Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion

PARNAUD, Géraldine, et al.

Abstract

In addition to promoting insulin secretion, an increase in cytosolic Ca(2+) triggered by glucose has been shown to be crucial for spreading of beta-cells attached on extracellular matrix (804G matrix). Calpains are Ca(2+)-dependent cysteine proteases involved in an extended spectrum of cellular responses, including cytoskeletal rearrangements and vesicular trafficking. The present work aimed to assess whether calpain is also implicated in the process of Ca(2+)-induced insulin secretion and spreading of rat pancreatic beta-cells. The results indicate calpain dependency of beta-cell spreading on 804G matrix. Indeed, treatment with three distinct calpain inhibitors (N-Ac-Leu-Leu-norleucinal, calpeptin, and ethyl(+)-(2S,3S)-3-[(S)-3-methyl-1-(3-methylbutylcarbamoyl)butyl-carbamoyl]-2-ox-iranecarboxylate) inhibited cell spreading induced by glucose and KCl, whereas cell attachment was not significantly modified. Calpain inhibitors also suppressed glucose- and KCl-stimulated insulin secretion without affecting insulin synthesis. Washing the inhibitor out of the cell culture restored spreading on 804G matrix and insulin [...]
Inhibition of calpain blocks pancreatic β-cell spreading and insulin secretion

Géraldine Parnaud,1 Eva Hammar,1 Dominique G. Rouiller,1 and Domenico Bosco2

1Department of Genetic Medicine and Development, University Medical Center; and 2Cell Isolation and Transplantation Center, Division of Surgical Research, Department of Surgery, University Hospital, Geneva, Switzerland

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Calpains are cytosolic neutral Ca2+-dependent cysteine proteases that catalyze the endoproteolytic cleavage of specific substrates and thereby regulate pathways that affect intracellular signaling (25, 28). Fourteen members of the calpain family have been identified, some of which are tissue specific and have been also identified in pancreatic islets (19). The extracellular matrix (ECM) plays a critical role in modulating the morphology, growth, migration, and differentiation of cells. The response of cells to matrix-adhesive proteins appears to be mediated primarily through the integrin family of adhesion receptors (24). The α3- and α6-integrins have been shown to be expressed in rat pancreatic β-cells (3, 18) and are known to be the principal receptors for the ECM protein laminin. Furthermore, rat islet β-cells spread in response to glucose when attached on the matrix produced by a rat bladder carcinoma cell line (804G), known to be rich in laminin 5. In a mixed population of cells, it has been previously observed that spread cells secrete more insulin acutely in response to glucose compared with cells that remain rounded (3). Glucose-induced spreading of β-cells on 804G matrix requires an increase in intracellular calcium concentration ([Ca2+]i) and is not a consequence of exocytotic processes that follow elevation of [Ca2+]i (2).

It is well known that Ca2+ plays a crucial role in insulin secretion from β-cells. A significant influx of Ca2+ occurs during the process of glucose-mediated insulin secretion. A wide variety of cytoplasmic enzymes, mostly kinases, are known to be activated or modulated by an elevation of [Ca2+]i and have been also identified in β-cells (17). These include calcium/calmodulin- and calcium/phospholipid-dependent kinases that have been shown to be involved in the initiation and maintenance of nutrient-stimulated insulin secretion. Whether calpain may also be implicated in the process of Ca2+-induced insulin secretion is not yet understood. In recent studies, calpain has been implicated in the process of exocytosis in the insulin-secreting cell line INS-1 or in mouse islet (23, 27, 38, 40). The extracellular matrix (804G matrix) is required for glucose-induced insulin secretion and spreading of rat pancreatic β-cells. It is therefore suggested that calpain could be a mediator of Ca2+-induced-insulin secretion and β-cell spreading.

extracellular matrix; intracellular calcium

Calpain activity has been shown to be crucial in a diverse spectrum of cellular responses. By making selective limited proteolytic cleavages, calpains modulate the activity of enzymes and induce specific cytoskeletal rearrangements, accounting for their roles in cell spreading and motility, vesicular trafficking, and structural stabilization (7, 9–11). Calpains are also expressed in rat pancreatic acinar cells and may participate in stimulus-secretion coupling of amylase (42).

