A 48-Hour Exposure of Pancreatic Islets to Calpain Inhibitors Impairs Mitochondrial Fuel Metabolism and the Exocytosis of Insulin

Yun-Ping Zhou, Seamus Sreenan, Chien-Yuan Pan, Kevin P.M. Currie, Vytautas P. Bindokas, Yukio Horikawa, Jean-Pyo Lee, Diane Ostrega, Noreen Ahmed, Aaron C. Baldwin, Nancy J. Cox, Aaron P. Fox, Richard J. Miller, Graeme I. Bell, and Kenneth S. Polonsky

Genetic variation in the gene for a cytosolic cysteine protease, *calpain-10,* increases the susceptibility to type 2 diabetes apparently by altering levels of gene expression. In view of the importance of altered β-cell function in the pathophysiology of type 2 diabetes, the present study was undertaken to define the effects on insulin secretion of exposing pancreatic islets to calpain inhibitors for 48 hours. Exposure of mouse islets to calpain inhibitors (ALLN, ALLM, E-64-d, MDL 18270, and PD147631) of different structure and mechanism of action for 48 hours reversibly suppresses glucose-induced insulin secretion by 40% to 80%. Exposure of islets to inhibitors of other proteases, ie, cathepsin B and proteasome, did not affect insulin secretion. The 48-hour incubation with calpain inhibitors also attenuates insulin secretory responses to the mitochondrial fuel α-ketoisocaproate (KIC). The same incubation also suppresses glucose metabolism and intracellular calcium ($[Ca^{2+}]_i$) responses to glucose or KIC in islets. In summary, long-term inhibition of islet calpain activity attenuates insulin secretion possibly by limiting the rate of glucose metabolism. A reduction of calpain activity in islet could contribute to the development of β-cell failure in type 2 diabetes thereby providing a link between genetic susceptibility to diabetes and the pathophysiologic manifestations of the disease.

RECENT GENETIC LINKAGE studies have suggested that genetic variation in the gene encoding the cysteine protease calpain-10 (the tenth member of the calpain family, see below) affects susceptibility to type 2 diabetes.[1,2] Specific combinations of single nucleotide polymorphisms (SNP) in the *calpain-10* gene are associated with increased risk for type 2 diabetes in a Mexican American population and 2 European populations.[2] In view of the central role of β-cell dysfunction in the pathophysiology of this common disease,[3-5] this unanticipated genetic finding has suggested the possibility that insulin secretion may be regulated in part by calpain-sensitive pathways.

Calpains or calcium-activated neutral proteases are a 12-member family of cytosolic endopeptidases.[6-8] Calpains are classified as conventional or atypical based on their protein structure and tissue distribution. Conventional calpains, such μ- and m-calpain (also called calpain I and II) have a cysteine protease domain and a calmodulin-like Ca^{2+}-binding domain,[6] and are expressed ubiquitously, whereas atypical calpains only contain a cysteine protease domain and are expressed more likely in a tissue-specific manner. Calpains are synthesized predominantly as inactive proenzymes.[7,8] They are activated by a rise in the intracellular calcium concentration ($[Ca^{2+}]_i$) and/or other factors. The mechanism of activation appears to involve autoproteolytic cleavage of their N-terminus peptide fragments[9] or through binding to the plasma membrane.[10] Once activated, calpains are believed to modulate a variety of intracellular signaling, cell proliferation and differentiation pathways by endoproteolytic cleavage of specific substrates.[6,7] Calpains have been implicated in the pathophysiology of various disease states, including Alzheimer's disease and cataracts.[11-14]

A role for calpains in the pathogenesis of type 2 diabetes or other metabolic disorders was not suspected until *calpain-10* was identified as a diabetes susceptibility gene. Calpains appear to be involved in adipocyte differentiation[15] and possibly insulin-induced downregulation of insulin receptor substrator-1.[16] A previous study also indicated that μ-calpain was present in pancreatic β cells.[17] The newly identified diabetes susceptibility gene, *calpain-10*, was highly expressed in rat and human islets when measured by reverse-transcription polymerse chain reaction (RT-PCR).[2] However, the potential effects of *calpain-10* or calpain-dependent pathways on β-cell function have not been determined. In a recent study,[18] we demonstrated that glucose-induced insulin secretion by mouse islets was increased by 4-hour exposure to calpain inhibitors, such as calpain inhibitor II (ALLM) and E-64-d. To further understand the potential role of calpains in the regulation of insulin secretion under both physiological and pathophysiological conditions, we have investigated the effect of inhibiting calpain activity in β cells for 48 hours.

MATERIALS AND METHODS

Islet Isolation and Treatment

Experiments were performed on islets obtained from nonfasted 9- to 13-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME).

From the Departments of Medicine, Neurobiology, Pharmacology and Physiology, Biochemistry and Molecular Biology, Human Genetics, and the Howard Hughes Medical Institute, University of Chicago, Chicago, IL; and the Department of Medicine, Washington University School of Medicine, St Louis, MO.

Submitted January 22, 2002; accepted November 21, 2002.

Supported by Grants No. DK-31842, DK-44840, and DK-20595 from the National Institutes of Health, the Blum-Kovler Foundation, the Jack and Dollie Galter Center of Excellence of the Juvenile Diabetes Research Foundation, and Howard Hughes Medical Institute.

Current address for Y.-P.Z.: Department of Genomics, Metabolex, Inc, 3876 Bay Center Place, Hayward, CA 94545; current address for S.S.: Department of Endocrinology, James Connolly Memorial Hospital, Blanchardstown, Dublin 15, Ireland.

