy (1969) except that for isolation of the nuclear fraction the first centrifugation of the homogenate at 600 $g$ was for 1.5 min instead of 5 min. Following isolation of the mitochondrial pellet (centrifugation at 3500 $g$ for 5 min) the granule pellet was isolated by centrifugation at 24,000 $g$ for 10 min, resuspended in 0.3 M sucrose (1 ml) and layered over a discontinuous sucrose gradient (1.7 M, 0.4 ml; 1.6 M, 0.6 ml; 1.55 M, 0.6 ml; and 1.4 M, 0.4 ml). Centrifugation at 105,000 $g$ for 60 min yielded fractions designated I to IV (Howell et al. 1969). In this study Fraction I was subdivided into 2 parts designated I and Ia to allow separation of the clear upper layer of the original suspending medium (0.3 M sucrose) from the gradient layer immediately below it.

The low concentration of phosphate buffer (5 mM) used to maintain a pH of 6.0 during subcellular fractionation caused no precipitation of Ca$^{2+}$ during particle isolation.

All glassware was siliconized before use.
**Assay procedures**

The protein in an aliquot of each fraction was precipitated with an equal volume of 10% trichloroacetic acid (TCA). The TCA supernatant (0.5 ml) containing $^{46}\text{Ca}^{++}$ was then placed directly into a scintillation vial and 15 ml of scintillant (50% toluene + 50% ethoxyethanol + 0.4% butyl PBD) was added. The radioactivity of the samples was counted in a Nuclear Enterprises Model 8100 scintillation spectrometer for 5-30 min to reduce the counting error to at least 5%. Small aliquots of untreated tissue fractions when added directly to the scintillation fluid gave counts comparable to aliquots of the TCA supernatants. Thus essentially all the $^{46}\text{Ca}$ in the various fractions appears in the supernatant after treatment with TCA.

Addition of $^{46}\text{Ca}^{++}$ to the samples prepared in the scintillant gave counts which averaged 103% of expected and showed no quenching. All data for the $^{46}\text{Ca}$ in subcellular fractions were normalized according to the amount of radioactivity present in the incubation medium during labelling with $^{46}\text{Ca}^{++}$. All data are based on $7.422 \times 10^6$ cpm/ml in the labelling medium.

Protein was precipitated with an equal volume of 10% TCA, washed twice with 5% TCA and dissolved in 1 N NaOH. Aliquots were taken for assay by the method of Lowry, Rosebrough, Farr & Randall (1951).

Insulin was assayed in aliquots of the fractions and in the incubation medium by radioimmunoassay (Phadebas Insulin test, Pharmacia Diagnostics).

Cytochrome oxidase was measured by oxidation of N-phenyl-p-phenylenediamine in incubates containing aliquots of the fractions (Pearl, Cascarino & Zweifach, 1963). The reaction was allowed to proceed for 60 min. Albumin labelled with $^{125}\text{I}$ was used to assess the amount of protein from the bovine serum albumin in the incubation medium that appeared in the final fractions. Approximately 5 µCi of $^{125}\text{I}$-labelled albumin were added to islets in the normal medium. The islets were then incubated for 30 min and subjected to the usual wash procedure. Contamination of the fractions with protein from the incubation medium was nil except for the supernatant where an average of 11.4% ($n = 2$) of the protein in the fraction was estimated to be from the medium. Some labelled albumin (amounting to no more than 14% of the total protein in these samples) was also found in the medium left above the density gradient (Fraction I).

**RESULTS**

Determination of the maximum capacity of any intracellular compartment for calcium depends not only on the store size but also upon the susceptibility of each subcellular pool to uniform and total isotopic replacement during a discrete time period. To avoid the hazards of excessively long periods of incubation no attempt was made in the present study to obtain an absolute isotopic equilibration of calcium in islet cells; rather comparative turnover of $\text{Ca}^{++}$ in individual subcellular fractions was assessed at definite time intervals on the basis of paired pulse-chase experiments.

