Calpains Play a Role in Insulin Secretion and Action

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Studies of the genetic basis of type 2 diabetes suggest that variation in the calpain-10 gene affects susceptibility to this common disorder, raising the possibility that calpain-sensitive pathways may play a role in regulating insulin secretion and/or action. Calpains are ubiquitously expressed cysteine proteases that are thought to regulate a variety of normal cellular functions. Here, we report that short-term (4-h) exposure to the cell-permeable calpain inhibitors calpain inhibitor II and E-64-d increases the insulin secretory response to glucose in mouse pancreatic islets. This dose-dependent effect is observed at glucose concentrations above 8 mmol/l. This effect was also seen with other calpain inhibitors with different mechanisms of action but not with cathepsin inhibitors or other protease inhibitors. Enhancement of insulin secretion with short-term exposure to calpain inhibitors is not mediated by increased responses in intracellular Ca(2+) or increased glucose metabolism in islets but by accelerated exocytosis of insulin granules. In muscle strips and adipocytes, exposure to both calpain inhibitor II and E-64-d reduced insulin-mediated glucose transport. Incorporation of glucose into glyogen in muscle was also reduced. These results are consistent with a role for calpains in the regulation of insulin secretion and insulin action. Diabetes 50:2013–2020, 2001

Type 2 diabetes is a complex metabolic disorder characterized by defects in both insulin secretion and insulin action (1). A combination of genetic predisposition and environmental factors influences its development. Recent studies have shown that variation in the gene encoding calpain-10 is associated with type 2 diabetes (2). In addition, the diabetes-associated DNA polymorphism in the calpain-10 gene is associated with decreased levels of calpain-10 mRNA in skeletal muscle and insulin resistance in nondiabetic subjects (3), suggesting one mechanism by which calpain-10 may increase susceptibility to type 2 diabetes.

Calpains, or calcium-activated neutral proteases, are a family of nonlysosomal cysteine proteases that catalyze the endoproteolytic cleavage of specific substrates and thereby regulate pathways that affect intracellular signaling, proliferation, and differentiation (4,5). As a first step in determining the biochemical pathways that may be controlled by calpain-10 and other calpains, we examined the effects of cell-permeable calpain inhibitors on insulin secretion and insulin action in isolated islets, muscle strips, and adipocytes.

RESEARCH DESIGN AND METHODS

Animals. Pancreatic islets were isolated from fasted 9- to 13-week-old C57BL/6J mice (Jackson, Bar Harbor, ME). Soleus muscle strips and adipocytes were isolated from 8- to 12-week-old male Harlan Sprague-Dawley rats (Indianapolis, IN). These studies were approved by the Animal Care and Use Committee of the University of Chicago.

Static incubation of isolated pancreatic islets. Isolation of mouse pancreatic islets was accomplished as described previously (6). After overnight incubation in RPMI-1640 medium (11.6 mmol/l glucose), islets were exposed to inhibitors in the same medium for 4 h at 37°C. The protease inhibitors used were ALLN (calpain inhibitor II, N-ac-Leu-Leu-methioninal, ALN (calpain inhibitor I, N-ac-Leu-Leu-norleucinal), cathepsin B inhibitor II, (Calbiochem-Novabiochem, San Diego, CA), E-64-d (ethyl ((+)(2S)-(S)-(3)-methyl-2-carboxamido)-butylcarbonyl]-2-oxiranecarboxylate, Matreya, Pleasant Gap, PA), PD147631 (a gift from Dr. Kevin Wang, Parke-Davis, Ann Arbor, MI), and CA074ME (Peptides International, Louisville, KY). GLP-1 (7–36 amide) was from Peninsula Laboratory (Belmont, CA). Other reagents were from Sigma Chemical (St. Louis, MO). The calpain inhibitors were dissolved in DMSO, which was also used to control cultured islets at the concentration of 0.1%. Islets were preincubated in Krebs-Ringer's buffer (KRB) containing 2 mmol/l glucose and similar concentrations of inhibitors for 60 min at 37°C. Triplicate groups of five islets then were incubated in borsolic tubes containing 1 mol of KRB with the same concentration of inhibitor and various insulin secretagogues for 1 h in a moving water bath at 37°C. Insulin concentrations were measured in aliquots of the incubation buffer by radioimmunoassay (7).