Y.-P. Zhou and S. Sreenan contributed equally to this work.

Address reprint requests to Kenneth S. Polonsky, MD, Department of Medicine, Washington University, 660 S Euclid, Campus Box 8066, St Louis, MO 63110.

0026-0495/03/5205-0003$30.00/0

doi:10.1053/meta.2003.50091

Animals were killed using methods approved by the Animal Care and Use Committee of the University of Chicago. Isolation of pancreatic islets was performed as previously described.[19] Following overnight incubation in RPMI 1640 medium (11.6 mmol/L glucose), islets were exposed to inhibitors of calpains and other proteases in the same medium for 48 hours at 37°C. The protease inhibitors were dissolved in dimethyl sulfoxide (DMSO), which was also added to control cultured islets at the final concentration of 0.1%.

Protease Inhibitors

Calpain inhibitor I (ALLN), calpain inhibitor II (ALLM), and calpain inhibitor III (MDL 28170) are cell-permeable peptidic calpain inhibitors[20,21] acting through binding to the active site on the cysteine protease domain. E-64-d (ethyl (+)-(2S,3S)-3-[(S)-3-Methyl-1-(3-methylbutylcarbamoyl) butyl-carbamoyl]-2-oxirane carboxylate) is a nonpeptidic inhibitor of calpain with a similar spectrum of action as ALLM and ALLN. PD147631 is a relatively specific small molecular inhibitor of calpain that binds to the Ca^{2+}-binding domain of the proteases. Its IC_{50} value for calpain is over 100 times less than that for cathepsin B and other cysteine proteases (personal communication, Kevin Wang). Multiple calpain inhibitors with different chemical structure and mechanisms of action were used to ensure that the effects observed resulted from the inhibition of calpain activity, rather than nonspecific toxic effects of the compounds. The inhibitors of calpain currently available to us are not absolutely specific to calpains. They may also inhibit other cysteine proteases to some extent. To test the possible effects of inhibiting other cysteine proteases, we also studied the effects on insulin secretion of 2 non-calpain protease inhibitors: cathepsin B inhibitor II, a small peptide that inhibits cathepsin B,[22] and lactacystin, a specific cell-permeable, irreversible inhibitor of the proteasome.[23] ALLN, ALLM, MDL 28170, cathepsin B inhibitor II, and lactacystin were obtained from Calbiochem-Novabiochem (San Diego, CA). E-64-d was obtained from Matreya (Pleasant Gap, PA). PD147631 was a gift from Dr Kevin Wang of Parke-Davis Pharmaceutical (Ann Arbor, MI). All other reagents were from Sigma Chemicals (St Louis, MO).

Static Incubation of Isolated Pancreatic Islets

Islets were preincubated in oxygenated Krebs-Ringers' bicarbonate medium (KRB) with the following composition (in mmol/L) Na^+ 143.5, K^+ 5.8, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 124.1, PO_4^{3-} 1.2. SO_4^{2+} 1.2, CO_3^{2-} 25, and 10 mmol/L Hepes (pH 7.4), 2 mg/mL bovine serum albumin (BSA), and 2 mmol/L glucose for 45 minutes at 37°C. Triplicate groups of 5 islets were then incubated in borosilicate tubes containing 1 mL of KRB with inhibitor and various insulin secretagogues for 1 hour in a moving water bath at 37°C.[24] Similar concentrations of inhibitors were included during the whole process of incubation. Insulin concentrations were measured in aliquots of the incubation buffer by radioimmunoassay as described.[25]

Measurement of $[Ca^{2+}]_i$, NAD(P)H and Calpain Activity in Islets

The NADH and $[Ca^{2+}]_i$ responses to glucose and other stimuli were measured in islets plated on glass coverslips. Isolated islets were cultured in RPMI 1640 medium for 2 to 4 days prior to the 48-hour treatment with inhibitors to allow for attachment to the coverslips. The coverslip was mounted into a microperifusion chamber on the specimen stage of a Nikon Diaphot 200 inverted microscope (Nikon, Melville, NY) equipped for epifluorescence. Islets were perifused with KRB (with or without secretagogues) at a rate of 2.5 mL/min at 37°C during the experiments.

To measure $[Ca^{2+}]_i$, islets attached to glass cover-slips were loaded with fura-2/AM (5 μmol/L, Molecular Probes, Eugene, OR) for 30 minutes at 37°C in the KRB medium. $[Ca^{2+}]_i$ was measured using dual-wavelength fluorescence video microscopy and expressed as the ratio of fura-2 fluorescence intensity (detected at 510 nm) excited at 340 and 380 nm (ratio, 340/380). The exchange of the excitation filters and the acquisition of images were controlled by the imaging software Metafluor 3.6 (Universal Imaging, Philadelphia, PA). The reduced forms of NAD and NADP, designated NAD(P)H, were measured using the same imaging system. The autofluorescence of NAD(P)H was excited at 365 nm and measured at 495 nm in dye-free islets.[26,27]

Calpain activity in islets was measured by monitoring the rate of proteolysis of the fluorogenic calpain substrate, *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethyl coumarin (10 μmol/L, Molecular Probes) as described previously.[28]

Glycolysis (glucose utilization) and Glucose Oxidation Rates

These measurement were determined by monitoring the production of ^3H_2O and $^{14}CO_2$ from [5-^{3}H]glucose and [U-^{14}C]glucose simultaneously by islets incubated in KRB medium with 2, 8.3, 16.7, and 27 mmol/L glucose together with trace amount of the radiolabeled glucose as described.[29]

Statistics

Data throughout are expressed as mean ± SE. Statistical significance for the comparison between vehicle- and drug-treated groups was examined with unpaired *t* test as appropriate.

