Endoplasmic reticulum Ca^{2+} is important for the proteolytic processing and intracellular transport of proinsulin in the pancreatic β-cell

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The role of intracellular Ca^{2+} in the proteolytic processing and intracellular transport of secretory granule proproteins was investigated by pulse–chase radiolabelling of isolated rat islets of Langerhans. The conversion of proinsulin was inhibited by depletion of medium Ca^{2+} with EGTA and by blocking the transport of Ca^{2+} into cells with the Ca^{2+}-channel antagonists verapamil, nifedipine and $NiCl_2$. Proinsulin conversion was also reduced by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, indicating that the process requires transport of Ca^{2+} into the endoplasmic reticulum. This was supported by the finding that proinsulin processing was inhibited when Ca^{2+} was depleted before or during pulse-labelling, but not after transport of the protein to post-endoplasmic-reticulum compartments. Similarly, the inhibition of proinsulin processing was reversed by re-introduction of medium Ca^{2+} around the time of radiolabelling, but not after 15 min of chase incubation. Ca^{2+} depletion also decreased proteolytic maturation of the prohormone convertases PC1, PC2 and carboxypeptidase H. Secretion experiments suggested that the rate and extent of proinsulin transport into secretory granules were inhibited marginally by Ca^{2+} depletion, whereas those of the convertases were markedly impeded. Inhibition of proinsulin conversion by Ca^{2+} depletion was thus not simply related to the Ca^{2+}-dependencies of mature PC1 and PC2, but also to a requirement for endoplasmic reticulum Ca^{2+} in proteolytic maturation of the convertases and in their transfer to secretory granules. The results also suggest that the Ca^{2+} required for prohormone processing in the granules enters the secretory pathway via the endoplasmic reticulum.

INTRODUCTION

The proteolytic conversion of human proinsulin in insulin secretory granules requires two Ca^{2+}-dependent endoproteases (designated type 1 and type 2), which cleave the precursor on the C-terminal side of residues Arg^{31}-Arg^{32} and Lys^{64}-Arg^{65} [1], and the Zn^{2+}-dependent carboxypeptidase H (CPH), which removes the exposed basic residues [2]. The endoproteases have been identified as the mammalian subtilisin-related proteins prohormone convertase 1 (PC1; also known as PC3) [3] and PC2 [4], which are broadly distributed in neuroendocrine cells, where they are involved in post-translational processing of other proproteins [5]. CPH is similarly involved in the processing of prohormones and neuropeptide precursors in a tissue-specific manner [6].

Biosynthetic studies have shown that each of these converting enzymes is subjected to post-translational proteolysis within the secretory pathway. ProCPH undergoes rapid proteolytic cleavage ($t_{\frac{1}{2}} = 30$–35 min) on the C-terminal side of amino acid 14 of the N-terminal propeptide, concomitant with its segregation to secretory granules [7–9]. This processing, however, is not required for catalytic activity of the enzyme [10]. ProPC1 conversion occurs by a two-step process, involving initial autocatalytic cleavage at the N-terminus of the protein soon after its translocation into the lumen of the endoplasmic reticulum (ER), followed by relatively slow ($t_{\frac{1}{2}} = 50$ min) C-terminal cleavage in the secretory granules, which may affect its sensitivity to Ca^{2+} [11–14]. ProPC2 undergoes similar two-step processing, although the initial N-terminal autocatalytic cleavage is relatively slow ($t_{\frac{1}{2}} = 140$ min), possibly due to retention of the protein in the ER [15–18]. Besides an essential Ca^{2+} requirement for the catalytic activity of mature PC1 and PC2 [1,3,18], Ca^{2+} may also be required for autocatalytic cleavage of proPC2 [19]. Autoproteolysis of proPC1, however, appears to be Ca^{2+}-independent in an *in vitro* translation/translocation system [20]. Further involvement of Ca^{2+} in proprotein conversion may occur in more distal compartments of the secretory pathway through activation of other endoproteases such as furin, a Ca^{2+}-dependent enzyme related to PC1 and PC2 [5], which may in turn play a role in activation of the proinsulin-converting enzymes or other proteins involved in granule function.

Previous studies on the role of intracellular Ca^{2+} in the proteolytic processing of proproteins have focused on viral glycoproteins [21] and a series of constitutively secreted proteins, including proalbumin and the complement precursors pro-C3 and pro-C4 [22]. These proteins are potential substrates for Ca^{2+}-dependent *trans*-Golgi network-resident endoproteases such as furin. The present study examines the role of Ca^{2+} in proinsulin processing in the islets of Langerhans and investigates in further detail the fate of the converting enzymes themselves.