Insulin secretion from perfused islets. Insulin secretion from perfused islets was measured using a modification of a previously described protocol (6,7).

Measurement of islet [Ca(2+)]i, NADPH, and calpain activity in isolated islets. [Ca(2+)]i, and NADPH responses to glucose or other secretagogues and calpain activity were measured in islets plated on glass coverslips and cultured in RPMI-1640 for 2–4 days to allow islets to adhere to the coverslips before the 4-h treatment with calpain inhibitors. We measured [Ca(2+)]i responses to glucose and other secretagogues using fura-2 as described (6). Intracellular NADPH responses to glucose were monitored by the changes in the autofluorescence at 460 nm (8). For measurements of calpain activity, islets were loaded with the fluorescent, membrane-permeant calpain substrate 1-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (Boc-Leu-
Met-UMAC [10 μM]; Molecular Probes, Eugene, OR) (9) in HEPES-buffered (10 mM) KRB with 2 mM glucose. The fluorescence emitted from the protein contained in the islets was measured with a combination of 480 and 520 nm excitation by light at 530 nm. Experiments were performed after a 4-h incubation in the presence of 200 μM ALLM, 200 μM E64-d, or vehicle.

During these measurements, islets on coverslips were perfused continuously with oxygenated KRB medium containing the inhibitors at 37°C. A computer-driven imaging system (MetaFluor 4.0; Universal Imaging, West Chester, PA) was used to capture and store images after ultraviolet excitation.

**Glucose and oxygen utilization and oxidation rates**. Glucose utilization and oxidation rates were measured (8,10) in mouse islets cultured in the presence or absence of calpain inhibitors for 4 h as described above. **Calpain current recording in dispersed pancreatic β-cells**. Isolated islets were dispersed into single β-cells by gentle trituration in Ca²⁺ – and Mg²⁺-free phosphate-buffered saline containing 0.25% trypsin-0.1 mM EDTA. Cells were plated on glass coverslips and maintained in culture in RPMI-1640 containing 11.6 mM glucose for 48–96 h before being treated with inhibitors. Calpain current measurements were obtained in the whole-cell patch-clamp configuration. Calpain currents were activated by step depolarizations to either +10 or +20 mV for either 20 or 100 ms, from a holding potential (HP) of −80 mV. All current records were corrected for leak and capacitance. The data were filtered at 2 kHz and then sampled every 100 μs. Pipette resistances were 1.5–2.5 MΩ. Series resistance was partially compensated with the P/6 compensation protocol of the Axopatch-1C amplifier.

Cells were incubated for 4 h in RPMI-1640 at 37°C in either 0.1% DMSO (control) or 100 μM ALLM and then transferred for an additional 1–2 h to KRB containing similar concentrations of DMSO or ALLM. For recording, cells were bathed in a solution containing (in mM): 145 NaCl, 2 KCl, 1 MgCl₂, 2 glucose, 10 HEPES, 10 CaCl₂ (pH 7.3; adjusted with NaOH), and either DMSO or ALLM. After the whole-cell configuration was established, the bath solution was exchanged for a tetrachloroquinuclidinium (TEA-) -based recording medium that contained (in mM): 5 NaCl, 100 CaCl₂, 2 glucose, 10 HEPES, 10 TEA-Cl, 1 and 100 μM TTX (pH 7.3; adjusted with TEA-OH), and either DMSO or ALLM. The intracellular pipette solution consisted of (in mM): 110 CsCl, 4 MgCl₂, 20 HEPES, 10 EGTA, 0.35 guanosine triphosphate, 4 ATP, and 14 creatine phosphate (pH 7.3; adjusted with CsOH).

**Membrane capacitance measurements**. Membrane capacitance changes in dispersed pancreatic β-cells were used to assess insulin exocytosis and were carried out in the perforated whole-cell configuration. For these experiments, the phase-tracking technique (11) was used, whereby a 60-nS peak-to-peak sine wave was superimposed on a holding potential of −80 mV (12), as described previously (13). β-cells were stimulated with a train of 10-step depolarizations to +20 mV (HP = −80 mV). Each step depolarization lasted 150 ms and separated by a 400-ms interstep duration. The data were collected at a 500-μs sampling rate and filtered at 5 kHz. Recordings with series resistances of >20 MΩ were discarded. Series resistance compensation was applied in all recordings.