RESULTS

Distinct Effects of Acute Versus Chronic Exposure to ALLN on Insulin Secretion in Normal Mouse Islets

To study the time course of calpain inhibition on islet function, we measured insulin secretion in mouse islets that had been incubated for 4, 24, and 48 hours with 100 μmol/L ALLN, a small peptide that competitively inhibits calpains of all classes. Consistent with the previous study,[18] a 4-hour incubation with ALLN enhanced insulin secretory response to 20 mmol/L glucose by 40% relative to control islets. In contrast, the insulin secretory response to 20 mmol/L glucose was reduced by 67% and 87% in islets incubated with the inhibitor for 24 hours (100 ± 55 *v* 301 ± 63 μU/5 islets/h) and 48 hours (46 ± 6 *v* 330 ± 40 μU/5 islets/h, n = 4, $P < .05$, Fig 1), respectively.

Inhibition of Insulin Secretion by Other Calpain Inhibitors But Not Inhibitors of Other Proteases

To test the association of impaired insulin secretion in long-term ALLN-treated islets with the inhibition of calpain activity, we then studied the effects of other cysteine protease inhibitors on islet function and islet calpain activity using a similar protocol.

The 48-hour exposure of mouse islets to 4 additional calpain inhibitors (ALLM, MDL28170, and PD147631 at 100 μmol/L; or E-64-d at 200 μmol/L) attenuated the insulin secretory response to 20 mmol/L glucose by 40% to 60% relative to control islets (n = 3-5, $P < .05$ or less for each inhibitor). The exposure to the calpain inhibitors did not significantly affect basal insulin secretion at 2 mmol/L glucose (Fig 2A), nor islet insulin content (Fig 2B), indicative of the general healthiness of the islets. Consistent with the expected mode of action of these compounds, exposure to calpain inhibitors significantly suppressed total calpain activity in islets. Following 48 hours of

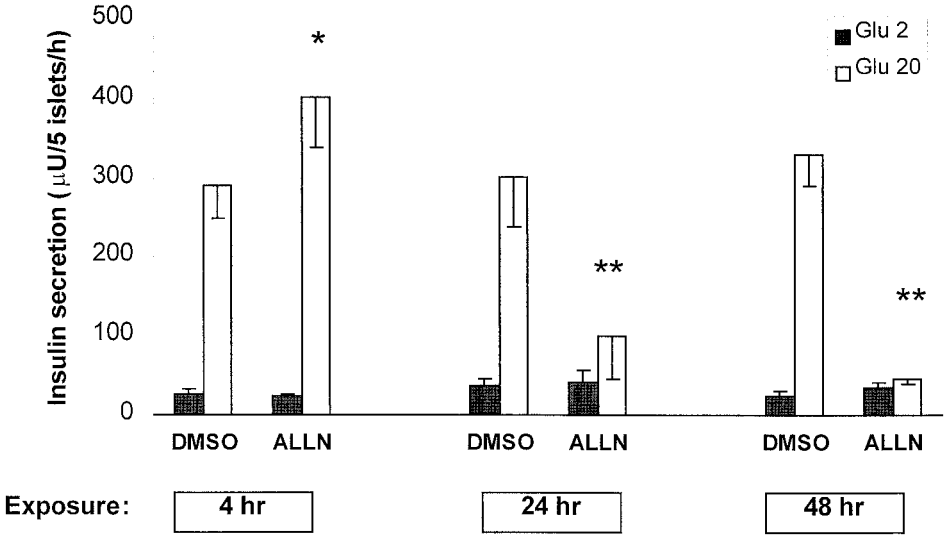

Fig 1. Time-dependent effect of ALLN on glucose-stimulated insulin secretion in mouse islets. Mouse islets were exposed to 100 μmol/L ALLN for 4, 24, and 48 hours in RPMI 1640 medium, and insulin secretion was determined in 60-minute static incubations in KRB with 2 and 20 mmol/L glucose in the continued presence of the inhibitor. Results represent mean ± SE of 3 experiments. *$P < .05$ and **$P < .01$ *v* DMSO-treated islets by unpaired *t* test.

treatment with 100 μmol/L ALLM or 200 μmol/L E-64-d, the rate of cleavage of a specific fluorogenic substrate of calpain within the 30 minutes of measurement was reduced by 54% ± 3% and 55% ± 4% (n = 5 for each group) relative to control treated islets (Fig 2C).

In contrast to the 5 calpain inhibitors described above, culturing islets with 100 μmol/L cathepsin B inhibitor II or 20 μmol/L lactacystin for 48 hours had no effect on either basal or glucose-stimulated insulin secretion.

Reversibility of the Inhibitory Effects of ALLM and E-64-d

To test the reversibility of the inhibitory effect of calpain inhibitors on insulin secretion, islets that had been exposed to 100 μmol/L ALLM or 200 μmol/L E-64-d for 48 hours were cultured for another 48 hours in RPMI 1640 medium with or without the inhibitors. Exposing islets to ALLM or E-64-d for 96 hours inhibited the insulin secretory response to 20 mmol/L glucose by 80% (Fig 3). In contrast, removal of the islets from the inhibitors for 48 hours restored insulin secretory responses to levels not different from control (Fig 3), indicating again that calpain inhibitors do not cause β-cell death or exert significant irreversible nonspecific toxic effects.

