Protein phosphorylation in permeabilized pancreatic islet cells

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A system of digitonin-permeabilized islet cells was developed to characterize Ca^{2+} - and calmodulin-dependent protein phosphorylation further and to determine whether activation of this membrane-bound process was sufficient for initiation of Ca^{2+} -stimulated insulin secretion. The efficacy of digitonin in permeabilizing the plasma membrane was assessed by Trypan Blue exclusion, by extracellular leakage of lactate dehydrogenase, and by permeability to $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This treatment did not detectably alter the ultrastructure of the permeabilized cells. Digitonin was equally effective when presented to islet cells that had been previously dispersed or directly to intact isolated islets. The Ca^{2+} - and calmodulin-dependent phosphorylation of endogenous membrane-bound substrates could be demonstrated in the permeabilized cells incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This activity displayed characteristics that were similar to those described for the protein kinase measured in subcellular fractions and was dependent on addition of exogenous calmodulin, indicating that calmodulin had been removed from the kinase by permeabilization of the cells. Ca^{2+} -dependent insulin release by the digitonin-permeabilized islet was demonstrated, with half-maximal release occurring at $0.1\ \mu\text{M}$ -free Ca^{2+} and maximal secretion at $0.2\ \mu\text{M}$ -free Ca^{2+} . Under these conditions, calmodulin did not further enhance insulin release, although a stimulatory effect of calmodulin was observed in the absence of free Ca^{2+} . These studies indicate that the permeabilized-islet model will be useful in dissecting out the factors involved in Ca^{2+} -activated insulin secretion.

The mechanism by which glucose stimulates the secretion of insulin from pancreatic islets remains unresolved. Various pieces of evidence suggest that alterations in cellular levels of Ca^{2+} are centrally involved in modification of the β -cell response. All known insulin secretagogues affect Ca^{2+} fluxes in intact islets; insulin secretion is dependent on extracellular Ca^{2+} , and alteration of extracellular Ca^{2+} concentrations can effect the release of insulin in the absence of glucose. Thus it appears that Ca^{2+} represents a final effector mechanism which initiates the secretory response (Wollheim & Sharp, 1981).

Many of the effects of Ca^{2+} are mediated by modification of protein kinase activities. We have fairly recently described a membrane-bound Ca^{2+}

and calmodulin-dependent protein kinase activity in subcellular fractions of pancreatic islets (Landt *et al.*, 1982). Various lines of evidence suggest that this protein kinase plays a role in insulin secretion. The protein kinase is activated by Ca^{2+} concentrations thought to be obtained in the stimulated β -cell (Colca *et al.*, 1983a) and is enriched in a microsomal fraction that contains a calcium pump capable of controlling Ca^{2+} concentrations in this range (Colca *et al.*, 1982). The Ca^{2+} - and calmodulin-dependent protein kinase phosphorylates endogenous islet-cell substrates of 57 and 54 kDa and is inactivated by alloxan under conditions where alloxan inhibits insulin secretion (Colca *et al.*, 1983b). Furthermore, studies performed with intact islets preloaded with $[\text{}^{32}\text{P}]\text{P}_i$ indicate that this protein kinase activity is correlated with glucose-stimulated insulin release (Colca *et al.*, 1983a).

However, experimental approaches that have studied protein phosphorylation in cell-free systems or after labelling of intact cells with $[\text{}^{32}\text{P}]\text{P}_i$ suffer from several theoretical and technical limitations. There is no assurance that an activity

Abbreviation used: SDS, sodium dodecyl sulphate.

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measured in cell homogenates or sonicated cells represents an interaction of an enzyme and substrate that occurs in the intact cell as opposed to being an artefact of the cell fractionation. On the other hand, studies with intact cells are limited by the fact that the experimental period must be preceded by a loading period with [^{32}P]P_i to label endogenous ATP pools; this also produces a large background of protein phosphorylation against which experimental changes must be measured. If labelling experiments are carried out in the presence of [^{32}P]P_i, experimental treatments may alter the labelled-nucleotide pool. Alternatively, if the [^{32}P]P_i is removed, some experimental treatments (e.g. elevation of medium glucose) may decrease the specific radioactivity of the ATP pool. Furthermore, it is not possible to define precisely changes in the cellular levels of Ca²⁺ that are presumed to occur in response to experimental treatments.