EXPERIMENTAL

Chemicals and reagents

[^{35}S]Methionine (1400 Ci/mmol) was purchased from NEN Research Products (Stevenage, Herts., U.K.). Dulbecco's modified Eagle's medium was obtained from Flow Laboratories (Rickmansworth, Herts., U.K.). All other analytical-grade biochemicals were obtained from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, U.K.), unless specified otherwise.

Abbreviations used: CPH, carboxypeptidase H; ER, endoplasmic reticulum; PC1 and PC2, prohormone convertases 1 and 2.

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Antibodies

A mouse monoclonal antibody (3B7) raised to human proinsulin, which recognizes rat insulin, proinsulin and processing intermediates, was purified and coupled to CNBr-activated Sepharose as described previously [23].

Antisera were raised in rabbits against glutathione S-transferase fusion proteins incorporating amino acids 111–137, 162–388 and 42–454 of rat PC1, PC2 and CPH respectively, which were produced using the bacterial expression vector pGEX-3X (Pharmacia, Stockholm, Sweden) essentially as described by Bennett et al. [4].

Islet isolation procedure

Islets of Langerhans were isolated from the pancreata of 10–12-week-old Wistar and New England Deaconess Hospital rats by collagenase digestion followed by purification on Histopaque density gradients as described previously [23]. Islets were cultured overnight in Dulbecco's modified Eagle's medium containing 11 mM glucose and 10 % (v/v) fetal calf serum, and then washed into an incubation medium consisting of modified Krebs bicarbonate buffer [20 mM Hepes (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, 0.1 % BSA and 16.7 mM glucose].

Radioisotopic labelling

Batches of 100 islets were preincubated in 0.3 ml of incubation medium at 37 °C under O_2/CO_2 (19:1) in 1.5 ml-capacity microcentrifuge tubes (Alpha, Eastleigh, Hants., U.K.), under the conditions described in the Figure legends. Islets were recovered by centrifugation for 10 s at 200 ***g*** (Heraeus Sepatech microcentrifuge; Kalkberg, Germany) and resuspended in 0.1 ml of pre-warmed (37 °C) medium containing 0.2 mCi of [^{35}S]methionine. Islets were recovered after 2–60 min by centrifugation as above and then incubated for various times in non-radioactive medium containing 2 mM methionine. Incubations were terminated by the addition of 1 ml of ice-cold incubation medium followed by centrifugation for 10 s at 2000 ***g*** and aspiration of the medium. Islets were washed by two further cycles of resuspension, centrifugation and aspiration, and then sonicated for 15 s at a amplitude of 3 μm (MSE Sonifier; Crawley, Surrey, U.K.) in 0.4 ml of a lysis buffer consisting of 25 mM $Na_2B_4O_7$ (pH 9), 3 % (w/v) BSA, 1 % Tween-20, 0.1 mM PMSF, 20 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 10 μM pepstatin A, 1 mM EDTA and 0.1 % NaN_3. The lysates were centrifuged for 3 min at 10000 ***g*** and the supernatants retained. Where secretion of radiolabelled proteins was monitored, media were removed from islets and clarified by centrifugation for 3 min at 10000 ***g***, and the supernatants were combined with 2 × concentrated lysis buffer.

Immunoprecipitation of insulin-related peptides

Islet lysate and medium samples were mixed end-over-end for 1 h at room temperature in microcentrifuge tubes with 20 μl of a 100 mg/ml suspension of Cowan-strain *Staphylococcus aureus* cells. Samples were centrifuged for 3 min at 10000 ***g*** and the supernatants were combined with 100 μl (packed gel) of 3B7 immunoadsorbent and mixed end-over-end overnight at 4 °C. The immunoadsorbent was recovered by centrifugation for 10 s at 10000 ***g*** and the supernatant was used for sequential immunoisolation of PC1, PC2 and CPH (see below). The 3B7 immunoadsorbent was washed with 4 × 1 ml of lysis buffer, 2 × 1 ml of 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 1 % Triton X-100, 1 % deoxycholate, 0.1 % SDS and 5 mM EDTA, and then with 2 × 1 ml of distilled water. The bound protein was eluted with 2 × 0.25 ml of 25 % (v/v) acetic acid, freeze-dried and reconstituted in 80 μl of 12.5 mM Tris/HCl (pH 8.6) containing 8 M urea and 0.005 % Bromophenol Blue. The samples were subjected to alkaline-urea/PAGE and fluorographic analysis as described previously [23]. Incorporation of radioactivity into specific protein bands was determined by densitometric scanning of suitably exposed fluorographs (Chromoscan III; Joyce-Loebl, Gateshead, U.K.).