**Distribution of insulin in subcellular fractions of rat pancreatic islets**

Insulin-containing granules were located primarily in Fractions II, III and IV of the sucrose density gradient, and glucose stimulation did not significantly alter insulin distribution in the subcellular fractions. Fractions III and IV were selected as granule-rich fractions in the data of this study since Fraction II was relatively rich in mitochondria (Fig. 1).

**Distribution of mitochondria in subcellular fractions of rat pancreatic islets**

Cytochrome oxidase activity was greatest in the mitochondrial fraction per se and in the uppermost layers of the density gradient (Fig. 2). These data are in agreement
Fig. 1. Distribution of insulin in subcellular fractions of rat islets of Langerhans. Islets were homogenized in 0.3 M sucrose with 5 mM phosphate buffer (adjusted to pH 6.0), and subcellular fractions separated by differential and density-gradient centrifugation. Fractions I–IV inclusive were obtained after sucrose density-gradient centrifugation. Fraction I is the 0.3 M sucrose medium remaining above the more dense layers of sucrose following centrifugation at 105,000 g for 60 min. Fractions III and IV were designated granule fractions throughout this study. Values given are means ± S.E. of 10 experiments except for the 'mitochondrial' fraction where n = 4. Stimulation with D-glucose 16.8 mM, or theophylline 10 mM did not significantly alter insulin distribution. Nuc = nuclei, unbroken cells, and cell debris; Mic = microsomal fraction; Sup = cell supernatant; Mito = mitochondria.

with those of Howell et al. (1969). Fractions designated IA and Mito were therefore taken as mitochondrial fractions throughout this study. Drug treatment did not affect the location of mitochondria in the subcellular fractions.

Exchangeability of calcium in subcellular fractions of rat pancreatic islets

Loss of 45Ca occurs from islets during incubation in 45Ca-free KRB solution. This is reflected by the time-dependent loss of label from individual subcellular fractions of unstimulated tissue (Fig. 3, Table 1). It is apparent that exchangeability of calcium in granule fractions is comparatively low as indicated by initial low levels of radioactivity and by slow loss of label (Fig. 3, Table 1). Whereas the loss of 45Ca from the microsomal fraction appears to be delayed in onset, it proceeds rapidly after 30 min of incubation in 45Ca-free medium (Fig. 3). The decrease of 45Ca from the mitochondrial pool is also rapid and approximates to 1st-order kinetics (Fig. 3). The crude nuclear pellet shows an intermediate rate of loss of label (not illustrated).
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Fig. 2. Distribution of cytochrome oxidase activity in subcellular fractions of rat pancreatic islets. Fractions were prepared and designated as in Fig. 1. Values given are means ± S.E. of 8 experiments. Fractions IA and Mito were designated mitochondrial-rich throughout this study. Neither D-glucose 16·8 mM nor theophylline 10 mM significantly altered the pattern of mitochondrial distribution within the subcellular fractions.

Effect of glucose stimulation on \(^{45}\text{Ca}\) in subcellular fractions of rat pancreatic islets

When stimulated for 5, 30 or 120 min with high glucose, islets released an average of 167 ± 52 (s.e.), 638 ± 221 and 1493 ± 337 μU more insulin respectively, than corresponding low-glucose controls. Mitochondrial \(^{45}\text{Ca}\) is significantly greater \((P < 0·05)\) by comparison of paired differences in glucose-stimulated islets when the 5- and 30-min glucose-stimulation periods are combined and compared to controls (Table 2). Changes in granule \(^{45}\text{Ca}\) are most obvious at 30 min of glucose stimulation (Table 2) where the gain is significant \((P < 0·05)\) by comparison of paired differences.

The increase of \(^{45}\text{Ca}\) in granules after exposure of islets to high glucose appears to be rapid but the elevated \(^{45}\text{Ca}\) level is lost after 120 min of glucose stimulation. This net loss at 120 min (Table 2) may be attributable to a release of granule content (i.e. insulin and calcium) by exocytosis.