Various forms of calpain have also been implicated in pathological processes, such as neurodegenerative disorders, Alzheimer’s disease, and muscular dystrophy (16, 26, 31, 40). Variation in CAPN10, the gene encoding the ubiquitously expressed cysteine protease calpain-10, has been associated with type 2 diabetes in Mexican Americans and in two northern-European populations, from Finland and Germany (15).

The extracellular matrix (ECM) plays a critical role in modulating the morphology, growth, migration, and differentiation of cells. The response of cells to matrix-adhesive proteins appears to be mediated primarily through the integrin family of adhesion receptors (24). The α3- and α6-integrins have been shown to be expressed in rat pancreatic β-cells (3, 18) and are known to be the principal receptors for the ECM protein laminin. Furthermore, rat islet β-cells spread in response to glucose when attached on the matrix produced by a rat bladder carcinoma cell line (804G), known to be rich in laminin 5. In a mixed population of cells, it has been previously observed that spread cells secrete more insulin acutely in response to glucose compared with cells that remain rounded (3). Glucose-induced spreading of β-cells on 804G matrix requires an increase in intracellular calcium concentration ([Ca2+]i) and is not a consequence of exocytotic processes that follow elevation of [Ca2+]i (2).

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MATERIALS AND METHODS

804G matrix preparation. Cells from a rat bladder carcinoma cell line (804G) were grown in DMEM containing 10% FCS and 5.6 mM glucose (32). At confluence, cells were washed and maintained for a further 3 days in the same medium in the absence of FCS. Conditioned

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medium (referred to hereafter as 804G matrix) was collected, centrifuged at 180 g for 5 min to remove any detached cells and debris, filtered through a 0.22-μm Millipore filter, and frozen at −20°C for later use.

Coating of plastic with 804G matrix and poly-L-lysine. Aliquots (60 μl) of 804G matrix or 100 μg/ml poly-L-lysine (PLL) were layered at the center of 35-mm adherent-culture Petri dishes. Dishes were kept in a damp box at 37°C for 18–20 h before being washed three times with sterile H2O and air dried. PLL-coated Petri dishes were used as a control.

Islet isolation. Islets of Langerhans were isolated by collagenase digestion of pancreases from male Wistar rats (weighing 180–200 g, Janvier, Le Genest-St-Ise, France) followed by Ficoll purification using a modification of the method of Sutton et al. (39). For cell preparation, the isolated islets were trypsinized and β-cells purified using a FACStar-Plus cell sorter (Becton-Dickinson) as previously described (35, 41).

Cell culture. Sorted β-cells were washed twice with sterile Dulbecco’s minimum essential medium (DMEM) (GIBCO, Invitrogen, Basel, Switzerland) containing 10% heat-inactivated FCS, 11.2 mM glucose, supplemented with 1 mM sodium pyruvate, 110 U/ml penicillin, 110 μg/ml streptomycin, and 50 μg/ml gentamicin (referred to hereafter as control medium), followed by centrifugation for 5 min at 180 g. Aliquots of 10⁵ cells were seeded in nonadherent 60-mm-diameter Petri dishes containing 3 ml of control medium. Cells were then incubated for 20 h at 37°C to allow full recovery of any cell surface molecules that might have been lost or damaged during islet isolation or cell purification. β-Cells were then centrifuged for 5 min at 180 g and resuspended at a density of 8 × 10⁵ cells/ml in control DMEM supplemented or not with a calpain inhibitor: ALLN (calpain inhibitor I, N-Ac-Leu-Leu-norleucinal; Calbiochem, Juro, Switzerland), calpeptin (Calbiochem), or EST (ethyl(-)-(2S,3S)-3-[(S)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxirane carboxylate, Calbiochem). Aliquots (50 μl) of this suspension were plated as droplets at the center of Petri dishes coated with 804G matrix or PLL and were incubated at 37°C. After 24 h, cells were analyzed for attachment and spreading under an inverted microscope. The calpain inhibitors were dissolved in DMSO, which was also added to control cultured cells at the same concentration. In addition, medium (~50 μl) was collected and centrifuged to remove any detached cells and debris and stored at −20°C for subsequent insulin measurement.

Actin staining. Actin was detected by staining with Alexa Fluor 546 phalloidin (Molecular Probes). After 24 h of incubation, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 4 min, and then stained with Alexa Fluor phalloidin. Images were collected using a Zeiss confocal laser-scanning microscope.