Measurement of protein phosphorylation in permeabilized cells offers advantages over the previously described systems. The permeabilized cells can be incubated directly with [γ - ^{32}P]ATP during a defined experimental time period. The concentrations of Ca²⁺ or other parameters can be controlled by direct addition to the external medium, since the plasma membrane is made freely permeable to these additions.

Detergent-permeabilized cells have been used to study pools of intracellular Ca²⁺ uptake (Hirata & Koga, 1982; Wakasugi *et al.*, 1982; Burgess *et al.*, 1983), DNA synthesis (Miller *et al.*, 1978), catecholamine secretion (Dunn & Holz, 1983; Wilson & Kirshner, 1983), and hormone action (Mooney *et al.*, 1983). Such studies have taken advantage of the ability of detergents such as digitonin to selectively permeabilize the cholesterol-rich plasma membrane. This study describes a technique to permeabilize dispersed islet cells as well as intact islets by a short incubation with digitonin. The digitonin-permeabilized islet cells are then used to measure Ca²⁺-activated protein phosphorylations and insulin secretion.

Experimental procedures

Materials

Male Sprague-Dawley rats (200–300g) were purchased from Sasco (O'Fallon, MO, U.S.A.). Collagenase (CLS IV) was obtained from Worthington, [γ - ^{32}P]ATP from New England Nuclear, Disperse from Godo Shusei (Tokyo, Japan), and calmodulin from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals, including digitonin, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Pancreatic islets

Pancreatic islets were obtained from fed rats by collagenase digestion (Lacy & Kostianovsky, 1967; McDaniel *et al.*, 1983), followed by purification on Ficoll gradients (Shibata *et al.*, 1976). The isolated islets were hand-picked under a dissecting microscope and then dispersed into single cells with Disperse (Ono *et al.*, 1977). Experiments either used these dispersed cells [(1–2) × 10⁴ cells/sample] or intact islets (20–40/sample). All incubation mixtures contained 300 μl unless otherwise noted.

Digitonin studies

Dispersed cells or intact islets were incubated in a modified Krebs buffer (115 mM-NaCl/24 mM-NaHCO₃/5 mM-KCl/1 mM-MgCl₂/1 mM-EGTA/20 mM-Hepes/0.1% bovine serum albumin, pH 6.8). Permeabilization was performed in the indicated buffer at 37°C with 0–40 μg of digitonin/ml for 15 min unless otherwise indicated. Dispersed cells were centrifuged at 500g for 5 min. Treated intact islets were allowed to sediment (30 s) and the medium was rapidly removed with a drawn Pasteur pipette.

Lactate dehydrogenase (EC 1.1.1.27) was measured in a 25 μl sample and fluorescence was read in a 1 ml sample (Lowry *et al.*, 1957). Total lactate dehydrogenase was measured in sonicated cells or islets (2 × 5 s at 18 W with a Branson sonifier). Total recovery of the lactate dehydrogenase by this method was 95–105%.

Trypan Blue exclusion was determined after a 5 min incubation with 4 mg of Trypan Blue/ml at room temperature and was determined for dispersed cells only.

Protein phosphorylation

Dispersed cells or islets pretreated with or without digitonin were washed one to three times with the modified Krebs buffer and then incubated at 37°C in the modified Krebs buffer containing 0–1.2 mM-CaCl₂, 1 mM-EGTA, 0–1 μM -calmodulin and 5–10 μCi of [γ - ^{32}P]ATP. For most incubations the cells were rapidly centrifuged or the islets were allowed to sediment and the radioactive medium was rapidly removed before addition of the stop solution [final concns. 3% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol]. The samples were then immediately placed in a boiling-water bath for 2 min. For studies in which the reaction time was varied or for short reaction times, i.e. 30 s, the assay was terminated by direct addition of the stop solution, followed by placing the sample in a boiling-water bath for 2 min. This protocol totally inactivates protein kinase activity. Phosphoproteins were then separated by electrophoresis on 10%-(w/v)-polyacrylamide slab gels.