Glucose stimulation caused an increase in microsomal \(^{45}\text{Ca}\) in 15 of 18 experiments. This effect also occurs soon after a glucose stimulus. The relative difference in labelling and Ca\(^{2+}\) exchangeability of the various subcellular compartments is summarized in Table 2.

The supernatant fraction contained cell cytoplasm and is the medium in which differential centrifugation is accomplished. Glucose stimulation did not significantly alter the \(^{45}\text{Ca}\), cpm/μg protein of the supernatant (263·8 ± 57 and 309·7 ± 56) in control and glucose stimulated respectively, including the 5- and 30-min incubations.
Fig. 3. Calcium exchangeability in rat pancreatic islet cells expressed as loss of $^{45}\text{Ca}$ from individual subcellular fractions. Islets were labelled in $^{45}\text{Ca}^{++}$ for 60 min and then incubated in a $^{45}\text{Ca}$-free medium (without stimulation) for the intervals indicated. The tissue was then homogenized in 0.3 M sucrose with 5 mM phosphate buffer (adjusted to pH 6.0), and subcellular fractions separated by differential and density gradient centrifugation. Values given are means ± S.E. of 6, 13, 9, and 5 experiments for 5, 30, 60, and 120 min of incubation respectively. ▲, mitochondria; ○, microsomal fraction; ●, secretory granule fraction.

Therefore elevated levels of $^{46}\text{Ca}$ in mitochondria, microsomes and granules after glucose stimulation are not due to accumulation from the medium during differential centrifugation. Furthermore, in 4 experiments in which $^{46}\text{Ca}$ was added directly to the homogenates of islets previously incubated in the absence or presence of 16.8 mM D-glucose for 30 min prior to homogenization, there was no significant difference ($P > 0.05$, using the comparison of paired differences, see Table 2) between the $^{46}\text{Ca}$ content of the fractions from the glucose-stimulated and non-stimulated islets, i.e., nuclear fraction 125 ± 23, mitochondrial fraction 98 ± 20, granule fraction 85 ± 16, and microsomal fraction 108 ± 32; each value expressed as a percentage of the corresponding non-stimulated control. Thus elevated levels of $^{46}\text{Ca}$ are maintained in glucose-stimulated tissue fractions from islets incubated in $^{46}\text{Ca}$ and little or no $^{46}\text{Ca}$
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Table 1. Calcium exchangeability in subcellular fractions of pancreatic islet cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A (5 min)</th>
<th>B (120 min)</th>
<th>B/A, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>63 ± 2</td>
<td>17 ± 4</td>
<td>27</td>
</tr>
<tr>
<td>Microsomes</td>
<td>49 ± 12</td>
<td>17 ± 3</td>
<td>35</td>
</tr>
<tr>
<td>Granules</td>
<td>12 ± 3</td>
<td>6 ± 1</td>
<td>50</td>
</tr>
</tbody>
</table>

Mean content $^{46}\text{Ca}/\mu\text{g protein} \pm \text{s.E.} \ (n = 5-6)$ after incubation in the absence of extracellular $^{46}\text{Ca}$ for 5 and 120 min.

Table 2. Effect of stimulation with D-glucose or theophylline on $^{46}\text{Ca}$ in subcellular fractions of labelled rat pancreatic islet cells

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>D-glucose, 16.8 mM</th>
<th>Theophylline, 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Nuclear</td>
<td>113 ± 56 (3)†</td>
<td>146 ± 24 (10)</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>158 ± 22 (5)</td>
<td>161 ± 27 (14)</td>
</tr>
<tr>
<td>Granule</td>
<td>130 ± 27 (4)</td>
<td>137 ± 15 (19)</td>
</tr>
<tr>
<td>Microsomal</td>
<td>174 ± 23 (3)</td>
<td>183 ± 35 (9)</td>
</tr>
</tbody>
</table>

Mitochondrial $^{46}\text{Ca}$ is significantly increased by glucose stimulation ($P < 0.05$ by comparison of paired differences). Granule $^{46}\text{Ca}$ is also increased significantly by glucose ($P < 0.05$ by comparison of paired differences) when the 5- and 30-min intervals are considered. The effect of glucose stimulation on microsomal $^{46}\text{Ca}$ borders on significance ($P < 0.16$), but nuclear $^{46}\text{Ca}$ is not significantly affected by glucose. Theophylline caused a significant decrease in $^{46}\text{Ca}$ in the mitochondrial fraction ($P < 0.05$ by group comparison of theophylline-treated, $n = 8$, with 1-h controls, $n = 22$).