Insulin secretion assay and insulin content. After 24 h of incubation, cells were washed three times with a Krebs-Ringer bicarbonate-HEPES buffer (KRBH; 125 mM NaCl, 4.74 mM KCl, 1 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 25 mM HEPES, pH 7.4, 0.1% BSA) supplemented with 2.8 mM glucose and preincubated with this same buffer for 1 h at 37°C. Cells were then incubated for 1 h at 37°C with KRBH containing 2.8 mM glucose and successively for 1 h at 37°C with KRBH containing 16.7 mM glucose or with 2.8 mM glucose supplemented with 10 μM mastoparan or 20 mM KCl. The incubation buffer was recovered and insulin extracted from cells with acid-ethanol. The amounts of insulin in the incubation buffer and cellular extracts were measured by classical radioimmunoassay using the charcoal separation technique. Rat insulin was used as the standard with a guinea-pig anti-porcine insulin antibody and ¹²⁵I-labeled insulin as the tracer. Insulin content is expressed as the sum of insulin extracted at the end of the stimulation test and insulin secreted during the first and second incubation periods. Data are expressed as means ± SE.

Quantification of insulin mRNA by real-time PCR. Total RNA of β-cells in the culture dish was isolated using the QIAshredder and RNeasy Mini Kit (Qiagen), according to the instructions of the manufacturer, and stored at −80°C. The RNA quality was verified by agarose gel electrophoresis. First-strand cDNA was synthesized by oligo(dT) priming of 1 μg of total RNA using the Superscript II RT kit (Invitrogen) according to the manufacturer’s protocol. PCR amplification was performed with the iCycler-iQ system (Bio-Rad). Primers were designed according to the Primer Express software.

![Fig. 1. Effect of calpeptin on pancreatic β-cell spreading. β-Cells were plated on poly-L-lysine (PLL; A–C) or 804G matrix (D–I) and incubated for 24 h under basal condition (2.8 mM glucose and 5 mM KCl; A and D), 11.2 mM glucose and 5 mM KCl (B and E), or 2.8 mM glucose and 25 mM KCl (C and F). Addition of 35 μg/ml calpeptin inhibited spreading induced both by glucose (H) or KCl (I) and had no effect on morphology of cells incubated under basal condition (G). Bar, 10 μm.](image-url)
qPCR core Kit SYBR Green I (Eurogentech) supplemented with fluorescein (Bio-Rad) was used in the PCR reaction to allow for quantitative detection of the PCR product. Rat insulin primers used were 5'-H11032-CTGCCCAGGCTTTTGTCAA-3' and 5'-H11032-TCCACTTCACGACGGGACTT-3' as sense and antisense primers, respectively. The temperature profile of the reaction was 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. An internal housekeeping gene control, ribosomal RNA L3, was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. All results were expressed as the ratios of the copy number of insulin to that of the housekeeping gene L3.

Measurement of [Ca\(^{2+}\)]. Cells attached on glass coverslips for 24 h were loaded in KRBH containing 2.8 mM glucose for 30 min at room temperature in the presence of 1.5 μM fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR). The coverslips were washed, placed in a thermostatic chamber at 37°C, and incubated in KRBH containing first 2.8 mM glucose and then 16.7 mM glucose, and finally 16.7 mM glucose supplemented with 20 mM KCl. Cells were illuminated alternatively at 340 and 380 nm using an Axiovert S100TV microscope (Carl Zeiss, Feldbach, Switzerland). Fluorescence emission at 510 nm was captured every 5 s using a cooled, back-illuminated, 16-bit, charge-coupled device, frame transfer camera (Princeton, Roper Scientific, Trenton, NJ) controlled by Metamorph/Metafluor 4.1.2 software (Universal Imaging, West Chester, PA). By use of established procedures, changes in the emission intensity of fura 2, expressed as a ratio of dual excitation, were used as indicators of changes in [Ca\(^{2+}\)]. Three independent experiments were performed on each group, with 40 cells examined for each experiment.

Calpain activity assay. Intracellular calpain activity was measured using a commercial kit (Calpain Activity Assay Kit; Oncogene, Juro, Switzerland) according to the manufacturer’s instructions. This kit is a fluorometric assay using a calpain substrate, the fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC).