Changes in $[Ca^{2+}]_i$ Signaling in ALLM- and E-64-d–Treated Islets

To determine if deficient insulin secretion in calpain inhibitor–treated islets is associated with alterations in intracellular Ca^{2+} signaling, we measured $[Ca^{2+}]_i$ responses to glucose and α-ketoisocaproic acid (KIC), which serves as a substrate for mitochondrial metabolism thereby generating adenosine

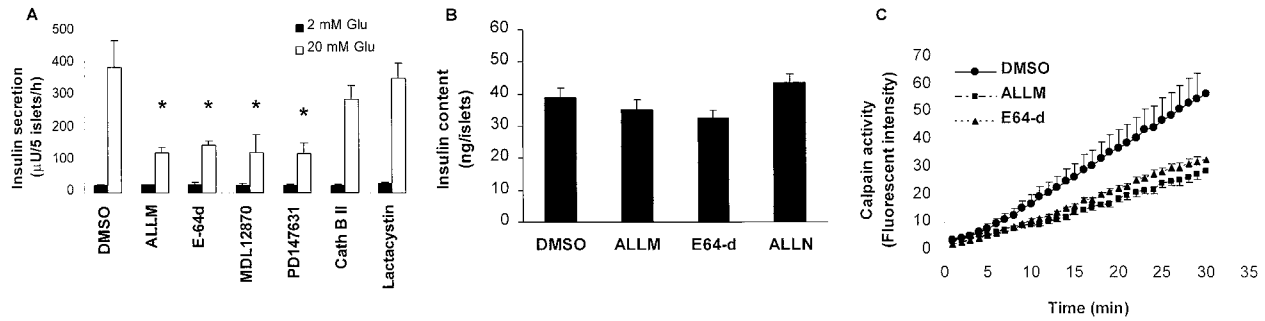

Fig 2. Effect of 48 hours exposure to various protease inhibitors on insulin secretion, islet insulin content, and calpain activity in mouse pancreatic islets. (A) Insulin secretion. Mouse islets were exposed to 4 calpain inhibitors (ALLM 100 μmol/L, E-64-d 200 μmol/L, MDL-28170 100 μmol/L MDL, and PD-147631 100 μmol/L), 100 μmol/L cathepsin B inhibitor II (Cath B II), 20 μmol/L lactacystin, or vehicle (DMSO) for 48 hours in RPMI 1640 medium, and insulin secretion was determined in 60-minute static incubations in KRB medium with 2 (□) and 20 mmol/L glucose (■) in the continued presence of the inhibitors. Data are mean ± SE of 4-6 experiments in each case. *$P < .05$ or less *v* DMSO-treated islets. (B) Islet insulin content. Islets were homogenized in acid ethanol (0.18 mol/L HCl in 75% ethanol), and insulin content was extracted with acid ethanol at 4°C for overnight before being measured by RIA after dilution. Insulin content in islets that had been treated with ALLM (100 μmol/L), E-64-d (200 μmol/L), or ALLN (100 μmol/L) for 48 hours was not different from that in DMSO-treated islets. (C) Calpain activity in ALLM- and E-64-d–treated mouse islets. Following the 48-hour exposure to 100 μmol/L ALLM or 200 μmol/L E-64-d, islets were incubated in KRB medium containing 10 μmol/L Boc-Leu-Met-CMAC (a calpain substrate) from 0 min and fluorescence emitted by the calpain proteolytic product was measured following excitation by light at 340 nm. Data represent mean ± SEM of 4 experiments.

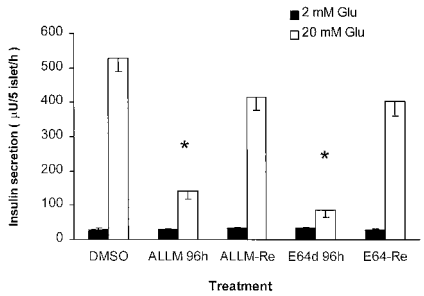

Fig 3. Reversibility of the inhibitory effect of ALLM and E-64-d on insulin secretion. Mouse islets that had been treated for 48 hours with 100 μmol/L ALLM or 200 μmol/L E-64-d were cultured for another 48 hours in either the continued presence (ALLM 96h and E64d 96h) or the absence (ALLM-Re and E64-Re) of the same concentrations of the inhibitors. Insulin secretion was then measured in 60-min static incubations as described in Fig 1.Data represent mean ± SE of 3 experiments. *$P < .01$ *v* DMSO-treated islets.

triphosphate (ATP) and other signaling molecules independent of the glycolytic pathway.[30] The viability of the islets was also assessed by measuring changes in $[Ca^{2+}]_i$ when islet cells were depolarized in the presence of 30 mmol/L of potassium chloride (KCl).[31]

Exposure to ALLM or E-64-d for 48 hours significantly delayed and reduced $[Ca^{2+}]_i$ responses to glucose and KIC (Fig 4A). The time interval necessary to elicit a half maximal $[Ca^{2+}]_i$ response to glucose ($t_{1/2}$, see Fig 4B) was 120 ± 14 seconds in control islets, and 319 ± 42 and 265 ± 65 seconds in ALLM- and E-64-d–treated islets, respectively ($P < .001$ for both groups, n = 7). The magnitude of the $[Ca^{2+}]_i$ responses to the 2 nutrients was also reduced in the inhibitor-treated islets. Thus, the area under the curves (AUC) of the $[Ca^{2+}]_i$ responses to 14 mmol/L glucose were reduced by 51% ± 5% and 47% ± 6%, respectively, in ALLM- and E-64-d–treated islets compared with control (n = 7, $P < .01$, Fig 4C). ALLM and E-64-d also delayed and reduced $[Ca^{2+}]_i$ responses to KIC to a similar extent as the responses to 14 mmol/L glucose. In contrast, $[Ca^{2+}]_i$ response to 30 mmol/L KCl was not decreased or delayed in ALLM- or E-64-d–treated islets relative to control. (Fig 4A through C).