The gels were dried, exposed to X-ray film, and the phosphoprotein bands of interest were cut from the gels for quantification of ^{32}P incorporation (Landt *et al.*, 1982). Ca^{2+} and calmodulin-dependent protein phosphorylation is defined as the amount of phosphorylation occurring in the presence of Ca^{2+} and calmodulin minus that occurring in the absence of either component. Quantification was generally of the 57kDa endogenous substrate, since it is more heavily phosphorylated (Colca *et al.*, 1983a).

Insulin secretion

Digitonin-treated islets were washed three times in Tris buffer (50mM-Tris/100mM-KCl/5mM- MgCl_2 /0.1% bovine serum albumin, pH6.8) and then incubated at 24°C for 15min in Tris/Pipes buffer (55mM-Tris/Pipes/111mM-KCl/5mM- MgCl_2 /1.1mM-EGTA/1mM-ATP/10mM-phosphocreatine/creatine kinase (10 units/ml)/0.1% bovine serum albumin, pH7.5) containing 0–1.2mM- CaCl_2 and 0–167 μg of calmodulin/ml. Islets were gassed with O_2/CO_2 (19:1). At the end of the incubation period, the medium was withdrawn and the insulin released was quantified by radioimmunoassay. Immunoreactive insulin was measured in triplicate by the method of Wright *et al.* (1971), with porcine insulin as standard.

Free Ca^{2+} concentrations were estimated by assuming an apparent stability constant for $\text{Ca}^{2+}\cdot\text{EGTA}$ of $10^{7.44}$ (24°C , 100mM-KCl, pH7.5), which was determined from an absolute stability constant of $10^{10.97}$ (20°C , 10.1) by interpolating for temperature and the H^+ activity coefficient as suggested by Martell & Smith (1974). Ionized- Ca^{2+} concentrations were then confirmed by measurement in the complete assay medium by using a Ca^{2+} -specific electrode (Orion 93-20, with a double-junction reference electrode 90-02) as previously described (Kotagal *et al.*, 1983).

Results

Permeabilization and phosphorylation in dispersed islet cells

Dispersed pancreatic-islet cells were washed in the modified Krebs buffer and incubated [(4–)

$\times 10^4$ cells/300 μl sample] with various concentrations of digitonin at 37°C . The cells were then sedimented and lactate dehydrogenase activity was determined in the medium and in sonicated resuspended pellets. Parallel sets of cells so treated were resuspended in medium without added digitonin, and incubated with Trypan Blue for 5min. Table 1 shows that the percentage of cells unable to exclude Trypan Blue was directly related to the percentage release of the cytosolic marker, lactate dehydrogenase, after pretreatment of cells with various concentrations of digitonin.

Analysis of the digitonin-treated islet cells by electron microscopy showed no significant ultrastructural differences when compared with non-treated islet cells (Fig. 1). In both groups, the ultrastructural appearance of the endoplasmic reticulum and the secretory granules was similar, although the mitochondria of permeabilized cells appeared slightly more condensed.

Studies were undertaken to determine whether Ca^{2+} and calmodulin-dependent protein kinase activity could be detected in the permeabilized cells. When cells were permeabilized with digitonin, washed, and then incubated with [γ - ^{32}P]-ATP for 10 min at 37°C , the extent of protein phosphorylation was proportional to the amount of permeabilization achieved during the digitonin pretreatment, as shown in Table 1. Whereas the addition of calcium alone (1mM buffered with EGTA; 4 μM -ionized Ca^{2+}) did not augment protein phosphorylation, the combined presence of Ca^{2+} and 1 μM -calmodulin increased the phosphorylation of 57kDa and 54kDa protein bands (Table 2; see also Fig. 2). This result indicated that endogenous calmodulin was depleted from or no longer available to the cellular kinase by the digitonin treatment.

Permeabilization and phosphorylation in intact islets

When intact islets were permeabilized with various concentrations of digitonin and then incubated with [γ - ^{32}P]-ATP with or without Ca^{2+} and calmodulin, results similar to those with the dispersed cells were obtained, namely the phosphorylation of the islet 57kDa and 54kDa proteins

Table 1. Effect of digitonin pretreatment on permeability of dispersed islet cells

Isolated pancreatic islets were dispersed into single cells as described in the Experimental procedures section. Samples of the cells were then incubated with the indicated concentrations of digitonin for 15min at 37°C . The cells were then sedimented and lactate dehydrogenase activity (LDH) was determined on samples of the supernatant and sonicated pellet as described in the text. A portion of the cell suspension from each sample was also tested for the ability of the cells to exclude Trypan Blue. Results are means $\pm$ S.E.M. for four experiments.