Immunoprecipitation of PC1, PC2 and CPH

The supernatants obtained after precipitation of insulin-related peptides were heated for 5 min at 100 °C in the presence of 0.5 % deoxycholate and 2 mM dithiothreitol to denature the radiolabelled proteins. After cooling on ice for 3 min, the samples were mixed end-over-end overnight at 4 °C with 10 μl of anti-PC1 serum and 50 μl (packed gel) of pre-swollen Protein A–Sepharose. The Protein A–Sepharose was recovered by centrifugation and the supernatants used for the sequential immunoprecipitation of PC2 and CPH. The Protein A–Sepharose was washed as above and the bound proteins were eluted with 2 × 100 μl of 20 mM HCl, freeze-dried and reconstituted in 80 μl of SDS sample loading buffer [125 mM Tris/HCl (pH 6.8) containing 2 % (w/v) SDS, 0.25 M sucrose, 5 mM EDTA, 65 mM dithiothreitol and 0.005 % Bromophenol Blue]. The samples were heated for 5 min at 100 °C and subjected to SDS/PAGE as described previously [23], and fluorography was performed as above. Molecular size calibration was achieved with ^{14}C-labelled lysozyme, β-lactoglobulin, α-chymotrypsin, ovalbumin, BSA, phosphorylase *b* and myosin heavy chain (BRL, Paisley, Scotland, U.K.).

RESULTS

Effect of Ca^{2+} depletion on proinsulin processing in the islets of Langerhans

Islets of Langerhans were cultured overnight prior to pulse–chase radiolabelling to allow their recovery after the isolation procedure. The basic experimental protocol incorporated a 2 h preincubation and 15 min of radiolabelling in 16.7 mM glucose to maximize the incorporation of [^{35}S]methionine into newly synthesized proteins (Figure 1A). This was followed by a 105 min chase incubation in 3.3 mM glucose to minimize exocytotic release of the radiolabelled proteins. The extent of proprotein conversion was then assessed by immunoisolation followed by PAGE and fluorography. Prolonged depletion of Ca^{2+} from the media did not affect islet integrity or viability, since islets incorporated [^{35}S]methionine into insulin and other proteins to the same extent whether or not Ca^{2+} was present.

Under control conditions, approx. 91 % of the islet proinsulin was converted into the processing intermediates (des-31,32- and des-64,65-proinsulin) and insulin by the end of the 2 h pulse–chase labelling period in 2.5 mM Ca^{2+} (Figure 1B). Substitution of 1 mM EGTA for Ca^{2+} in the media markedly inhibited this process, with only 18 % of the proinsulin being converted (Figure 1B). Inhibition of proinsulin processing required the presence of EGTA in the media, since simply omitting Ca^{2+} had little effect (Figure 1B). Islets incubated in the presence of 1 mM EGTA with excess Ca^{2+} (2 mM) processed proinsulin to a normal extent (86 %) (Figure 1B), suggesting that the effect of EGTA was due to chelation of Ca^{2+} and not to toxicity or chelation of other ions, such as Zn^{2+}, which conceivably play a role in proinsulin sorting and hexamer formation.