Consistent with the above $[Ca^{2+}]_i$ response data, insulin secretory responses to 15 mmol/L KIC were significantly reduced in both ALLM- and E-64-d–treated islets (Fig 4D). On the other hand, the insulin secretion stimulated by 30 mmol/L KCl (in the presence of 2 mmol/L glucose) was normal in ALLM-treated islets, but was reduced by 53% in E-64-d–treated islets (Fig 4D).

Glucose Metabolism in ALLM- and E-64-d–Treated Islets

Consistent with the attenuated insulin secretory and $[Ca^{2+}]_i$ responses to glucose and KIC, rates of glycolysis (Fig 5A) and glucose oxidation (Fig 5B) at concentrations of glucose that stimulate insulin secretion (>8 mmol/L) were significantly reduced in ALLM- and E-64-d–treated islets compared to controls. For example, the rate of glucose utilization at 27 mmol/L glucose was inhibited by 53% in ALLM-treated and by 64% in E-64-d–treated islets ($P < .01$ for both, n = 4), and the rate of glucose oxidation at 27 mmol/L glucose was suppressed by 35% and 46% ($P < .01$ for both groups, n = 4), respectively, in the inhibitor-treated islets. The rates of glycolysis and glucose oxidation measured at 2 mmol/L glucose were unaffected by the exposure to calpain inhibitors.

The metabolism of KIC (which occurs only in mitochondria) was also markedly reduced in islets exposed to ALLM and E-64-d for 48 hours. Thus, the generation of NADH following KIC stimulation, measured by NAD(P)H autofluorescence, was reduced by over 60% in the inhibitor-treated islets relative to control (Fig 5C). The NADH responses to glucose were indistinguishable between control and ALLM- or E-64-d–treated islets, possibly due to the lower sensitivity of this assay to detect NAD(P)H responses to glucose relative to the responses to mitochondrial substrates such as KIC and methyl pyruvate. We observed similar pattern of NAD(P)H responses in a model of islet mitochondrial defect.[26]

DISCUSSION

A broad spectrum of proteins including cytoskeletal proteins, histones, enzymes, receptor proteins, and transcription factors can serve as substrates for calpains. Calpains regulate the functional activities of these molecules by modifying their chemical structures. The activity of calpain itself is tightly controlled by $[Ca^{2+}]_i$ and the endogenous calpain inhibitor calpastatin. It appears that this class of proteases regulates a variety of biological processes, although the precise mechanisms of control are yet to be uncovered.

Our results clearly demonstrate that 48 hours exposure of islets to calpain inhibitors attenuates insulin secretory responses to glucose. The effects of calpain inhibitors on insulin secretion depend on the time of exposure. Our previous studies demonstrated enhanced insulin secretory responses to glucose following incubation of pancreatic islets with calpain inhibitors for 4 hours.[18] This time-dependent effect of compounds on insulin secretion is not without precedent. For example, it is well known that free fatty acids acutely enhance, and then chronically inhibit glucose-stimulated insulin secretion.[24] Since calpains may act on a potentially broad spectrum of substrates, the current data could be explained if calpain inhibition affects a substrate(s) in one signaling pathway in the short-term and yet another substrate(s) in a different pathway in the longer term. It is also possible that different duration of calpain inhibition affects the function of different cell types in islets thereby yielding distinct consequences in terms of insulin secretion. Since both of our studies used intact islets rather than purified β cells, any changes in the secretion of glucagon and/or somatostatin could affect the results on insulin secretion.

The inhibition of insulin secretion by calpain inhibitors is due to a reduction in islet calpain activity. Inhibitors of cathepsin and proteasome at biologically effective concentrations[22,23] did not affect insulin secretion, whereas all 5 calpain inhibitors tested in this study (ALLN, ALLM, MDL 28170, E-64-d, and PD147631) suppressed glucose-induced insulin secretion. The calpain inhitors at the doses used in the present study would be expected to have at most only minor inhibitory effects on other cysteine proteases such as cathepsin or proteasome. This is especially true with PD147631, which is over 100 times more