The extracellular solution for the capacitance measurements contained (in mM): 130 NaCl, 3 glucose, 10 Na-Hepes, 1 M KCl, 2 CaCl₂, and 5 CaCl₂ (pH 7.3) with NaOH. The pipette solution contained (in mM): 135 Cs-glutamate, 10 NaCl, 0.5 CaCl₂, 5 MgCl₂, 5 NaAc, 0.5 MgCl₂ (pH 7.3) with CsOH. Pipettes were backfilled with an identical solution to that of amphotericin B (final concentration of 0.5 mg/ml) was added and then sonicated. The amphotericin B stock solution (125 mg/ml) was kept frozen at −20°C and used for 1 week. ALLM pretreatment was as described above. All electrophysiological recordings were carried out at room temperature (22–24°C).

**Glycogen synthesis rates in skeletal muscle**. Measurement of glycogen synthesis rates was performed using a modification of a previously described protocol (14). Soleus muscle strips were isolated from nonfasted male rats and incubated in KRB-5 mM NaF-10 mM HEPES, 0.2% BSA in the presence and absence of 100 μM ALLM, 200 μM E64-d, or vehicle.

**2-Deoxyglucose uptake into skeletal muscle and adipose tissue**. 2-deoxy-glucose transport in muscle and adipose tissue was measured using a modification of a previously described protocol (14). After a 30-min preincubation in KRB containing no glucose, 2 mM pyruvate, 10 mM Na-Hepes, 0.2% BSA, and 100 μM ALLM or 200 μM E64-d, the muscle strips were transferred to identical medium containing 0.1 mM 2-deoxy-[2-3H]glucose (6.5 μCi/ml) and 0.1 mM [14C]glucose (0.2 μCi/ml) and incubated for 30 min at 37°C. The uptake of 2-deoxy-[3H]glucose was calculated as described previously (14).

Adipocytes were isolated from epididymal fat pads (15). For the measurement of basal and insulin-stimulated transport of glucose, aliquots of 200 μl of adipocytes (2 × 10⁶ cells/ml) were incubated for 120 min at 37°C in KRB with different concentrations of insulin either with or without 100 μM ALLM or E64-d. Then, another 50 μl of KRB containing 5 mM 2,4-DNP (final concentration 1 mM), 0.5 μM of 2-deoxy-[2-3H]glucose was added and cells were incubated for an additional 5 min at 37°C. The transport was stopped by addition of cytochalasin B (final concentration 50 μM) and insulin strips run through 250 μl of dioxyn phenylphthalein oil (Fisher Scientific, Pittsburgh, PA). Cells were then transferred to scintillation vials for counting.

**Measurement of 3-O-methyl-D-glucose uptake**. Epithelial cells were isolated from overnight fasted rats and were incubated for 1 h at 37°C in 3 ml of oxygenated Krebs-Henseleit buffer (KRB) supplemented with 5 mM glucose, 15 mM mannitol, 0.1% BSA, with or without 100 μM ALLM. Epithelial cells were then incubated in identical media with or without 15 mM of insulin or were transferred to KRB medium gassed with 5% CO₂, 25% N₂, 5% CO₂ for 30 min. Muscle glucose transport activity was measured using minor modifications of previously described methods (16). After the initial incubations, all cells were washed for 15 min at 37°C in 3 ml of KRB containing 20 mM mannitol and 0.1% BSA to remove glucose from the extracellular space. Muscles then were transferred to 1.5 ml of KRB containing 0.3 mM 3-O-methyl-D-glucose (3-MG, 2 μM/ml), 12 mM [14C]mannitol (0.2 μCi/ml), and 0.1% BSA for 30 min. When present during the previous incubations, insulin and ALLM also were added to the media for the wash step and transport assay. Extracellular space and intracellular 3-MG concentrations (μmol · ml intracellular water⁻¹) were determined as described previously (16).