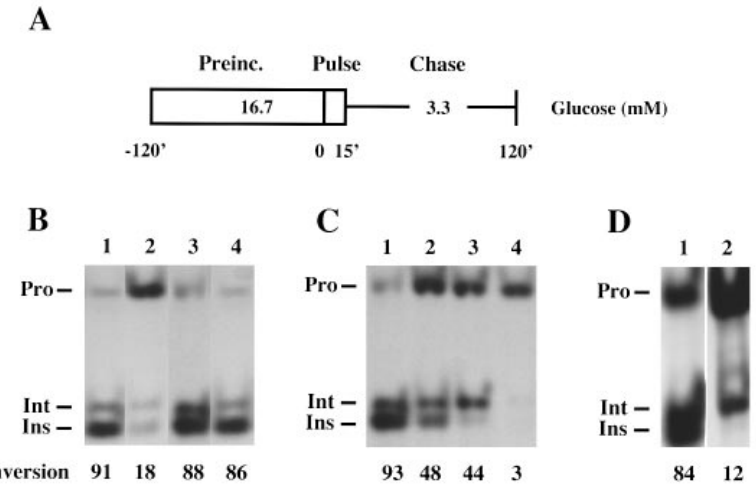

Figure 1 Effect of Ca^{2+} depletion on islet proinsulin conversion

(**A**) Diagram of pulse–chase radiolabelling protocol. Islets were preincubated for 120 min and pulse-labelled for 15 min with 0.2 mCi of [^{35}S]methionine in 16.7 mM glucose, and then chase-incubated for 105 min in non-radioactive medium containing 3.3 mM glucose and 2 mM methionine. (**B**) Islets were pulse–chase-radiolabelled in medium containing 2.5 mM $CaCl_2$ (track 1), 1 mM EGTA (no $CaCl_2$; track 2), no addition (no $CaCl_2$; track 3) or 2 mM $CaCl_2$ + 1 mM EGTA (track 4). (**C**) Islets were pulse–chase-radiolabelled in medium containing no $CaCl_2$ with no other addition (track 1), or in medium containing no $CaCl_2$ but with 0.1 mM nifedipine (track 2), 1 mM $NiCl_2$ (track 3) or 0.1 mM verapamil (track 4). (**D**) Islets were pulse–chase-radiolabelled in medium containing either no $CaCl_2$ (track 1) or no $CaCl_2$ with 2 μM thapsigargin (track 2). The cellular forms of insulin were immunoprecipitated and subjected to PAGE and fluorography as described in the Experimental section. The positions of migration of proinsulin (Pro), intermediates (Int) and insulin (Ins) are indicated. The extent of conversion (shown below each track) was determined by densitometric scanning of the bands on fluorographs, and is expressed as the amount of insulin and conversion intermediates as a percentage of the total insulin-related radioactivity. Similar results were obtained on at least three separate occasions.

Addition of the L-type Ca^{2+}-channel blockers nifedipine (0.1 mM) and verapamil (0.1 mM) to medium lacking Ca^{2+} resulted in a decrease in proinsulin processing to 48 and 3% respectively, and the non-specific Ca^{2+}-channel antagonist $NiCl_2$ (1 mM) reduced conversion to approx. 44% (Figure 1C). Addition of thapsigargin (2 μM), an inhibitor of the ER Ca^{2+}-ATPase [24], reduced proinsulin processing to approx. 12%, compared with 84% observed for control islets (Figure 1D).

Effect of Ca^{2+} depletion on the conversion of islet prohormone convertases

Conversion of the precursor forms of the proinsulin processing enzymes CPH, PC1 and PC2 was also examined in islets depleted of Ca^{2+} with 1 mM EGTA. The extent of proCPH processing in islets after a 2 h chase in the presence of Ca^{2+} approximated that of proinsulin, with around 93% undergoing conversion from the 57 kDa precursor to the 54 kDa mature form (Figure 2A). Substitution of 1 mM EGTA for Ca^{2+} in the media markedly inhibited this process, with only 30% of the proCPH being converted.

The extent of conversion of PC1 from its 94 kDa precursor to the 70 kDa mature granule form reached 62% after the 2 h chase incubation in the presence of Ca^{2+} (Figure 2B). Depletion of Ca^{2+} with 1 mM EGTA resulted in conversion of only 19% to the mature form. In addition, there was an accumulation of a slightly lower-molecular-mass form (92 kDa) after the longer periods of Ca^{2+} depletion. This is comparable in size with an early precursor form observed in pulse-labelling experiments in other neuroendocrine cells, and conceivably corresponds to the initial core-glycosylated translation product which appears to be rapidly modified by post-translational proteolysis and addition of terminal carbohydrate residues [13,14].

An effect of Ca^{2+} depletion on the processing of proPC2 was also seen (Figure 2C), although this was less dramatic than with the other islet convertases, since proPC2 conversion normally follows a slow time course [15]. Approx. 38% of the proPC2 was converted into the mature form in control islets, and 4% was converted in Ca^{2+}-depleted islets.

Effect of timing of Ca^{2+} depletion on proinsulin conversion

Depletion of Ca^{2+} with 1 mM EGTA immediately following radiolabelling and throughout the chase incubation had no effect on proinsulin processing (92% conversion) (Figure 3). Significantly less proinsulin was processed (71%) when Ca^{2+} was removed from the medium at the start of the radiolabelling period. The extent of processing decreased progressively when Ca^{2+} was removed 1 h (36%) and 2 h (18%) prior to the addition of radiolabel to the medium.