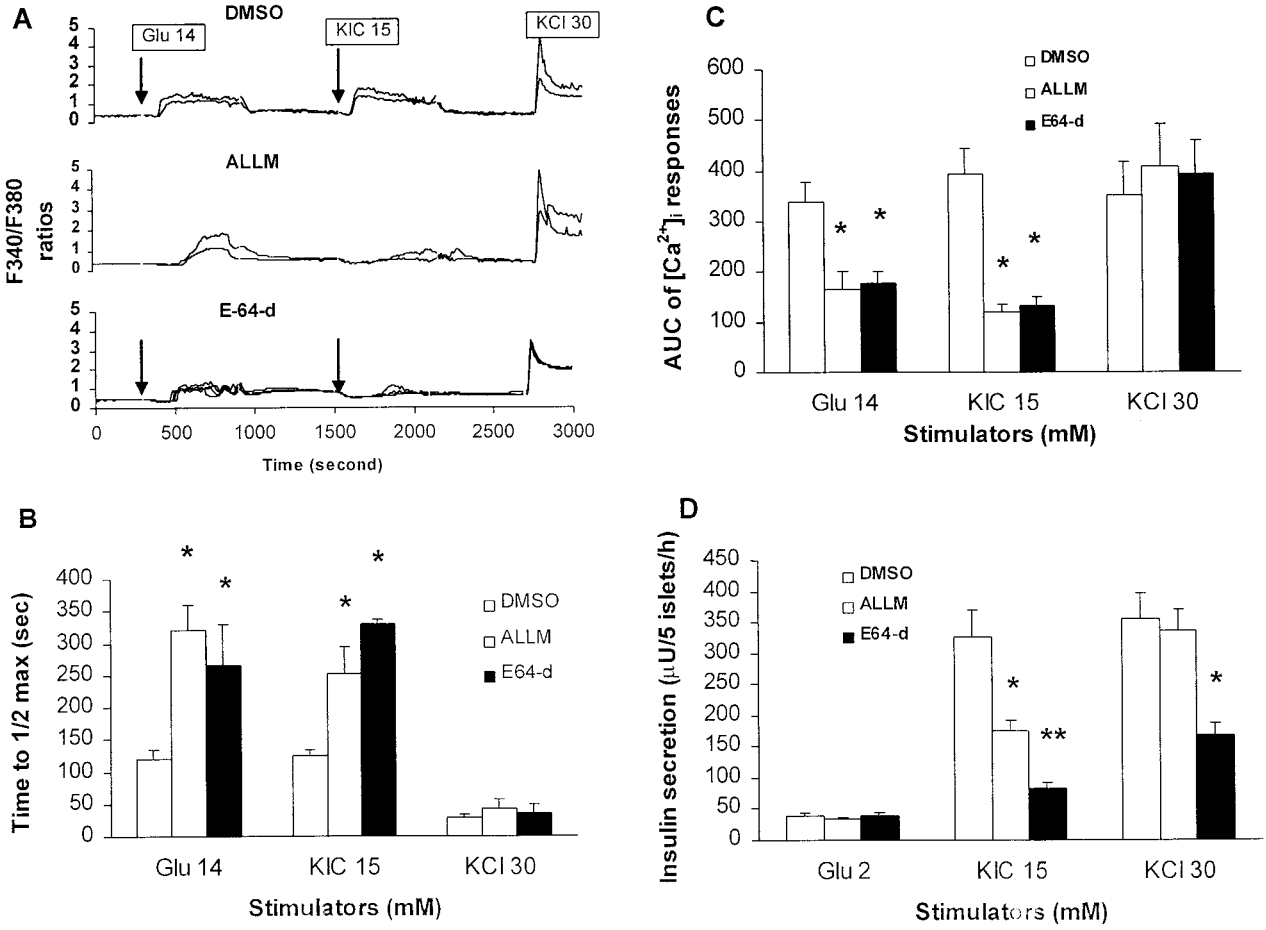

Fig 4. Effect of calpain inhibitors on $[Ca^{2+}]_i$ in mouse islets. (A) Representative records of $[Ca^{2+}]_i$ responses to glucose, KIC, and KCl in islets treated with ALLM and E-64-d. Islets attached to glass coverslips were treated with 100 μmol/L ALLM or 200 μmol/L E-64-d for 2 days. $[Ca^{2+}]_i$ responses to 14 mmol/L glucose, 15 mmol/L KIC, and 30 mmol/L KCl were measured with Ca^{2+} indicator Fura-2-AM and a computer-driven digital imaging system. Representative records of the responses in 3 groups of islets are shown. (B) Time lapse between the application of the secretagogues and the point of half maximal response in control and inhibitor-treated islets. Data represent mean ± SE of 7 experiments. *$P <$ .01 compared with DMSO-treated islets. (C) Comparison of the $[Ca^{2+}]_i$ response AUC in the 3 groups of islets. Data represent mean ± SE of 7 experiments. *$P <$ .01 *v* DMSO-treated islets. (D) Insulin secretory responses to KCl and KIC in ALLM- and E-64-d–treated islets. Islets were treated with 100 μmol/L ALLM or 200 μmol/L E-64-d for 48 hours, and insulin secretory responses to KIC (15 mmol/L) or 30 mmol/L KCl were determined by static incubation in KRB medium containing 2 mmol/L glucose in the continued presence of the inhibitors. Data represent mean ± SE of 6 experiments. *$P <$ .01 and **$P <$ .001 *v* DMSO-treated islets.

effective in inhibiting calpain than other cysteine proteases. An assay of calpain activity demonstrated that 48 hours exposure to ALLM and E-64-d suppressed islet calpain activity by more than 50%. In contrast, inhibitors of cathepsin and other proteases did not inhibit calpain activity by the assay used in the present study.[28] Taken together these observations all indicate that the impairment of insulin secretion seen following 48 hours exposure to calpain inhibitors is due to the inhibition of calpain activity and not the inhibition of the activity of other proteases. The isozyme(s) of calpain involved in the control of insulin secretion has not been determined. This is due to the fact that isozyme-specific calpain inhibitors are not available and animal models in which expression of various calpain forms have been selectively altered, have not been developed.