**Statistical analyses**. Data are expressed as mean ± SE. The statistical significance of differences was assessed at the 5% level. Differences between two groups were tested using the nonpaired Student's t test when the data were normally distributed or the Wilcoxon's rank-sum test when the data were not normally distributed. A paired t test was used when differences between responses in two aliquots of islets from the same mouse injected under different conditions were being tested. Analysis of variance (ANOVA) was used to test differences among more than two groups.

**RESULTS**

A 4-h exposure to calpain inhibitors stimulates insulin secretion. Exposure to calpain inhibitor II (ALLM, 250 μM) and E64-d (200 μM) enhanced the insulin secretory responses to 20 mM glucose in islets 1.97 ± 0.3-fold (n = 5, P < 0.01, paired t test) and 1.77 ± 0.1-fold (n = 6, P < 0.001), compared with islets exposed to 20 mM glucose without the inhibitors. No effect was observed on basal insulin secretion (at 2 mM glucose). Significant effects of ALLM and E64-d on the insulin secretory response to 20 mM glucose were seen at inhibitor concentrations >100 μM (Fig. 1A and B, Table 1). Pretreatment of islets with calpain inhibitor I (ALLM, 100 μM) resulted in a 2.7-fold increase and with the nonpeptide α-mercaptoacryl acid derivative PD 147631 (a relatively specific nonpeptidic inhibitor of calpain with an IC₅₀ value for calpain that is >100 times less than that for cathepsin B and other cysteine proteases; K. Wang, personal communication) resulted in a 1.8-fold increase in the response to 20 mM glucose compared with control experiments performed in the absence of inhibitors (Table 1).

Enhancement of the insulin secretory response to glucose by ALLM was observed only at glucose concentrations >8 mM (Fig. 1C) and also was observed in a dynamic islet perfusion system (Fig. 1D). Islet insulin content was unchanged after a 4-h exposure to 100 μM ALLM in the presence of either 2 or 20 mM glucose: 30.9 ± 1.6 vs. 31.7 ± 3.0 ng/islet in ALLM and control, respectively; 20 mM glucose: 28.3 ± 4.7 vs. 32.4 ± 2.0 ng/islet in ALLM- and control-treated islets, respectively.

ALLM produced a 1.55 ± 0.2-fold (n = 6, P < 0.05, paired t test) increase in the insulin secretory response to 50 mM GLP-1, an agent that promotes insulin secretion through its effects on adenyl cyclase and resulting increase in cAMP (17). ALLM did not, however, significantly increase the insulin secretory responses to 30 mM KCN.

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an agent that directly depolarizes the β-cell or 100 μmol/l carbachol that mobilizes Ca^{2+} from intracellular stores (18) (data not shown).

We also examined the effects of other protease inhibitors on insulin secretion. Insulin secretory responses to 20 mmol/l glucose were not altered in the presence of pepstatin A (100 μmol/l), an aspartyl protease inhibitor, or cathepsin B inhibitor II (100 μmol/l), a lysosomal cysteine protease inhibitor, indicating that the stimulatory effects of ALLM and E-64-d on insulin secretion are specific to these protease inhibitors (Table 1).

**A 4-h exposure to calpain inhibitors does not affect glucose utilization or NADPH levels.** Exposure of islets to calpain inhibitors for 4 h did not have any significant effects on glucose utilization and oxidation rates and islet NADPH measurements. Rates of glucose utilization at basal (2 mmol/l glucose) and stimulatory glucose concen-