[Digitonin] ($\mu\text{g}/\text{ml}$)	0	4	10	20
Percentage of LDH released	23 $\pm$ 9	31 $\pm$ 10	63 $\pm$ 11	84 $\pm$ 7
Percentage of cells positive for Trypan Blue staining	8 $\pm$ 2	33 $\pm$ 6	66 $\pm$ 9	87 $\pm$ 5

Fig. 1. *Ultrastructure of permeabilized islet cells*
Pancreatic islets were incubated in modified KRB medium with or without 20 μg of digitonin/ml for 15 min at 37°C as described in the text. The islets were then fixed in 1.7% (v/v) glutaraldehyde/1.3% (v/v) *p*-formaldehyde/0.1 M-cacodylate buffer, post-fixed with 1% OsO_4 , dehydrated, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead nitrate. (a) Control; (b) digitonin-permeabilized. Detailed examination of the photomicrographs showed no difference between the groups.

Fig. 2. *Protein phosphorylation in digitonin-permeabilized islets*

Pancreatic islets were permeabilized by 15 min incubations (37°C) with the indicated concentration of digitonin. Lactate dehydrogenase (LDH) was then determined in the supernatants as well as in sonicated resuspended pellets. Parallel groups of islets (25/sample) were then washed twice with albumin-free medium and incubated for 5 min with 10 μCi of [γ -³²P]ATP with or without 1 μM -calmodulin and/or 10 μM -Ca²⁺ as described in the text. The autoradiogram and associated lactate dehydrogenase results shown are representative of three experiments of this design. Similar results (shown in Tables 1 and 2) were obtained with digitonin treatment of dispersed islet cells.

was dependent on the addition of both Ca²⁺ and calmodulin and proportional to the percentage of lactate dehydrogenase released during the digitonin pretreatment (Fig. 2). Although the digitonin treatment did not disrupt the ultrastructure of the β -cells (Fig. 1), the extent of phosphorylation obtained after pretreatment of islets with 20 or 40 μg of digitonin/ml was similar to that occurring after disruption of the intact islets by sonication, i.e. the cells were made sufficiently permeable to digitonin (20 μg /ml). It is noteworthy that 49 $\pm$ 14% of the total islet Ca²⁺- and calmodulin-dependent protein kinase was recovered from the intact islets after digitonin treatment. Additional studies were undertaken to characterize protein phosphorylation in these permeable islets. The time course for the Ca²⁺ and calmodulin-dependent protein kinase in pre-permeabilized islets is shown in Table 3. Interestingly, a similar time course for the activity was obtained after preincubation of untreated islets with Ca²⁺, calmodulin, and ATP and initiation of the intracellular phosphorylation with digitonin (Table 3). That is, the speed of permeabilization of the islets by digitonin

Table 2. *Ca²⁺- and calmodulin-dependent phosphorylation of permeabilized islet cells*

Dispersed pancreatic islet cells were permeabilized with various concentrations of digitonin as described in Table 1. The respective cells were then washed and incubated with [γ -³²P]ATP (5 μ M, 10 μ Ci/tube) with and without Ca²⁺ (1 mM total, 4 μ M free) and calmodulin (1 μ M) for 10 min as described in the Experimental procedures section. Results show the mean phosphorylation of the 57 kDa membrane-bound protein obtained in two experiments with islet cells.