Calpain activity of individual β-cells was determined by incubating the cells with the calpain substrate BOC-Leu-Met-CMAC. This substrate readily permeates biological membranes and, once inside the cell, conjugates with glutathione to form BOC-Leu-Met-CMAC-SG (34). Calpain cleaves the conjugate between methionine and CMAC-SG, resulting in the fluorescent product CMAC-SG, which has an emission maximum at 410 nm when excited at 360 nm. Cells were plated on coated Petri dishes and incubated for 24 h in the presence or absence of calpeptin. The cells were then incubated for 20 min in the presence of 50 μM BOC-Leu-Met-CMAC (Molecular Probes). The...
RESULTS

\(\beta\)-Cell spreading on 804G is inhibited by calpain inhibitors. Calpeptin, a calpain inhibitor, was used to define the role of calpain in the adhesion and spreading of pancreatic \(\beta\)-cells on the 804G matrix. \(\beta\)-Cells rapidly spread out (2–3 h), developing lamellipodia and continuously expressed this phenotype when attached on 804G matrix and incubated in the presence of 11.2 mM glucose (Figs. 1E and 2A) or 25 mM KCl (Fig. 1F), conditions known to increase \([Ca^{2+}]_i\). Under these conditions, addition of calpeptin largely inhibited cell spreading but did not affect cell attachment (Figs. 1, H and I, and 2, B–D). This effect was observed after 2–3 h (not shown), 24 h (Figs. 1, G–I, and 2, B–D), or 48 h (Fig. 2F) of treatment and was dose dependent (Fig. 2, B–D). \(\beta\)-Cell spreading was recovered within 24 h after the calpain inhibitor was removed from the medium, indicating that this inhibition of spreading is reversible (Fig. 2G).

Spreading did not occur when \(\beta\)-cells were cultured on the 804G matrix at low glucose (2.8 mM; Fig. 1D) or when attached under any condition on PLL (Fig. 1, A–C). Under these conditions, treatment with calpeptin had no effect on \(\beta\)-cell adhesion or morphology (Fig. 1G). The organization of actin filaments in round and spread cells was examined by staining with fluorescent phalloidin. Spread cells contained many stress fibers and microfilaments that were concentrated in lamellipodia (Fig. 3, C and D). In the presence of calpeptin, the organization of actin filaments was modified so that stress fibers were absent (Fig. 3, E and F). Round \(\beta\)-cells cultured on the 804G matrix at low glucose show the same actin organization as cells treated with calpeptin (Fig. 3, A and B). Similar results were observed when the calpain inhibitors ALLN or EST were used instead of calpeptin (data not shown).

To establish the efficacy of calpeptin as an inhibitor of intracellular calpain in \(\beta\)-cells, we evaluated its ability to inhibit calpain cleavage of the calpain substrate Suc-LLVY-AMC. Under control conditions, calpain activity was detected in \(\beta\)-cells; after addition of calpeptin, a significant decrease of calpain activity was observed (41.2 ± 12.7% of control in calpeptin-treated \(\beta\)-cells, \(P < 0.01\)). The intracellular calpain activity in individual \(\beta\)-cell was determined by using the calpain substrate BOC-Leu-Met-CMAC. Heterogeneous labeling with strongly and weakly fluorescent cells was observed (Fig. 4A). However, in \(\beta\)-cells treated with calpeptin, only...
occasional, weak labeling could be observed (Fig. 4B). These results demonstrate the efficacy of calpeptin to inhibit calpain in intact β-cells.

**Insulin secretion is inhibited by chronic exposure of β-cells to calpain inhibitors.** To study the role of calpain on β-cell function, β-cells were plated on PLL or 804G matrix and preincubated for 24 h with calpeptin, and short-term insulin secretion was then measured after 1-h of incubation at 2.8 mM glucose and 1 h at 16.7 mM glucose. Compared with control, calpeptin dose-dependently inhibited the insulin secretory response to 16.7 mM glucose of β-cells attached on either 804G matrix or PLL (Fig. 5A). The inhibitory effect of calpeptin on insulin secretion thus seems to be independent of the substrate on which the cells are plated. Significant effects on insulin secretion were also observed when ALNN or EST were used (Fig. 5, C and D).