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**FIG. 1.** Effect of protease inhibitors on the insulin secretory response to glucose in mouse pancreatic islets. A and B: Effect of increasing concentrations (μmol/l) of E-64-d (A) and ALLM (B) on the insulin secretory response to 2 mmol/l glucose (□) and 20 mmol/l glucose (●). Results are mean ± SE of five to six experiments in each group. *P < 0.05 compared with islets incubated in the absence of calpain inhibitors. C: Insulin secretion by islets incubated at various glucose concentrations in the absence (□) and presence (●) of 100 μmol/l ALLM. Results are mean ± SE of four to seven experiments per group. *P < 0.05 compared with islets incubated in the absence of ALLM (using paired t test comparing data from islets incubated in the presence of ALLM with those incubated in the absence of the inhibitor at each glucose concentration). D: Insulin secretion by perfused islets in response to stimulation with 20 mmol/l glucose. The perfusate contained 2 mmol/l glucose except where shown. Islets were preincubated for 4 h. In experiments involving inhibitors, ALLM (100 μmol/l) was present throughout the experiment, but E-64-d (200 μmol/l), which is an irreversible calpain inhibitor, was present only during the preincubation. Results are mean ± SE of four to five experiments in each group. The AUC of insulin secretion was compared between groups using ANOVA and post hoc Duncan test. Responses were 2.4-fold greater in ALLM-treated islets compared with control (P < 0.05) and, although the AUC was 2-fold higher in E-64-d-treated islets, this difference was not statistically significant.

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**TABLE 1**

Effect of protease inhibitors on insulin secretory response of isolated mouse islets to 20 mmol/l glucose

<table>
<thead>
<tr>
<th>Inhibitor (concentration)</th>
<th>Control (ng insulin · islet⁻¹)</th>
<th>Protease inhibitor-treated (ng insulin · h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-64-d (100 μmol/l)</td>
<td>1.59 ± 0.21</td>
<td>2.30 ± 0.20*</td>
</tr>
<tr>
<td>ALLM (100 μmol/l)</td>
<td>1.41 ± 0.28</td>
<td>2.29 ± 0.16*</td>
</tr>
<tr>
<td>ALL (100 μmol/l)</td>
<td>1.10 ± 0.11</td>
<td>3.00 ± 0.30*</td>
</tr>
<tr>
<td>PD 17681 (100 μmol/l)</td>
<td>1.74 ± 0.11</td>
<td>3.04 ± 0.28*</td>
</tr>
<tr>
<td>Cathepsin B inhibitor II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(100 μmol/l)</td>
<td>1.36 ± 0.34</td>
<td>1.11 ± 0.46</td>
</tr>
<tr>
<td>Pepstatin A (100 μmol/l)</td>
<td>1.20 ± 0.13</td>
<td>1.30 ± 0.14</td>
</tr>
</tbody>
</table>

Data are mean ± SE of n = 3–6 experiments. The amount of insulin secreted during the course of a 1-h static incubation in KRB containing 20 mmol/l glucose is shown. *P < 0.05.
trations (20 mmol/l) in the presence of 100 μmol/l ALLM (14.5 ± 3.8 and 89.5 ± 3.0 pmol·islet⁻¹·h⁻¹, respectively) or 200 μmol/l E-64-d (15.5 ± 4 and 79.5 ± 9.5 pmol·islet⁻¹·h⁻¹, respectively) were not significantly different from those in islets incubated in their absence (14.5 ± 2.1 and 76.5 ± 6.5 pmol·islet⁻¹·h⁻¹; n = 3 in each case). Similarly, there was no significant difference in the glucose oxidation rates at basal or stimulatory glucose concentrations in the presence of ALLM (6.0 ± 0.7 and 39.5 ± 4.1 pmol·islet⁻¹·h⁻¹) and E-64-d (5.2 ± 0.4 and 40.0 ± 2.4 pmol·islet⁻¹·h⁻¹) compared with those measured in the absence of inhibitor (4.4 ± 0.8 and 32.5 ± 6.5 pmol·islet⁻¹·h⁻¹; n = 3 in each case). Consistent with a lack of effect of ALLM and E-64-d on β-cell glucose metabolism, the NADPH response to an increase in the glucose concentration from 2 to 14 mmol/l in the presence of 100 μmol/l ALLM (2.7 ± 0.4-fold increase; n = 4) and E-64-d (2.8 ± 0.3-fold increase; n = 2) was not significantly different from controls (2.6 ± 0.2-fold increase; n = 4).

A 4-h exposure to calpain inhibitors does not increase [Ca²⁺], or calcium current density. The stimulatory effects of ALLM and E-64-d on insulin secretion in response to high glucose were not associated with increases in [Ca²⁺] (Fig. 2A and B). Calcium currents were similar in control and ALLM-treated cells (Fig. 2C); no differences in amplitude or kinetics were apparent. In addition, no shifts in voltage dependence were observed (data not shown), and the average peak calcium current density obtained in control and ALLM-pretreated cells was comparable (Fig. 2D).