Phosphorylation conditions	[Digitonin] (μ g/ml) . . .	Protein phosphorylation (fmol of P _i /57 kDa band)			
		0	4	10	20
No additions		38	86	139	156
Ca ²⁺		44	94	159	133
Ca ²⁺ + calmodulin		111	298	440	320

Table 3. *Time course of Ca²⁺- and calmodulin-dependent protein phosphorylation in digitonin-permeabilized pancreatic islets*

Incubation mixtures contained pancreatic islets permeabilized with 20 μ g of digitonin/ml (15 min, 37°C) and then washed free of detergent (protocol A) or untreated islets (protocol B). For protocol A the permeabilized islets were pre-incubated with and without 300 μ M-free Ca²⁺ and 1 μ M-calmodulin (2 min, 37°C): the reaction was initiated with 5–10 μ Ci of [γ -³²P]ATP (total volume 100 μ l) and stopped at the indicated times with stop solution as described in the Experimental procedures section. For protocol B, untreated islets were preincubated with 5–10 μ Ci of [γ -³²P]ATP with and without 300 μ M-free Ca²⁺ and 1 μ M-calmodulin (2 min, 37°C) and the reaction was initiated by the addition of 20 or 40 μ g of digitonin/ml (total volume 100 μ l) and then stopped at the indicated times as discussed above. Data are the means $\pm$ S.E.M. for Ca²⁺- and calmodulin-dependent P_i incorporation into the 57 kDa and 54 kDa islet-cell substrates (i.e., minus phosphorylation in the absence of Ca²⁺) for three experiments, each performed in duplicate.

Protocol	Incubation time (min) . . .	Ca ²⁺ - and calmodulin-dependent P _i incorporation (c.p.m.)		
		0.5	1	5
(A) Pre-permeabilized (20 μ g of digitonin/ml)		725 $\pm$ 104	1317 $\pm$ 88	2158 $\pm$ 248
(B) Started with digitonin at:				
20 μ g/ml		944 $\pm$ 382	1210 $\pm$ 407	2288 $\pm$ 768
40 μ g/ml		807 $\pm$ 232	1829 $\pm$ 271	2522 $\pm$ 576

was not such as to rate-limit the measurement of protein phosphorylation in this system.

To determine if the protein kinase activity in the permeabilized-islet system was similar to that described previously for the kinase activity in sub-cellular fractions, the concentration of free Ca²⁺ or exogenous calmodulin was varied. The calmodulin requirement for this protein kinase activity is shown in Fig. 3(a). Maximal stimulation of the rate of phosphorylation occurred at approximately 10 μ g of calmodulin/ml. The Ca²⁺ dependence of the protein kinase activity (Fig. 3b) showed that maximal activation was present at approx. 2–3 μ M-Ca²⁺. The characteristics of this protein kinase activity as measured in permeabilized islets were similar to those described for the activity measured in subcellular fractions (Colca *et al.*, 1983a). Additional studies have also been performed to address this question. Ca²⁺ and calmodulin-dependent protein phosphorylation assays were performed in digitonin permeabilized islets as described in Fig. 2. The islets were then homogenized in fractionation buffer at 4°C containing EDTA to chelate

Mg²⁺ and minimize subsequent dephosphorylation. The particulate membranes and cytosol were then separated by ultracentrifugation, the phosphoproteins being by autoradiography. The Ca²⁺- and calmodulin-dependent phosphoproteins as described in the present study were totally associated with the protein pellet as opposed to the cytosol. These studies are consistent with the other supportive data indicating that the Ca²⁺- and calmodulin-dependent protein kinase system in digitonin-permeabilized islets exists in the membrane fraction.

Insulin secretion in permeabilized islets

Insulin release by digitonin-treated islets was examined at 37°C under conditions similar to those described for protein phosphorylation. However, the addition of Ca²⁺ (0.1–40 μ M-free Ca²⁺) with or without calmodulin did not augment the release of insulin (results not shown). Since it seemed likely that the failure to demonstrate Ca²⁺-induced insulin secretion may have resulted from technical limitations of the assay, modifications were made

Fig. 3. Ca^{2+} - and calmodulin-dependence of the protein kinase activity measured in digitonin-permeabilized islets. Pancreatic islets were permeabilized with $20 \mu\text{g}$ of digitonin/ml as described in the text. The islets were then washed in albumin-free medium and incubated with $10 \mu\text{M}$ - Ca^{2+} and various concentrations of calmodulin (a) or $1 \mu\text{M}$ ($17 \mu\text{g/ml}$)-calmodulin and various concentrations of Ca^{2+} (b). Reactions, initiated by $10 \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were terminated after 30 s and processed as described in the text.