As shown in Table 1, insulin content was significantly increased in β-cells treated with 35 μg/ml calpeptin compared with control. However, there were no significant differences in insulin mRNA levels. Insulin secreted in the medium during the 24-h incubation was significantly reduced in calpeptin-treated cells, suggesting a blockage of the exocytosis step.

To test whether the inhibitory effect of calpeptin on insulin secretion could be reversed, β-cells that had been exposed to 35 μg/ml calpeptin for 24 h were cultured for another 24 h in control medium. Removal of the inhibitor from the cells for 24 h restored insulin secretory responses to levels not different from control (Fig. 5B), indicating that calpain inhibitors do not cause β-cell death or irreversible nonspecific toxic effects. By contrast, exposing cells to calpeptin continuously for 48 h did inhibit the insulin secretory response to 16.7 mM glucose ($P = 0.038$).

To further determine the specificity of calpeptin on Ca$^{2+}$-induced insulin secretion, β-cells were stimulated with 25 mM KCl or 10 μM mastoparan instead of 16.7 mM glucose. As expected, depolarization of the β-cells by KCl stimulated insulin secretion, and the effect was higher in β-cells attached on the 804G matrix compared with PLL. Calpeptin treatment inhibited the effect of KCl on insulin secretion in β-cells plated on either PLL or 804G matrix (Fig. 6B). Mastoparan, known to activate exocytosis downstream of [Ca$^{2+}$]i elevation, strongly increased insulin secretion in β-cells attached on PLL and to a lesser extent in β-cells attached on the 804G matrix (Fig. 6C). Interestingly, calpeptin treatment did not affect mastoparan-induced insulin secretion in β-cells attached on PLL or 804G matrix (Fig. 6C). These results suggest that calpain acts upstream of the exocytotic process but downstream of the rise in intracellular calcium.

**Table 1. Effect of calpeptin on insulin mRNA expression, insulin contents, and insulin secretion after 24 h of culture**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin mRNA, insulin/insulin mRNA ratio</th>
<th>Insulin Content, ng/20,000 cells</th>
<th>Secreted Insulin Content, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>1,863.17 ± 177.24</td>
<td>439.24 ± 18.29</td>
<td>1,408.37 ± 190.79</td>
</tr>
<tr>
<td>PLL + CALP</td>
<td>1,985.43 ± 233.16</td>
<td>1,036.13 ± 35.36†</td>
<td>76.01 ± 22.61*</td>
</tr>
<tr>
<td>Mx</td>
<td>1,949.18 ± 116.75</td>
<td>289.14 ± 32.51†</td>
<td>1,884.87 ± 395.7</td>
</tr>
<tr>
<td>Mx + CALP</td>
<td>1,724.74 ± 424.44</td>
<td>978.96 ± 65.87†</td>
<td>123.57 ± 46.97*</td>
</tr>
</tbody>
</table>

Data are means ± SE of 3 independent experiments; PLL, poly-L-lysine; Mx, 804G matrix; CALP, calpeptin. *P < 0.01 vs. its respective control; †P < 0.001 vs. its respective control.

**Fig. 6.** Effect of calpeptin on the insulin secretory response to glucose, KCl, and mastoparan. β-Cells were attached on either PLL or 804G matrix (Mx) and incubated for 24 h with 35 μg/ml calpeptin; insulin secretion in response to 1 h with 16.7 mM glucose (A), 25 mM KCl (B), or 10 μM mastoparan (C) was then measured. Results are means ± SE of 4–7 independent sets of experiments.

**Short exposure of β-cells to calpeptin inhibited insulin secretory responses to KCl and glucose.** To test the effect of a short-term exposure of β-cells to calpeptin on insulin secretion, we added the calpain inhibitor to the buffer during the insulin secretion assay. Calpeptin significantly inhibited the insulin
secretory response to 16.7 mM glucose or 25 mM KCl (Fig. 7), whereas no significant effect was observed on basal insulin secretion (2.8 mM glucose). The inhibition of insulin secretion was observed on cells plated on both the 804G matrix and PLL. Insulin content was similar in control and in calpeptin-treated cells (data not shown).