* Values are means ± s.E.
† Number of observations shown in parentheses.

Redistribution occurs following homogenization and centrifugation using the paired experiment technique.

Effect of theophylline on $^{46}\text{Ca}$ in subcellular fractions of rat pancreatic islets

Table 2 shows that 10 mM theophylline causes depletion of mitochondrial $^{46}\text{Ca}$ but other calcium pools are much less affected by theophylline under these conditions. This contrasts with the results of experiments in which glucose was used as the stimulus, where as already described an elevation of $^{46}\text{Ca}$ was seen in mitochondrial, granule and microsomal calcium pools. Insulin release in response to theophylline amounted to $230 ± 76$ (s.E.) μU more than the corresponding controls in the 60-min incubation.
DISCUSSION

Stimulation of prelabelled islets by glucose increases $^{45}\text{Ca}^{2+}$ in mitochondrial, granule and microsomal fractions of the $\beta$-cell (Table 2). With sustained stimulation (120 min) the distinction between stimulated and non-stimulated cells is lost as $\text{Ca}^{2+}$ redistribution and exchange between the various intracellular pools, as well as across the cell membrane, contributes to a progressive loss of label from the cells.

It is important to note that subcellular particles from pancreatic islets display differences in exchangeability not only with respect to degree of labelling but also in terms of rate of loss of label. For example, Fig. 3 or Table 1 indicates that although mitochondria show quantitatively the greatest accumulation of $^{45}\text{Ca}$, they lose it more rapidly, whereas the granule fraction takes up much less $^{45}\text{Ca}^{2+}$ but correspondingly loses it less rapidly.

As pointed out by Howell, Montague & Tyhurst (1975), of the 3 major subcellular components which might contribute to intracellular calcium sequestration, each possesses a large surface area when compared with the plasma membrane and an appropriate volume for sequestration. Thus, whilst the mean surface area (Dean, 1973, 1976) of the $\beta$-cell plasma membrane is 973 $\mu$m$^2$ and the organelle free distribution space of the cytoplasmic ground substance constitutes 53% of the total cellular volume, the equivalent surface area (and % cell volume) of smooth endoplasmic reticulum amounts to 2539 $\mu$m$^2$ (6%); rough endoplasmic reticulum, 6158 $\mu$m$^2$ (14%); mitochondria (inner membranes; estimated from outer), 3638 $\mu$m$^2$ (4%), and secretory granules, 3435 $\mu$m$^2$ (12%). It is likely therefore, that both surface area and volume for sequestration are important in determining $^{45}\text{Ca}$ exchangeability.

The increase in $^{45}\text{Ca}$ labelling of mitochondria and microsomes within the first 30 min of stimulation with D-glucose suggests that the rise in free [Ca], which occurs either by influx of $\text{Ca}^{2+}$ across the $\beta$-cell membrane or displacement from its inner surface (Matthews, 1979) rapidly triggers the uptake of $\text{Ca}^{2+}$ into these intracellular compartments, an effect enhanced by any initial decrease in cellular $\text{Ca}^{2+}$ extrusion (Malaisse et al. 1975). The acceleration of mitochondrial calcium uptake may therefore limit the level of free [Ca], responsible for activating insulin release and serve to maintain a fine control of $\text{Ca}^{2+}$ gradients (see Matthews, 1979); similarly the endoplasmic reticulum may participate in the short-term regulation of free [Ca], by uptake and sequestration. Longer periods of stimulation will obliterate these more immediate effects especially if the islet loading with $^{45}\text{Ca}$ is prolonged (Hellman et al. 1979). Our observations in islet cells are in contrast to those in pancreatic exocrine cells where stimulation of secretion causes a decrease in mitochondrial labelled calcium. However, in exocrine cells the influx of Na$^+$ may be instrumental in raising free $\text{Ca}^{2+}$ indirectly by an action on mitochondrial $\text{Ca}^{2+}$ stores (see Clementi & Meldolesi, 1975).