Table 4. *Effect of Ca^{2+} and calmodulin on insulin release in digitonin-permeabilized pancreatic islets*
Digitonin-permeabilized ($20 \mu\text{g/ml}$, 20 min, 37°C) pancreatic islets were washed free of detergent in Tris buffer (50 mM -Tris/ 100 mM -KCl/ 5 mM -MgCl₂/ 0.1% bovine serum albumin, pH 6.8) and then incubated at 24°C in a Tris/Pipes buffer mimicking intracellular conditions [55 mM -Tris/Pipes/ 111 mM -KCl/ 5 mM -MgCl₂/ 1.1 mM -EGTA/ 10 mM -phosphocreatine/creatine kinase (10 units/ml)/ 0.1 bovine serum albumin, pH 7.5] containing no or 0.95 mM -CaCl₂ and 0 – $167 \mu\text{g}$ of calmodulin/ml. After 15 min incubation, insulin released in the media was measured by radioimmunoassay. Results are expressed as means $\pm$ S.E.M. of three to four experiments each performed in quadruplicate.

[Calmodulin] ($\mu\text{g/ml}$)	[Free Ca^{2+}] (μM)	Insulin release ($\mu\text{units/min per islet}$)	
		0	0.2
0		0.48 ± 0.05	0.96 ± 0.13
1.7		1.04 ± 0.14	1.02 ± 0.18
8.4		0.93 ± 0.15	0.89 ± 0.15
16.7		0.82 ± 0.10	0.77 ± 0.09
167.2		1.11 ± 0.30	0.94 ± 0.27

to optimize the possibility of demonstrating Ca^{2+} -dependent secretion. Assays were performed at room temperature to minimize background insulin release and at pH 7.5 in the presence of EGTA in order to obtain submicromolar concentrations of free Ca^{2+} . In the presence of an ATP-regenerating system and in a media mimicking intracellular conditions, Ca^{2+} -induced secretion by digitonin-treated islets was successfully demonstrated (Fig. 4). Under these conditions, a basal rate of $0.64 \pm 0.06 \mu\text{unit}$ of insulin/min per islet was obtained in the absence of Ca^{2+} and maximal insulin release ($1.08 \pm 0.09 \mu\text{unit/min per islet}$, $n = 20$) was observed at $0.2 \mu\text{M}$ -free Ca^{2+} . The effect of calmodu-

lin (0 – $167 \mu\text{g/ml}$) on insulin release in the absence or presence of $0.2 \mu\text{M}$ -free Ca^{2+} is detailed in Table 4. In the absence of Ca^{2+} , exogenous calmodulin significantly enhanced insulin secretion, whereas, in the presence of $0.2 \mu\text{M}$ -free Ca^{2+} , no further effect on insulin release could be demonstrated. Ca^{2+} - and calmodulin-dependent protein kinase activity similar to that demonstrated in the experiments defining this permeabilized-islet system was also observed under these identical assay conditions, i.e. pH 7.5 and room temperature (e.g. basal activity $0.250 \pm 0.019 \text{ fmol of } \text{P}_i/10 \text{ min per islet}$, $0.471 \pm 0.06 \text{ fmol of } \text{P}_i/10 \text{ min per islet}$ at $1.1 \mu\text{M}$ - Ca^{2+} and $1 \mu\text{M}$ -calmodulin).

Fig. 4. Ca^{2+} -induced insulin secretion by digitonin permeabilized islets

Pancreatic islets were permeabilized with 20 μg of digitonin/ml as described in the text. Islets were washed three times in Tris buffer (50 mM-Tris/100 mM-KCl/5 mM-MgCl₂/0.1% bovine serum albumin, pH 6.8) and then incubated for 15 min at 24°C in a Tris/Pipes buffer mimicking intracellular conditions [55 mM-Tris/Pipes/111 mM-KCl/5 mM-MgCl₂/1.1 mM-EGTA/10 mM-phosphocreatine/creatine kinase (10 units/ml)/0.1% bovine serum albumin, pH 7.5]. Free- Ca^{2+} concentrations were obtained by addition of 0–1.2 mM- CaCl_2 as described in the Experimental procedures section and then confirmed by measurement with a Ca^{2+} -specific electrode. Results are means $\pm$ S.E.M. for five experiments each performed in quadruplicate.