$[\text{Ca}^{2+}]_{i}$ is not directly affected by calpeptin in $\beta$-cells. To exclude the idea that the deficient insulin secretion and the inhibition of spreading in calpeptin-treated cells are associated with an alteration in intracellular $[\text{Ca}^{2+}]_{i}$ signaling, we measured $[\text{Ca}^{2+}]_{i}$ responses to KCl and glucose in absence or presence of calpeptin. $\beta$-Cells plated on the 804G matrix showed a rise in $[\text{Ca}^{2+}]_{i}$ in response to KCl similar to that of $\beta$-cells plated on PLL (Fig. 8, A and B). The magnitude of the $[\text{Ca}^{2+}]_{i}$ response to KCl was not reduced in calpeptin-treated cells plated on 804G matrix or on PLL. Similarly, exposure to calpeptin did not reduce the number of cells that showed a rise in $[\text{Ca}^{2+}]_{i}$ in response to KCl (Table 2). When stimulated with glucose, calpeptin-treated cells still showed an increase of $[\text{Ca}^{2+}]_{i}$, even if the magnitude of $[\text{Ca}^{2+}]_{i}$ response (Fig. 8, C and D) and the number of cells showing a rise in $[\text{Ca}^{2+}]_{i}$ (Table 2) were lower compared with control. This effect of calpeptin on glucose-induced $[\text{Ca}^{2+}]_{i}$ elevation suggests that calpeptin may be involved in some pathway coupling glucose to $[\text{Ca}^{2+}]_{i}$ elevation.

**DISCUSSION**

In the present study, we investigated the role of calpain in the process of $\text{Ca}^{2+}$-induced insulin secretion and spreading of primary rat $\beta$-cells. Our data provide evidence that calpain is necessary for glucose- and KCl-induced insulin secretion and...
spreading of rat β-cells. Indeed, three distinct, synthetic cell-penetrating calpain inhibitors, calpeptin, ALLN, and EST, block both glucose- and KCl-induced insulin secretion and cell spreading in a dose-dependent manner. The calpain peptidyl inhibitors used in these studies, EST, ALLN, and calpeptin, were chosen on the basis of their specificities. All of these inhibitors inhibit only cysteine proteases. Calpeptin is a hydrophobic cell-permeable peptidyl aldehyde, containing a benzoxycarbonyl residue at the NH2 terminus. The mechanism of action probably involves a covalent interaction between the active-site cysteine of calpain and an electrophilic center of the inhibitor (31). A number of our results point strongly to an inhibition of calpain as the mechanism underlying the observed effects. First, different calpain inhibitors (calpeptin, ALLN, and EST) that work by different mechanisms have similar effects on Ca2\(^{2+}\)-induced spreading and insulin secretion. Second, hydrolysis of the calpain-specific substrate Suc-LLVY-AMC was inhibited in calpeptin-treated cells. The possibility that nonspecific toxic effects were involved in the impairment of insulin secretion in β-cells exposed to calpain inhibitors for 24 h was also considered. We believe that this possibility can be excluded for the following reasons. First, removal of the calpain inhibitors from the medium resulted in the recovery of the insulin secretory response to glucose, making it unlikely that decreased β-cell viability is responsible for the defects in insulin secretion. Second, inhibitor-treated cells had normal Ca2\(^{2+}\) responses to the direct depolarization induced by 25 mM KCl. Whether inhibitors used in this study act exclusively on calpain activity was not studied. For example, calpeptin has been shown to inhibit cathepsin B (36). Consequently, we cannot exclude the possibility that other proteases may also contribute in part to the process.

Cleavage of cytoskeletal/membrane attachments is the most thoroughly documented function of the calpains. Work on platelets has shown that the cysteine protease calpain is one of the signaling molecules activated following integrin-ligand interactions (37), suggesting that it may modulate intracellular events required for effective cell adhesion and spreading. The present work suggests that calpain is involved in the spreading of β-cells on the 804G matrix. A role of calpain in cell spreading has been described previously in other cell types. Studies in which calpain was inhibited with membrane-permeable inhibitors or by an overexpression of the endogenous inhibitor calpastatin all showed inhibition of cell spreading and motility (7, 8, 21, 30, 33). A role of calpain in inside-out signaling, through integrin, has been described previously. Calpain proteolytically modifies proteins associated with the integrin-mediated focal adhesion formation, such as focal adhesion kinase (4). The β1-integrin cytoplasmic domain is also a target of calpain (29). Interestingly, this integrin is expressed by β-cells and has been shown to be involved in β-cell spreading and insulin secretion (3). Furthermore, it has been demonstrated that calpain associates with β1-integrin-containing membrane lipid rafts (14). This would represent an ideal target for calpain to modulate cell morphology subsequent to signaling from the extracellular matrix.

