Muscarinic Control of MIN6 Pancreatic β Cells Is Enhanced by Impaired Amino Acid Signaling*

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Background: Depletion of the GPCR T1R1/T1R3 increased calcium and ERK1/2 signaling by carbachol.
Results: T1R3 depletion or reducing amino acids overnight increased M3 muscarinic receptor expression and altered calcium responses.
Conclusion: M3 receptor expression in β cells is up-regulated by reduced amino acid availability.
Significance: The M3 muscarinic receptor is a potential therapeutic target in β cells with impaired amino acid sensitivity.

We have shown recently that the class C G protein-coupled receptor T1R1/T1R3 taste receptor complex is an early amino acid sensor in MIN6 pancreatic β cells. Amino acids are unable to activate ERK1/2 in β cells in which T1R3 has been depleted. The muscarinic receptor agonist carbachol activated ERK1/2 better in T1R3-depleted cells than in control cells. Ligands that activate certain G protein-coupled receptors in pancreatic β cells potentiate glucose-stimulated insulin secretion. Among these is the M3 muscarinic acetylcholine receptor, the major muscarinic receptor in β cells. We found that expression of M3 receptors increased in T1R3-depleted MIN6 cells and that calcium responses were altered. To determine whether these changes were related to impaired amino acid signaling, we compared responses in cells exposed to reduced amino acid concentrations. M3 receptor expression was increased, and some, but not all, changes in calcium signaling were mimicked. These findings suggest that M3 acetylcholine receptors are increased in β cells as a mechanism to compensate for amino acid deficiency.

The essential function of the pancreatic β cell is to secrete insulin in response to increases in circulating glucose. Other nutrients, hormones, and paracrine agents influence pancreatic β cell functions and insulin secretion to optimize glucose homeostasis. Ligands for several G protein-coupled receptors (GPCRs) are among the most significant in tuning insulin secretion from β cells, which express several different classes of GPCRs, including muscarinic acetylcholine receptors (mACHRs) (1).

Five mACHRs subtypes, M1-M5, have been identified (2). The M1, M3, and M5 subtypes are Gq-coupled receptors, whereas M2 and M4 are Gi-coupled receptors that are inhibited by pertussis toxin (3). β cell muscarinic receptors are Gq-coupled because binding of acetylcholine to these receptors results in the well-characterized action of Gq to activate phospholipase C β. The resulting hydrolysis of phosphatidylinositol 4,5-bisphosphate generates the second messenger inositol 1,4,5-trisphosphate, which binds to its receptor on the ER and induces calcium release from intracellular stores (4). Muscarinic agonist-induced mobilization of intracellular calcium (Ca2+) was absent in mice selectively lacking two members of the β cell Gq protein family, Goαq and Go11 (5).

The M3 mACHR (M3R) is the predominant receptor subtype expressed in β cells and insulin-secreting cell lines (6, 7). Parasympathetic nerve endings that innervate the pancreas release acetylcholine during the preabsorptive and absorptive phases of feeding (8) to activate this receptor. Studies utilizing M3R knockout mice have implicated M3R as the receptor subtype responsible for cholinergic potentiation of glucose-stimulated insulin secretion (9, 10). Furthermore, mice selectively deficient in β cell M3Rs demonstrated impaired glucose tolerance and reduced insulin release, whereas mice overexpressing M3Rs in β cells exhibited a significant increase in glucose tolerance and insulin release (11). Similar observations were made in mice overexpressing constitutively active β cell M3Rs (1).

Signaling by the M3R also activates ERK1/2 in β cells, most likely downstream of elevated intracellular calcium (12–14). ERK1/2 activation enhances insulin gene transcription following nutrient-induced insulin secretion (15–18). We have reported previously that the GPCR complex T1R1/T1R3 is an early amino acid sensor in the MIN6 pancreatic β cell line and in other cell types (12). Similar to M3R, T1R1/T1R3 activation leads to a rise in Ca2+ and ERK1/2 phosphorylation that is partially dependent upon phospholipase C β activation. Reduced expression of T1R3 in MIN6 cells resulted in a decrease of amino acid-induced ERK1/2 and mammalian target of rapamycin complex 1 activation. Signaling defects in cells in which the receptor had been depleted included a reduction in the ability of amino acids to induce changes in Ca2+ (12).