A 4-h exposure to calpain inhibitors affects β-cell membrane capacitance. Membrane capacitance measurements confirmed the large enhancement of insulin secretion observed after ALLM pretreatment (Fig. 2E and F). Representative capacitance changes, from control (top) and ALLM-pretreated cells (bottom) are shown in Fig. 2E. Stimulation induced much larger average changes in peak membrane capacitance in ALLM-pretreated cells in comparison with control cells (Fig. 2F).

A 4-h exposure to calpain inhibitors is associated with a reduction in calpain activity in pancreatic islets. To document that ALLM and E-64-d were inhibiting...
Calpains rather than other cysteine proteases, we measured calpain activity in isolated islets using the fluorogenic calpain-specific substrate Boc-Leu-Met-CMAC (Fig. 3) (9). Although this compound does not distinguish between different calpain isozymes, it does seem to be specific for calpains and not for other lysosomal proteases under physiological conditions (9). In islets incubated in the presence of 200 μmol/l ALLM or 200 μmol/l E-64-d, the rate of generation of the fluorescent signal was lower than in islets incubated in the absence of the calpain inhibitors (Fig. 3A). The area under the curve (AUC), which is a measure of the rate of generation of the fluorescent product, was reduced to 35 ± 4% (n = 3, P < 0.01) and 45 ± 5% (n = 4, P < 0.03, using ANOVA and post hoc Duncan test) of control values in the presence of ALLM (200 μmol/l) and of E-64-d (200 μmol/l), respectively (Fig. 3B).

**Calpain inhibitors affect insulin action in skeletal muscle and adipocytes.** We measured insulin-stimulated 2-DG uptake in adipocytes and muscle strips in the presence of 100 μmol/l ALLM and 200 μmol/l E-64-d. In adipocytes, insulin (12 nmol/l) increased uptake of 2-DG from 457 ± 59 pmol · 2 × 10^5 cells^−1 · 5 min^−1 to 1,384 ± 178 pmol · 2 × 10^5 cells^−1 · 5 min^−1 (P < 0.01, n = 4, paired t test; Fig. 4A). In the presence of 100 μmol/l ALLM, insulin increased 2-DG uptake from 598 ± 102 pmol · 2 × 10^5 cells^−1 · 5 min^−1 to 751 ± 71 pmol · 2 × 10^5 cells^−1 · 5 min^−1. This change represented an 84% reduction in the increase in 2-DG uptake in response to insulin (P < 0.01) compared with experiments performed in the absence of calpain inhibitors. In the presence of E-64-d, 2-DG uptake was 361 ± 29 and 749 ± 129 pmol · 2 × 10^5 cells^−1 · 5 min^−1 in the absence and presence of insulin, respectively (n = 4), a 58% reduction in insulin-stimulated 2-DG uptake (P < 0.03).

Insulin-mediated 2-DG transport into strips of soleus muscle also was reduced by 100 μmol/l ALLM or 200 μmol/l E-64-d (Fig. 4B). Insulin (12 nmol/l) increased 2-DG uptake into soleus muscle strips from 0.26 ± 0.01 to 0.47 ± 0.03 μmol · ml H_2O_1 · 30 min^-1 (P < 0.001, n = 5, paired t test). In the presence of ALLM, 2-DG uptake was 0.28 ± 0.04 and 0.34 ± 0.05 μmol · ml H_2O_1 · 30 min^-1 in the absence and presence of insulin, respectively (n = 5), whereas the presence of E-64-d, 2-DG uptake was 0.31 ± 0.02 and 0.36 ± 0.02 μmol · ml H_2O_1 · 30 min^-1 in the absence and presence of insulin, respectively (n = 5). The magnitude of the increase in 2-DG uptake in response to insulin was reduced by 71 and 75% in the presence of ALLM and E-64-d, respectively (P < 0.001 in each case using ANOVA and post hoc Duncan test). Similar results were observed with the measurement of 3-MG uptake in epitrochlearis muscles (Fig. 4C). Thus, after a 1-h treatment with 100 μmol/l ALLM, the rate of 3-MG uptake in epitrochlearis stimulated by 12 nmol/l insulin was reduced by 61% (0.44 ± 0.06 vs. 1.13 ± 0.07 μmol · ml^-1 · 10 min^-1, for n = 5 muscles/group; P < 0.01).