Calpains also play a role in signal transduction pathways by coupling calcium influxes to important structural alterations of membrane proteins associated with fusion and secretory events. Thus calpain activation has been implicated in protein secretion of neuroblastoma cells (5), rat pancreatic acinar cells (42), and alveolar epithelial cells (44). As in other studies (23, 27, 38, 43), our results show an involvement of calpain in insulin secretion. Some works (23, 43), including ours, show that chronic exposure to calpain inhibitors decreases insulin secretion. However, the effect of an acute exposure to calpain inhibitors on insulin secretion is still controversial. Some studies demonstrated enhancement of glucose-induced insulin secretion in islets exposed for 4 h to calpain inhibitors (38, 43), whereas other studies (27), including ours, showed that calpain inhibitors decreased insulin secretion after an acute treatment. The reason for these differences is still unknown.

Calpain inhibitors suppressed glucose- and KCl-stimulated insulin secretion without affecting mastoparan-induced insulin secretion. Mastoparan, a tetradecapeptide from wasp venom, is thought to stimulate insulin release by activating small G proteins of the Rho family, such as Cdc42 and Rac (1, 20). Our observation that calpeptin did not affect mastoparan-induced insulin secretion is consistent with the hypothesis that calpain acts at a point upstream of Rho family members (21, 22).

Calpeptin did not affect the KCl-induced [Ca2\(^{2+}\)]\(_{i}\) increase of β-cells; however, calpeptin did decrease the Ca2\(^{2+}\) response of β-cells stimulated with glucose. Zhou et al. (43) demonstrated that calpain inhibition induced a decrease in glucose metabolism that may account for the calpeptin effect on the glucose-induced Ca2\(^{2+}\) increase in INS-1 cells (23). Furthermore, it has been demonstrated that calpain-10 is a regulator of exocytosis in INS-1 cells (23). Therefore, inhibition of calpain could have effects both on the coupling of glucose metabolism to calcium mobilization and on the exocytotic events induced by calcium.

Calpains may be implicated in different events occurring at different time points. This is the case for platelet secretion and

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**Table 2. Effect of calpeptin on percentage of β-cells showing a [Ca2\(^{2+}\)], response to glucose and KCl**

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>KCl</th>
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<tbody>
<tr>
<td>PLL</td>
<td>96.4±5.0</td>
<td>100±0</td>
</tr>
<tr>
<td>PLL + CALP</td>
<td>56.5±25.1*</td>
<td>99±2</td>
</tr>
<tr>
<td>Mx</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>Mx + CALP</td>
<td>69±13.1*</td>
<td>98±2.7</td>
</tr>
</tbody>
</table>

Data are averages ± SE of 3 independent experiments. [Ca2\(^{2+}\)], intracellular Ca2\(^{2+}\) concentration. *P < 0.01 vs. its respective control.
spreading: calpain activity was implicated in Ca^{2+}-associated secretion events occurring during the first 30 s of platelet activation by the thrombin receptor agonist peptide, and calpain activity also regulated later platelet activation events involving integrin function as well as actin remodeling (6). A rise in [Ca^{2+}]], may not be a consequence of spreading in response to glucose, because a rise in [Ca^{2+}]]; in response to glucose has been observed in β-cells in suspension (13), and it has been routinely observed in β-cells attached to plastic or glass, where they do not spread. A rise in [Ca^{2+}]], was also observed in calpeptin-treated β-cells, suggesting that calpains may play an important, early role in insulin secretion and regulates later events associated with spreading. In addition, insulin secretion and spreading of β-cells, responses inhibited by calpeptin, could each be regulated by different types of calpains.

In conclusion, our results suggest a role of calpains in the regulation of spreading and insulin secretion of primary pancreatic β-cells. The effect of calpains on insulin secretion seems independent of β-cell spreading, since insulin secretion of nonspread β-cells attached on PLL was also inhibited by calpeptin. Because many steps could be modulated by calpains, further experiments are needed to clarify the molecular and physiological mechanisms associating calpains with insulin secretion and β-cell spreading.

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