Effect of calcium replacement on proprotein processing and secretion

The reversibility of the effect of Ca^{2+} depletion was determined after a 2 h preincubation in the presence of 1 mM EGTA. Replacement of Ca^{2+} at the beginning of the biosynthetic labelling period resulted in considerable restoration of proinsulin processing, with approx. 72% being converted through to the conversion intermediates and insulin (Figure 4). By contrast, reintroduction of Ca^{2+} at the beginning of the chase incubation resulted in only partial restoration of proinsulin conversion, with 47% being processed. Replacement of Ca^{2+} at times greater than 15 min into the chase incubation resulted in only 23–24% proinsulin processing, similar to the value of 20% observed in islets maintained in the absence of Ca^{2+}.

To determine whether Ca^{2+} depletion disrupted the transport of proteins to the secretory granules, islets were pulse–chase-radiolabelled using the standard 15 min labelling period in 16.7 mM glucose, followed by a further 105 min chase incubation

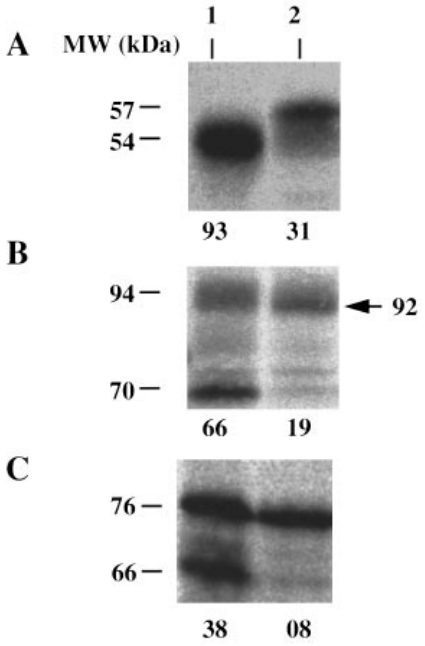

Figure 2 Effect of Ca^{2+} depletion on processing of islet prohormone convertases

Islets were pulse–chase radiolabelled as described in the legend to Figure 1(A) and incubated throughout either in 2.5 mM $CaCl_2$ (track 1) or in Ca^{2+}-free medium containing 1 mM EGTA (track 2). The cellular forms of (**A**) CPH, (**B**) PC1 and (**C**) PC2 were immunoprecipitated and subjected to PAGE and fluorography as described in the Experimental section. Only the relevant sections of each fluorograph, containing the immunoprecipitated enzymes, are shown. Preliminary experiments showed that each antiserum recognized specifically its respective enzyme, with no detectable cross-reactivity. The positions of migration of the precursor and mature forms of the convertases (kDa; as determined from the migration of ^{14}C-labelled molecular size standards) are indicated. The additional 92 kDa form of PC1 which appears following Ca^{2+} depletion is indicated by an arrow. The extent of conversion (shown below each track) was determined by densitometric scanning of the bands on fluorographs, and is expressed as the amount of the processed forms of each protein as a percentage of the total related radioactivity in each track. Similar results were obtained on three separate occasions.

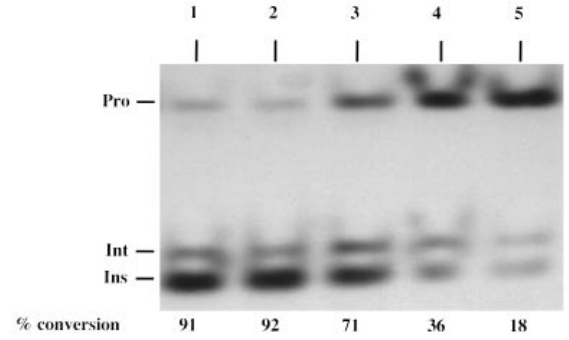

Figure 3 Effect of timing of Ca^{2+} depletion on proinsulin conversion

Islets were pulse–chase-radiolabelled as described in the legend to Figure 1(A), and either incubated throughout with 2.5 mM $CaCl_2$ (track 1) or transferred to Ca^{2+}-free medium containing 1 mM EGTA at the beginning of the chase period (+15 min; track 2), at the beginning of the radiolabelling period (0 min; track 3), 60 min prior to radiolabelling (−60 min; track 4) or 120 min prior to radiolabelling (−120 min; track 5). The cellular forms of insulin were immunoprecipitated and subjected to PAGE and fluorography as described in the Experimental section. The positions of migration of proinsulin (Pro), intermediates (Int) and insulin (Ins) are indicated. The extent of conversion (shown below each track) was determined by densitometric scanning of the bands on fluorographs, and is expressed as the amount of the processed forms of insulin and conversion intermediates as a percentage of the total insulin-related radioactivity. Similar results were obtained on three separate occasions.