Calpain inhibitors impair insulin secretion by inhibiting glucose metabolism (perhaps mainly in mitochondria) in β cells. Exposing islets to ALLM or E-64-d for 48 hours significantly reduced the insulin secretory response not only to glucose, but also to the mitochondrial substrate KIC. More importantly, the 2 inhibitors profoundly suppressed the rates of glucose metabolism and NADH response to KIC in islets. It is currently not known how the cytosolic calpains regulate mitochondrial function, or how calpain inhibition disturbs mitochondrial metabolism. The coupling of impaired insulin secretion to the alteration in $[Ca^{2+}]_i$ and glucose metabolism may explain why the suppression of insulin secretion was only seen when incubation exceeded 4 to 6 hours, and why basal insulin secretion was not affected. In the case of short-term (4 to 6 hours) calpain inhibition,[18] no significant changes in glucose metabolism and $[Ca^{2+}]_i$ were observed in calpain inhibitor–treated islets. The only change in β-cell signaling in these islets was the significant acceleration of insulin granule exocytosis associated with

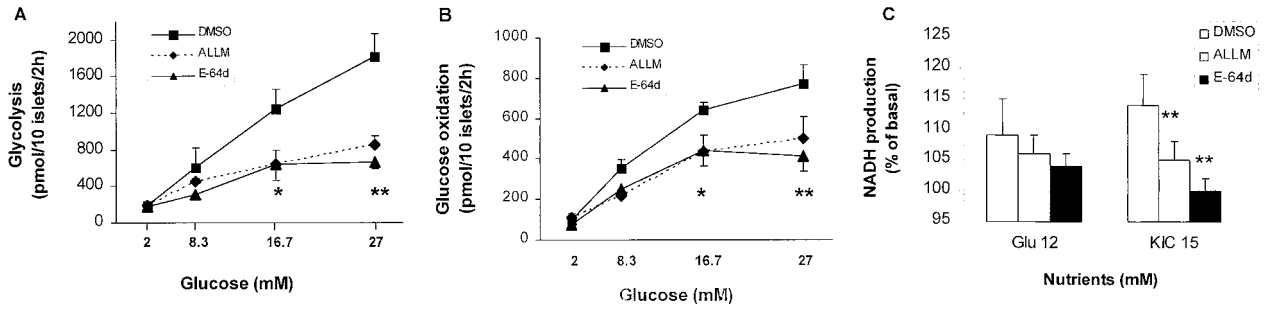

Fig 5. Glucose metabolism in islets treated by calpain inhibitors. (A & B) Following a 48-hour exposure to 100 μmol/L ALLM or 200 μmol/L E-64-d in RPMI 1640 medium, glycolysis (^3H_2O production from [5-^{3}H]glucose, A) and glucose oxidation ($^{14}CO_2$ production from [U-^{14}C]glucose, B) in islets were measured in 2-hour incubations in KRB media with 2, 8.3, 16.7, and 27 mmol/Lglucose. Data are mean ± SE of 4 experiments. *$P < .05$ and **$P < .01$ *v* DMSO-treated islets. (C) NAD(P)H responses to glucose and ketoisocaproic acid (KIC). Islet autofluorescence at 460 nm (excited by 350 nm) were measured during perifusion with medium containing 14 mmol/L glucose or 15 mmol/L KIC following the 48-hour treatment with ALLM and E-64-d. The fluorescence intensity was normalized with the basal values of each islet obtained at 2 mmol/L glucose and expressed as % increase over the basal. Data are mean ± SE of 5 separate experiments. *$P < .01$ *v* DMSO-treated islets.

enhanced insulin secretion. Consistent with normal basal secretion (at 2 mmol/L glucose) in calpain inhibitor–treated islets, long-term calpain inhibition did not affect basal glycolysis and glucose oxidation and basal $[Ca^{2+}]_i$ levels in islets. Basal insulin secretion in normal islets is apparently not heavily dependent on glucose metabolism and a rise of $[Ca^{2+}]_i$.[32]

Whether calpain inhibition also affects other processes in β cells, such as the exocytosis of insulin granules, awaits further investigation. In this context, the different effects of ALLM and E-64-d on KCl-induced insulin secretion (Fig 4D) may be relevant. Because the Ca^{2+} responses to KCl were normal in both ALLM- and E-64-d–treated islets, the observed differences in the effects on secretion of these 2 calpain inhibitors likely occur in signaling events after the rise of $[Ca^{2+}]_i$, most likely in the exocytotic process. One possibility is that E-64-d leads to a greater impairment of mitochondrial metabolism than does ALLM (this tendency can be seen in the measurement of glucose oxidation and NAD(P)H responses to KIC, see Fig 5). The decrease in ATP production in E-64-d–treated islets might have reached a critical threshold that will limit insulin exocytosis, as several steps of the exocytotic process utilize ATP.[33]

The possibility that nonspecific toxic effects were involved in the impairment of insulin secretion in islets exposed to calpain inhibitors for 48 hours was also considered. We believe that this possibility can be excluded for the following reasons: (1) removal of the calpain inhibitors from the islet cultures resulted in the recovery of the insulin secretory response to glucose making it unlikely that decreased islet viability is responsible for the defects in insulin secretion, and (2) inhibitor-treated islets had normal Ca^{2+} responses to the direct depolarization induced by 30 mmol/LKCl, despite reduced Ca^{2+} responses to glucose and KIC. This suggests that the impaired Ca^{2+} responses (to glucose and KIC) were not a result of nonspecific toxicity of calpain inhibitors.

In summary, inhibition of calpain activity for 48 hours leads to deficient insulin secretion in pancreatic islets possibly due to defects in mitochondrial metabolism. Together with our previous study on calpain inhibitors, these data suggest the existence of a novel pathway in the β cell, involving calpains, which affects insulin secretion in response to a variety of stimuli and that a long-term reduction in calpain activity could result in impaired insulin secretion by pancreatic β cells. The existence of this pathway could explain the observation that genetic variation in the gene for at least one calpain confers susceptibility to type 2 diabetes.