Discussion

A system has been described for effectively permeabilizing the plasma membrane of pancreatic-islet cells by using low concentrations of digitonin. The digitonin treatment is equally as effective in permeabilizing either dispersed islet cells or intact islets. Studies are greatly expedited by using the intact islets, which can be manipulated under a microscope without centrifugation. It should be noted that incubation of the islets in a modified Krebs–Ringer bicarbonate buffer supplemented with EGTA with or without digitonin increased the intercellular space in the islets as determined by microscopy and this may in fact augment the effectiveness of digitonin in the non-dispersed islets. Although digitonin caused leakage of the cytosolic content from the cells (as judged by release of lactate dehydrogenase), the ultrastructure of the cells remained intact. Furthermore, at the digitonin concentration employed in the present study, cholesterol-poor membranes such as are found in the endoplasmic reticulum (Korn, 1966) are not affected by this treatment (Gogelein & Huby, 1984). The system is thus useful for studying protein phosphorylation in the structurally undisturbed intracellular membranes, and to determine the requirements for insulin secretion.

The demonstration of Ca^{2+} - and calmodulin-dependent protein kinase activity in the per-

meabilized cells with kinetic characteristics similar to those described in subcellular membrane fractions (Landt *et al.*, 1982; Colca *et al.*, 1983a) supports the hypothesis that this enzymic system functions in the intact cell. As the cell membranes were permeable to Ca^{2+} and calmodulin, it was possible to define the conditions for measurement of the kinase activity by controlled additions to the extracellular medium and to initiate the reactions with [γ -³²P]ATP. In contrast with studies with impermeable cells, where these parameters cannot be controlled directly, the experiments support the existence of this protein kinase system in the structurally intact membranes. The need for addition of exogenous calmodulin in this system implies that the source of calmodulin for the protein kinase activity is depleted by the loss of cytosolic calmodulin. It is possible, therefore, that alteration of soluble-calmodulin concentrations may play a role in this protein kinase activity *in vivo*.

Several groups of investigators have shown that the addition of Ca^{2+} to digitonin-permeabilized adrenal chromaffin cells directly augments the release of catecholamine (Dunn & Holz, 1983; Wilson & Kirshner, 1983). Pace *et al.* (1980) and Yaseen *et al.* (1982) have shown that intact islets permeabilized by electric discharge release insulin in proportion to the concentration of Ca^{2+} present in the incubation medium. In those studies there was not a depletion of cytosolic contents, although after a period of time these cells permeabilized by electric discharge may become depleted of ATP (Pace *et al.*, 1980). These results obtained with islets made permeable by electric discharge are essentially similar to those described initially by Baker & Knight (1978, 1981) for adrenal chromaffin cells made permeable by electric discharge. Thus the basic features of Ca^{2+} -stimulated secretion from the pancreatic islets and adrenal chromaffin cells may be similar.

We have demonstrated that Ca^{2+} -dependent insulin release also can occur in digitonin-permeabilized islets. The failure to show Ca^{2+} -stimulated insulin release at 37°C is due to the large non-specific background release of insulin (10 times the basal rate observed at 24°C). However, at room temperature and under conditions mimicking intracellular requirements, Ca^{2+} -dependent insulin release was present as well as the Ca^{2+} - and calmodulin-dependent phosphorylation of the 57 kDa endogenous substrate. The effect of calmodulin on insulin secretion in this system was unexpected. The lack of further enhancement of Ca^{2+} -dependent insulin release with calmodulin could be due to the experimental conditions, such as free Ca^{2+} concentration, resulting in already-maximal insulin release. However, calmodulin in

the absence of added Ca^{2+} , over a wide range of concentrations, stimulated insulin release. This phenomenon could not be accounted for by Ca^{2+} contamination of the calmodulin preparation, as confirmed by theoretical and Ca^{2+} -electrode determinations (see the Experimental procedures section). The possibility that calmodulin *per se* acts on an unknown link of the exocytosis process cannot be excluded. In any event, the development of the digitonin-permeabilized islet system will allow detailed testing of these hypotheses under various conditions with inhibitors of the protein kinase activity such as calmodulin antagonists, diphenylhydantoin and alloxan (Landt *et al.*, 1982; Norling *et al.*, 1984; Colca *et al.*, 1983b).