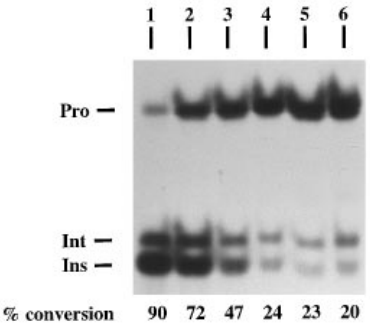

Figure 4 Effect of Ca^{2+} replacement on proinsulin conversion

Islets were pulse–chase-radiolabelled as described in the legend to Figure 1(A) and incubated throughout either in 2.5 mM $CaCl_2$ (track 1) or in Ca^{2+}-free medium containing 1 mM EGTA (track 6), or they were incubated initially in Ca^{2+}-free medium containing 1 mM EGTA and then transferred to medium containing 2.5 mM $CaCl_2$ at the beginning of the radiolabelling period (track 2), at the beginning of the chase period (track 3), at 15 min of chase incubation (track 4) or at 30 min of chase incubation (track 5). The cellular forms of insulin were immunoprecipitated and subjected to PAGE and fluorography as described in the Experimental section. The positions of migration of proinsulin (Pro), intermediates (Int) and insulin (Ins) are indicated. The extent of conversion (shown below each track) was determined by densitometric scanning of the bands, and is expressed as the amount of the processed forms of insulin and conversion intermediates as a percentage of the total insulin-related radioactivity. Similar results were obtained on three separate occasions.

in 3.3 mM glucose to permit transfer of the newly synthesized proteins from the ER to the granules. Ca^{2+} was then re-introduced together with 16.7 mM glucose for a further 2 h to stimulate the exocytotic release of the granule contents (Figure 5A). Both Ca^{2+}-depleted and control islets showed a strong insulin secretory response to the higher glucose concentration in the presence of Ca^{2+} (Figure 5B). The molecular composition of the secreted peptides reflected that of the cells; Ca^{2+}-depleted islets released predominantly proinsulin and islets incubated in normal Ca^{2+} released mainly insulin. Densitometric scanning showed that the rate of release of insulin-related peptides from the Ca^{2+}-depleted islets was marginally lower than that from control islets (8.6% and 11.6% respectively). The peptides were not released during the chase incubation in 3.3 mM glucose, suggesting that constitutive release due to failure of sorting in the *trans*-Golgi network had not occurred (results not shown).

Quantitative analyses of the secretion of radiolabelled CPH, PC1 and PC2 from Ca^{2+}-depleted and control islets (Figures 5C–5E) were precluded because of the low cellular abundance of these proteins. Nevertheless it was clear that, in comparison with (pro)insulin, Ca^{2+} depletion resulted in a smaller percentage of the cellular content of these proteins being released from the islets upon stimulation by glucose. In the case of PC2, it was noted that control islets released predominantly the mature form of the protein (65 kDa), in spite of the fact that the intracellular content was composed of approximately equal amounts of the precursor and mature forms.

DISCUSSION

A number of interacting regulatory processes operating at both the enzymic and cellular levels ensure that the proteolytic conversion of proinsulin into insulin in the pancreatic β-cell is confined to the secretory granules [15,25]. The concentration and condensation of proinsulin and the converting enzymes during