To determine whether the observed effects were specific for calpain inhibitors, we used the cathepsin inhibitor compound CA074ME (a cell permeable peptide inhibitor of cathepsin B (19)) in similar experiments. Maximal insulin-stimulated glucose uptake (12 nmol/l insulin) in epitrochlearis muscle strips was 1.1 ± 0.14 μmol · ml H_2O_1 · 10 min^-1 (n = 8) in the absence and 1.1 ± 0.14 μmol · ml H_2O_1 · 10 min^-1 (n = 6) in the presence of 1 μmol/l CA074ME.

ALLM also inhibited hypoxia-induced 3-MG uptake in skeletal muscle (Fig. 4D). An 80-min incubation of epitrochlearis muscle in hypoxic conditions caused an increase in 3-MG uptake in the absence of ALLM (0.26 ± 0.05 vs. 1.21 ± 0.2 μmol · ml^-1 · 10 min^-1), and a 1-h preexposure to 100 μmol/l ALLM suppressed the hypoxia-induced 3-MG uptake by 58% (0.50 ± 0.09 vs. 1.21 ± 0.2 μmol · ml^-1 · 10 min^-1 for n = 4 muscles/group; P < 0.05). The basal 3-MG uptake rate also was significantly inhibited by ALLM (0.26 ± 0.05 vs. 0.07 ± 0.02 μmol · ml^-1 · 10 min^-1 for n = 4 muscles/group; P < 0.05).

Insulin (6 nmol/l) increased the rate of muscle glycogen synthesis in soleus muscle strips from 0.58 ± 0.08 to 1.55 ± 0.20 nmol glucose · mg^-1 · h^-1 (n = 6, P < 0.005, using paired t test; Fig. 5). In the presence of 100 μmol/l ALLM, the glycogen synthesis rates were 0.27 ± 0.03 and 0.40 ± 0.05 nmol glucose · mg^-1 · h^-1 in the absence and presence of insulin, respectively (n = 6), whereas in the presence of 200 μmol/l E-64-d, the glycogen synthesis rates were 0.49 ± 0.08 and 0.80 ± 0.14 nmol glucose · mg^-1 · h^-1 in the absence and presence of insulin, respectively (n = 6). The magnitude of the increase in glycogen synthesis in response to insulin was 87 and 68% lower in
the presence of ALLM and E-64-d, respectively (P < 0.01 in each case using ANOVA and post hoc Duncan test).

DISCUSSION

Genetic studies have shown that variation in the gene encoding calpain-10 is associated with type 2 diabetes (2). Low levels of calpain-10 mRNA in skeletal muscle also are associated with insulin resistance, suggesting that calpain-10 may have a role in determining the normal response of skeletal muscle to the effects of insulin (3). The presence of calpain-10 mRNA in pancreatic islets, muscle, and liver, and the three most important tissues that control blood glucose levels, suggests that calpain-10 may regulate pathways that affect insulin secretion, insulin action, and hepatic glucose production, each of which is altered in patients with type 2 diabetes. As a first step in identifying the biochemical pathways that may be regulated by calpain-10 and/or other calpains, we studied the effects of calpain inhibitors on insulin secretory responses to glucose in islets and insulin action in muscle and adipocytes.

The present studies provide some insight into the mechanism by which ALLM and E-64-d alter the insulin secretory responses to glucose. After a 4-h exposure to calpain inhibitors, the increase in glucose-induced insulin secretion was not associated with increased [Ca^{2+}], rates of glucose oxidation and utilization, or NADPH generation. Thus, pathways in the β-cell that are responsible for the uptake and metabolism of glucose were unaffected. We believe that the primary site(s) of calpain action in the regulation of insulin secretion may be in the pathways that regulate the movement or fusion of insulin secretory granules with the plasma membrane. This hypothesis is supported by the observation that membrane capacitance is increased in islets after short-term exposure to ALLM, in the absence of effects on glucose metabolism and mitochondrial function.