The present studies demonstrate the usefulness of the digitonin-permeabilized cells in the study of protein phosphorylation and insulin release in intact cellular membranes. This system can complement studies with subcellular fractions or cells loaded with [^{32}P]P_i and offers certain advantages over both of these techniques. It is also applicable to the direct study of other protein kinases such as cyclic AMP-dependent protein kinase and protein kinase C and the impact of these activities on insulin secretion.

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References

- Baker, P. F. & Knight, D. E. (1978) *Nature (London)* **276**, 620–622
- Baker, P. F. & Knight, D. E. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 83–103
- Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W., Jr. (1983) *J. Biol. Chem.* **258**, 15336–15345
- Colca, J. R., McDonald, J. M., Kotagal, N., Patke, C., Fink, C. J., Greider, M. H., Lacy, P. E. & McDaniel, M. L. (1982) *J. Biol. Chem.* **257**, 7223–7228
- Colca, J. R., Brooks, C. L., Landt, M. & McDaniel, M. L. (1983a) *Biochem. J.* **212**, 819–827
- Colca, J. R., Kotagal, N., Brooks, C. L., Lacy, P. E., Landt, M. & McDaniel, M. L. (1983b) *J. Biol. Chem.* **258**, 7260–7263
- Dunn, L. A. & Holz, R. W. (1983) *J. Biol. Chem.* **258**, 4989–4993
- Gogelein, H. & Huby, A. (1984) *Biochim. Biophys. Acta* **733**, 32–38
- Hirata, M. & Koga, T. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1544–1549
- Korn, E. D. (1966) *Science* **153**, 1491–1498
- Kotagal, N., Colca, J. R. & McDaniel, M. L. (1983) *J. Biol. Chem.* **258**, 4808–4813
- Lacy, P. E. & Kostianovsky, M. (1967) *Diabetes* **16**, 35–39
- Landt, M., McDaniel, M. L., Bry, C. G., Kotagal, N., Colca, J. R., Lacy, P. E. & McDonald, J. M. (1982) *Arch. Biochem. Biophys.* **213**, 148–154
- Lowry, O. H., Roberts, N. R. & Kapphahn, J. I. (1957) *J. Biol. Chem.* **224**, 1047–1064
- Martell, A. E. & Smith, R. M. (1974) *Critical Stability Constants*, vol. 1 (*Amino Acids*), Plenum Press, New York
- McDaniel, M. L., Colca, J. R., Kotagal, N. & Lacy, P. E. (1983) *Methods Enzymol.* **98**, 182–200
- Miller, M. R., Castellot, J. J., Jr. & Pardee, A. B. (1978) *Biochemistry* **17**, 1073–1080
- Mooney, R. A., Ebersohl, R. D. & McDonald, J. M. (1983) *Eur. J. Biochem.* **136**, 603–608
- Ono, J., Takaki, R. & Fukuma, M. (1977) *Endocrinol. Jpn.* **24**, 265–270
- Norling, L. L., Colca, J. R., Brooks, C. L., Kloepper, R. F., McDaniel, M. L. & Landt, M. (1984) *Biochim. Biophys. Acta* **801**, 197–205
- Pace, C. S., Tarvin, J. T., Neighbors, A. S., Pirkle, J. A. & Greider, M. H. (1980) *Diabetes* **29**, 911–918
- Shibata, A., Ludvigsen, C. W., Naber, S. P., McDaniel, M. L. & Lacy, P. E. (1976) *Diabetes* **25**, 667–672
- Wakasugi, H., Kimura, T., Haase, W., Kribben, A., Kaufmann, R. & Schulz, I. (1982) *J. Membr. Biol.* **65**, 205–220
- Wilson, S. P. & Kirshner, N. (1983) *J. Biol. Chem.* **258**, 4994–5000
- Wollheim, C. B. & Sharp, G. W. G. (1981) *Physiol. Rev.* **61**, 914–973
- Wright, P. H., Makulu, P. R., Vichick, D. & Sussman, K. E. (1971) *Diabetes* **20**, 33–45
- Yaseen, M. A., Pedley, K. C. & Howell, S. L. (1982) *Biochem. J.* **206**, 81–87