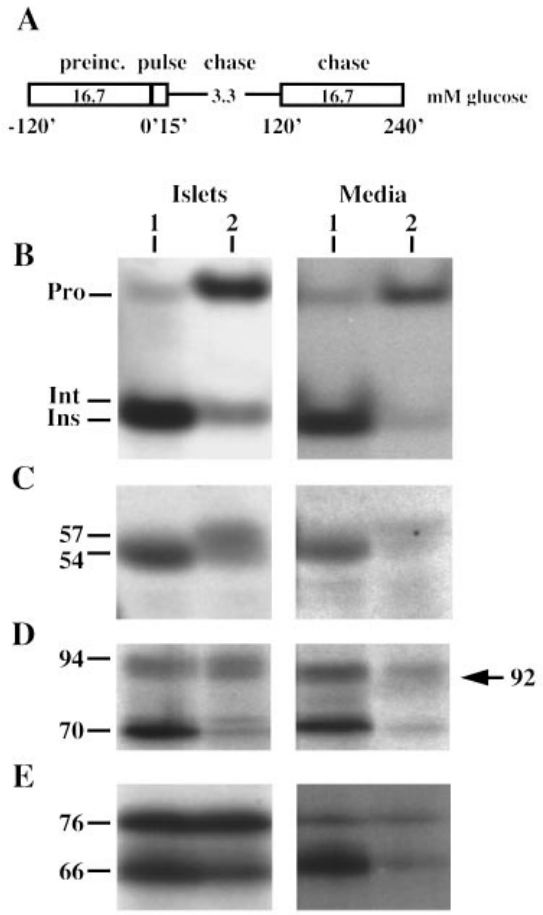

Figure 5 Effect of Ca^{2+} replacement on the secretion of proinsulin and the prohormone convertases

(**A**) Diagram showing extended pulse–chase radiolabelling protocol. Islets were preincubated for 120 min and pulse-labelled for 15 min with 0.2 mCi of [^{35}S]methionine in 16.7 mM glucose, chase-incubated for 105 min in non-radioactive medium containing 3.3 mM glucose and 2 mM methionine, and then chased for a further 120 min with 16.7 mM glucose. The islets were either incubated throughout with 2.5 mM $CaCl_2$ (track 1) or incubated initially in Ca^{2+}-free medium containing 1 mM EGTA followed by transfer to medium containing 2.5 mM $CaCl_2$ for the extended 2 h incubation with 16.7 mM glucose (track 2). The final islet and 2–4 h (extended chase period) medium forms of (**B**) insulin, (**C**) CPH, (**D**) PC1 and (**E**) PC2 were immunoprecipitated and subjected to PAGE and fluorography as described in the Experimental section. The relevant sections of each fluorograph, containing the immunoprecipitated proteins, are shown. The positions of migration of proinsulin (Pro), intermediates (Int), insulin (Ins), and the precursor and processed forms of CPH, PC1 and PC2 are indicated (numbers are kDa). The position of migration of the 92 kDa form of PC1 is indicated by an arrow. Similar results were obtained on three separate occasions.

transport from the ER to the storage granules favours processing in distal compartments of the secretory pathway. Acidification and accumulation of Ca^{2+} in the nascent granule appear to be necessary for the activity of the processing endoproteases [1]. The processing enzymes themselves undergo proteolytic cleavage at sites marked by clusters of basic amino acids, which may be responsible for their activation or targeting to the granules [7–9,13,15,26]. Further regulation may be imposed by the co-expression of other proteins, such as 7B2, which may act as an endogenous inhibitor and chaperonin of PC2 that affects its targeting or rate of delivery to the granules [27,28].

The present finding that depletion of Ca^{2+} in isolated islets of Langerhans resulted in a marked decrease in proinsulin conversion shows that Ca^{2+} plays an essential role in one or more aspects of the conversion process. It is likely that inhibition of proinsulin processing resulted from depletion of compartmental Ca^{2+} stores within the secretory pathway, since prolonged preincubation of islets in Ca^{2+}-depleted media was required. The β-cell mitochondria, ER and secretory granules contain large intracellular pools of Ca^{2+} [29,30], with the granules exhibiting the lowest rate of turnover [31,32]. Re-introduction of Ca^{2+} into the medium of Ca^{2+}-depleted islets has been observed to rapidly restore cytosolic Ca^{2+} levels, as shown by fluorimetric measurements of intracellular Ca^{2+} [33,34] and by the rapid re-activation of insulin secretion [35,36]. The present results suggest that the Ca^{2+} pool that is critical for normal proinsulin processing can be reconstituted during translocation of the prohormone into the lumen of the ER. As this interval is short relative to the time required for the transit of proinsulin from the ER to the granule compartment (approx. 30 min) and to the half-life of the proinsulin-to-insulin conversion (approx. 45 min), it is likely that many of the observed Ca^{2+}-related effects are mediated at the level of the ER. Further support for this comes from the observations that proinsulin processing was blocked by the ER Ca^{2+}-ATPase inhibitor thapsigargin and that conversion was not affected when Ca^{2+} was depleted after radiolabelling, when the bulk of the labelled proinsulin was likely to be in post-ER compartments.