Glucose transport is the major rate-limiting step in the metabolism of glucose in muscle and fat tissue, and impaired glucose uptake by these tissues is an important feature of insulin resistance (20). Treatment of muscle...
FIG. 5. Effect of ALLM and E-64-d on glycogen synthesis rates in skeletal muscle. Muscle strips were incubated in the absence or presence of insulin and in the absence and presence of inhibitors as shown. Results are mean ± SE of six separate experiments. *P < 0.05 compared with muscles incubated in the absence of insulin; #P < 0.05 compared with muscles incubated in the absence of inhibitor (using ANOVA and post hoc Dunnet test in each case).

strips with ALLM and E-64-d resulted in a significant reduction in the rate of insulin-stimulated glucose uptake, as measured by two different assays (2-DG and 3-MG uptake) and the incorporation of glucose into glycogen. The two calpain inhibitors also inhibited 2-DG uptake, into adipocytes. Although the exact site where calpains act in the signaling pathways of insulin action needs further study, the results of the effects of calpain inhibitors on hypoxia-induced 3-MG uptake may shed light on this issue. The hypoxia-associated increase in glucose uptake is not mediated by the insulin receptor-IRS-phosphatidylinositol 3-kinase system (21,22). The inhibition of hypoxia-stimulated glycogen uptake by ALLM suggests that the calpain-regulated step is distal to this system.

The specific calpain isozyme(s) or cysteine protease(s) implicated in the control of insulin secretion and insulin action in the studies described above is unknown. Because isozyme-specific calpain inhibitors are not available, it is not possible to alter selectively the activity of calpain-10 using inhibitors. ALLM and E-64-d are not specific inhibitors of calpains; they also inhibit cathepsins, and for that reason we cannot be certain that the effects that we are attributing to calpain inhibition are due to inhibition of these proteases. However, a number of factors point strongly to calpain inhibition as the mechanism underlying the effects observed. First, the inhibition of hydrolysis of the calpain-specific substrate Boc-Leu-Met-CMAC by ALLM and E-64-d in pancreatic islets is consistent with the hypothesis that ALLM and E-64-d affect insulin secretion by inhibiting calpain activity rather than lysosomal cysteine proteases such as the cathepsins. Second, different calpain inhibitors that work by different mechanism, such as ALLM and the specific calpain inhibitor PD 147631, have similar effects on glucose-induced insulin secretion as ALLM and E-64-d. Third, cathepsin inhibitors and the aspartyl protease inhibitor pepstatin A showed no effect on insulin secretion. The identification of the specific calpain(s) involved must await the development of specific inhibitors or animal models of either increased or decreased expression of various forms of calpains in different tissues, including the pancreatic β-cell and muscle and adipocyte tissue, to determine which calpains are involved in the regulation of insulin action and insulin secretion.

The possibility that the enhancement in insulin secretion, observed in islets exposed to calpain inhibitors, results from nonspecific toxic effects of the calpain inhibitors on the islets was considered. We do not believe that this is the mechanism for the following reasons. First, the effects are seen with calpain inhibitors of different structures that act by different mechanisms. Second, toxic effects on islets would be expected to result in nonspecific release of insulin from islets, but both basal insulin secretion and islet insulin content were unaffected by exposure to calpain inhibitors.

The present studies demonstrate enhancement of glucose-induced insulin secretion in pancreatic islets exposed to calpain inhibitors for 4 h. The mechanism(s) responsible for this change seems to include effects on the insulin secretory machinery responsible for movement of insulin secretory granules to and fusion with the β-cell membrane. Calpain inhibition also results in reduced insulin-stimulated glucose uptake into adipocytes and muscle and reduced glycogen synthesis rates in muscle. These latter results are consistent with physiological studies in nondiabetic subjects in which low levels of calpain-10 mRNA were associated with insulin resistance (3). Taken together, the in vitro studies presented here and in vivo studies in human subjects (3) support the hypothesis that calpains are involved in the regulation of insulin secretion and insulin action.

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