REFERENCES

1. Hanis CL, Boerwinkle E, Chakraborty R, et al: A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. Nat Genet 13:161-166, 1996

2. Horikawa Y, Oda N, Cox NJ, et al: Genetic variation in the calpain 10 gene (*CAPN10*) is associated with type 2 diabetes mellitus. Nat Genet 26:163-175, 2000

3. Polonsky KS, Sturis J, Bell GI: Non-insulin-dependent diabetes mellitus—A genetically programmed failure of the beta cell to compensate for insulin resistance. N Engl J Med 334:777-783, 1996

4. Bell GI, Polonsky KS: Diabetes mellitus and genetically programmed defects in beta-cell function. Nature 414:788-791, 2001

5. Porte D Jr: Beta cells in type II diabetes mellitus. Diabetes 40: 166-180, 1991

6. Sorimachi H, Ishihura S, Suzuki K: Structure and physiological function of calpains. Biochem J 328: 721-732, 1997

7. Carafoli E, Molinari M: Calpain: A protease in search of a function? Biochem Biophys Res Commun 247:193-203, 1998

8. Croall DE, DeMartino GN: Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. Physiol Rev 71: 813-847, 1991

9. Shields DC, Banik NL: Pathophysiological role of calpain in experimental demyelination. J Neurosci Res 55:533-541, 1999

10. Suzuki K, Sorimachi H: A novel aspect of calpain activation. FEBS Lett 433:1-4, 1998

11. Richard I, Broux O, Allamand V, et al: Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 81:27-40, 1995

12. Chen M, Fernandez HL: The Alzheimer's plaques, tangles and memory deficits may have a common origin—Part IV: Can calpain act as alpha-secretase? Front Biosci 3:A66-A75, 1998

13. Blomgren K, Kawashima S, Saido TC, et al: Fodrin degradation and subcellular distribution of calpains after neonatal rat cerebral hypoxic-ischemia. Brain Res 684:143-149, 1995

14. Yokota M, Saido TC, Tani E, et al: Three distinct phases of fodrin proteolysis induced in postischemic hippocampus. Involvement of calpain and unidentified protease. Stroke 26:1901-1907, 1995

15. Patel YM, Lane MD: Role of calpain in adipocyte differentiation. Proc Natl Acad Sci USA 96:1279-1284, 1999

16. Smith LK, Rice MR, Garner WG: The insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes is mediated by a calcium-dependent thiol protease. Mol Cell Endocrinol 122:81-92, 1996

17. Kitahara A, Ohtsuki H, Kirihata Y, et al: Selective localization of calpain I (the low-Ca^{2+}-reqiring form of Ca^{2+}-dependent cysteine proteinase) in B-cells of human pancreatic islets. FEBS Lett 185:120-124, 1985

18. Sreenan SK, Zhou YP, Otani K, et al: Calpains play a role in insulin secretion and action. Diabetes 50:2013-2020, 2001

19. Lacy PE, Kostianovky M: Method for the isolation of intact islet of Langerhans from the rat pancreas. Diabetes 16:35-39, 1967

20. Mehdi S: Cell-penetrating inhibitors of calpain. Trends Biochem Sci 16: 150-153, 1991

21. Wang KK, Yuen PW: Calpain inhibition: An overview of its therapeutic potential. Trends Pharmacol Sci 15:412-419, 1994

22. McConnell RM, York JL, Frizzell D, et al: Inhibition studies of some serine and thiol proteinases by new leupeptin analogues. J Med Chem 36:1084-1089, 1993

23. Lee DH, Goldberg AL: Proteasome inhibitors: Valuable new tools for cell biologists. Trends Cell Biol 8:397-403, 1998

24. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J Clin Invest 93:870-876, 1994

25. Sturis J, Pugh W, Tang J, et al: Alterations in pulsatile insulin secretion in the Zucker diabetic fatty rat. Am J Physiol 267:E250-E259, 1994

26. Zhou Y-P, Pena JC, Roe MW, et al: Overexpression of Bcl-x_L in β-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am J Physiol Endocrinol Metab 278:E340-E351, 2000

27. Duchen MR, Smith PA, Ashcroft FM: Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. Biochem J 294: 35-42, 1993

28. Rosser BG, Powers SP, Gores GJ: Calpain activity increases in hepatocytes following addition of ATP. Demonstration by a novel fluorescent approach. J Biol Chem 268:23593-23600, 1993

29. Zhou Y-P, Priestman DA, Randle PJ, et al: Fasting and decreased B cell sensitivity: Important role for fatty acid-induced inhibition of PDH activity. Am J Physiol 270:E988-E994, 1996

30. Martin F, Sanchez-Andres JV, Soria B: Slow $[Ca2^+]_i$ oscillations induced by ketoisocaproate in single mouse pancreatic islets. Diabetes 44:300-305, 1995

31. Rorsman P: The pancreatic beta-cell as a fuel sensor: An electrophysiologist's viewpoint. Diabetologia 40:487-95, 1999

32. Zhou YP, Cockburn B, Pugh W, et al: Basal insulin hypersecretion in insulin-resistant Zucker diabetic and Zucker fatty rats: Role of enhanced fuel metabolism. Metabolism 48:857-864, 1999

33. Wollheim CB, Maechler P: β-cell mitochondria and insulin secretion, messenger role of nucleotides and metabolites. Diabetes 51:S37-S41, 2002 (suppl 1)