Ca^{2+} has important functions at many levels within the secretory pathway. While it is an essential cofactor for the endoproteases PC1 and PC2 in secretory granules [1], it could exert its effects at a number of other levels. For example, it is required for the activity of the chaperonin calnexin in the ER [37] and promotes the aggregation and membrane association of some secreted proteins, and as such has been implicated in the sorting of proteins to the regulated pathway in the *trans*-Golgi network [38]. Studies *in vitro* have shown that fusion of carrier vesicles mediating ER-to-Golgi transport in permeabilized NRK cells requires Ca^{2+} in the physiological range (0.1 μM) [39] and is prevented in buffers containing EGTA. A global disruption of vesicular traffic did not appear to account for the present observations, in so far as proinsulin appeared to be sorted efficiently over a typically normal time course into a cellular compartment with the secretory characteristics of granules. However, the proteases appeared to be less efficiently sorted or secreted than the proinsulin-related peptides. This may relate to previous findings that these enzymes can behave as peripheral membrane proteins [40,41] and that the convertases, unlike proinsulin, appear to undergo a series of post-translational modifications prior to sorting into granules. Of particular importance may be the proteolytic processing of the pro- forms of the enzymes at sites marked by clusters of basic amino acids, since it has been demonstrated in the case of the related protease furin that mutation of the propeptide junctional sequence prevents its export from the ER [42]. In the case of PC2, processing can be achieved by an autocatalytic intermolecular reaction which is Ca^{2+}-sensitive [19,20]. PC1 may undergo similar autoproteolytic activation, but not necessarily in a Ca^{2+}-dependent manner [20]. The enzyme involved in proCPH processing has not been identified, although the primary sequence of the cleavage site suggests that it also belongs to the same Ca^{2+}-dependent subtilisin family as PC1, PC2 and furin. Such an enzyme could also be responsible for cleavage of PC1 or PC2 through an intermolecular mechanism, although little evidence exists for this at the present time. It was clear from our results that the decreases in secretion of PC1, PC2 and CPH were accompanied by inhibition of the initial processing of these proteins, which is consistent with such a hypothesis.

A consequence of the Ca^{2+}-related retardation of processing

enzyme movement would be the formation of granules with a normal (pro)hormone content but a relative deficiency in the levels of the enzymes. This effect might be compounded further by the fact that the processing enzymes that reach the granule compartment would be predominantly in their pro- forms. Although proCPH is reported to be catalytically active [10], proPC2 appears to be inactive [26] and the question of the activity of proPC1 is unresolved. Any changes in the specific activities of the endopeptidases in the nascent granule are likely be reflected in changes in the rate of proinsulin conversion, since these enzymes, in particular PC1, are rate-limiting for proinsulin conversion [1,43,44].

Given that Ca^{2+} taken up at the ER is essential for the proteolytic processing of proproteins in the more distal secretory granule compartment, the question arises as to whether Ca^{2+} that enters the secretory pathway through the ER is carried forward to the granules either by bulk flow or by association with the secretory proteins themselves. The relative concentrations of Ca^{2+} and (pro)insulin in isolated secretory granules [45] are such that insulin could act as a carrier for the cation. The apparent gradient of lumenal Ca^{2+} between the ER and the secretory granule compartment might be accounted for by the progressive concentration of the hormone along the pathway. There is no direct experimental evidence for active Ca^{2+} uptake by purified insulin granules [45,46]. In chromaffin granules there is a Na^{+}–Ca^{2+} exchange mechanism which could possibly concentrate the cation in such organelles [47]. A mechanism whereby accumulation of Ca^{2+} in the granule occurred via the ER would offer an alternative explanation for the currently observed difficulty of restoring proinsulin conversion unless the cation was added back before or during translation of the protein.

Studies on the release of insulin in response to cholinergic stimulation or to other agonists that affect intracellular Ins(1,4,5)P_3 levels have shown that the β-cell ER Ca^{2+} pool is readily mobilized [48–51]. The extent to which such mobilization affects the the Ca^{2+} concentration in the ER lumen is not clear, but the possibility should be considered that this might be of sufficient magnitude to have an impact on the regulation of prohormone processing. The hypothesis that the abnormalities in insulin secretion and processing of proinsulin in fasting and diabetic subjects [52,53] are related to changes in Ca^{2+} handling by the β-cell [54,55], rather than to alterations in the synthesis of the enzymes, is worthy of further investigation in this context.

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