The results of the present study agree in part with those of Howell & Tyhurst (1976) who exposed islets to $^{45}\text{Ca}^{2+}$ and measured the cellular distribution of label by electron-microscopic autoradiography. Both studies show accumulation of $^{45}\text{Ca}$ in mitochondria and to a lesser extent in granules. An increased microsomal binding of
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$^{45}$Ca disclosed by the present experiments would be difficult to demonstrate by the method of Howell and Tyhurst. Our finding of a delay in loss of $^{45}$Ca from microsomal elements during incubation in a label-free medium suggests the possibility that calcium is transferred from subcellular particles to membrane structures; similar results have been obtained in smooth microsomal material from exocrine pancreas (Clementi & Meldolesi, 1975).

Howell et al. (1975) concluded that storage granules do not appear to constitute a labile pool but the results of the present study, as well as those of others (Ravazolla et al. 1976; Schäfer & Klöppel, 1974b) using ultracytological techniques, show that granule calcium does undergo some change in response to glucose stimulation. On the other hand, Bloom, Hellman, Sehlin & Täljedal (1977) showed a marked uptake of $^{45}$Ca into the insulin secretory granule fraction loaded with $^{45}$Ca$^{2+}$ during glucose stimulation. These results contrast with ours and the difference is probably due to incorporation of large amounts of $^{45}$Ca$^{2+}$ into newly synthesized granules in tissues labelled with $^{45}$Ca during glucose stimulation in the study of Bloom et al. The slow rate of calcium accumulation and relative stability of the granule pool during the chase-stimulation incubations of our experiments suggests that despite the high total calcium content (as demonstrated by X-ray microanalysis and pyroantimonate fixation) and potentially large capacity (as indicated by ultrastructural morphometry) this calcium pool is not in close equilibrium with the rest of the intracellular calcium and unlike the mitochondria and endoplasmic reticulum does not participate in the rapid control of $[Ca]$ required for regulation of secretion. In line with this observation, mitochondria and microsomes isolated from pancreatic β-cells rapidly take up $^{45}$Ca in the presence of ATP (Sehlin, 1976) but secretory granules do not (Howell et al. 1975). The secretory granules of the islet cell therefore appear analogous to storage granules in other cells (Clementi & Meldolesi, 1975) and may be important in the long term rather than immediate post-stimulus regulation of calcium levels in secretory cells.

In the present study the mitochondrial turnover of Ca$^{2+}$ was also influenced by theophylline which, in contrast to D-glucose, decreased $^{45}$Ca in this fraction. Theophylline may therefore release Ca$^{2+}$ from mitochondria, or it may act indirectly by diminishing calcium influx without altering efflux in the mitochondrial compartment. Either action would raise cytosolic Ca$^{2+}$ and so enhance secretion; similar effects have been noted in other tissues (Batra, 1974; Borowitz, Leslie & Baugh, 1975).

Viewed overall our results are compatible with the conclusion that the control of insulin secretion involves mechanisms for the intracellular uptake, sequestration and extrusion of calcium. On stimulation with D-glucose $[Ca]$, rises, mitochondrial turnover is accelerated (Carafoli & Crompton, 1976) together with an increased calcium uptake by the endoplasmic reticulum. In contrast the secretory granules (derived from the endoplasmic reticulum via the Golgi and already containing a high concentration of Ca$^{2+}$) constitute a relatively stable pool and once formed show less tendency to take up or exchange calcium.
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