Despite the impaired ability of amino acids to stimulate ERK1/2 in T1R3-depleted MIN6 cells, carbachol, a muscarinic
receptor agonist, activated ERK1/2 better in T1R3-depleted cells than in control cells (12). We explored the underlying mechanisms for the enhanced carbahol response in MIN6 cells to determine whether similar mechanisms were enlisted to compensate for amino acid deficiency.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2/AM was purchased from Molecular Probes. Nifedipine was purchased from Calbiochem. 2-Amino-ethoxydiphenyl borate (2-ABP) was purchased from Sigma. Thapsigargin was purchased from Santa Cruz Biotechnology.

**Cell Culture**—MIN6 cells were cultured, and stable cell lines with T1R3 expression reduced following expression of a short hairpin were created and maintained as described previously (12).

**Calcium Assays**—Cells were plated at 80% confluency in white-walled, 96-well plates (Costar 3903). After 48 h, the cells were washed twice with PBS (0.2 ml/well) and incubated with 5 μM Fura-2/AM diluted in Krebs-Ringer bicarbonate solution (KRKH) containing 115 mM NaCl, 5 mM KCl, 24 mM NaHCO3, 1 mM MgCl2, 2.5 mM CaCl2, 25 mM HEPES (pH 7.4), 0.1% BSA, and 4.5 mM glucose for 1 h (0.1 ml/well). Cells were then washed twice with KRKH (0.2 ml/well) and equilibrated in the same buffer for 30 min (0.1 ml/well). Agents were applied (0.1 ml/well) to triplicate wells at 2× concentrations using injectors at a rate of 225 μl/s. Changes in Ca2+ were assessed every 0.74 s by dual excitation of Fura-2 at 340/11 and 380/20 nm (center/bandpass) and emission at 508/20 nm using the SynergyTM 2 multimode microplate reader (BioTek) with Gen5TM software.

Cells were pretreated with the indicated inhibitors for 30 min prior to stimulation. For experiments performed in the absence of calcium, cells were loaded, washed, and equilibrated with calcium-free KRKH in which MgCl2 was substituted for 2.5 mM CaCl2. To assess store-operated calcium entry (SOCE), intracellular stores were depleted using 10 μM thapsigargin. Calcium was then replenished with a second injection of KRKH containing 12.5 mM CaCl2 (5× concentration, 50 μl/well). To assess receptor-operated calcium entry (ROCE), after calcium repletion, a third injection was required to apply 0.6 μM carbahol (6× concentration, 50 μl/well). Final concentrations of all agents were 1×. For experiments involving nifedipine or 2-APB, cells were pretreated with inhibitors for 30 min prior to stimulation. All steps in each assay were performed at room temperature.

**Nutrient Deprivation**—MIN6 cells were plated as above for calcium assays or in 12-well plates for RNA or protein isolation. To examine the effects of reduced amino acids, cells nearing confluency were washed twice with PBS and incubated with KRKH supplemented with 10% dialyzed serum, 4.5 mM glucose, and either 1.0× or 0.1× amino acids for 16 h at 37 °C and 10% CO2 prior to stimulation with carbahol or cell lysis. Calcium was measured as above with reduced amino acids throughout. To examine the effects of reduced glucose, cells were incubated as above in KRKH containing 10% dialyzed serum, 1× amino acids, and either 25 or 2 mM glucose. Human islets were provided by the Integrated Islet Distribution Program. Islets were washed twice in KRKH and then once in KRKH containing 10% dialyzed serum, 4.5 mM glucose, and either 0.1× or 1× amino acids prior to treatment overnight.

**Immunoblotting**—Cells were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.2 mg/ml PMSF, 100 mM NaF, and 2 mM Na3VO4. For lysates from stable cell lines, 40 μg of protein, as determined by BCA assay (Pierce), was resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C. Antibodies were diluted in 5% milk/TBST as follows: M3R muscarinic receptor (1:250, Millipore, catalog no. AB9018, rabbit, polyclonal) and ERK1/2 (1:2000, Abcam, catalog no. ab54230, mouse, monoclonal). For the carbahol time course, pERK1/2/ERK1/2 blots used 20 μg of protein. Antibodies were diluted in 5% milk/TBST as follows: pERK1/2 (1:1000, Sigma, catalog no. M8159, mouse, monoclonal) and ERK1/2 (1:1000, 691 rabbit (19)). The membranes were then washed with TBST and incubated for 1 h at room temperature with secondary antibodies: donkey anti-rabbit IRDye 680RD or donkey anti-mouse IRDye 800CW (1:10,000, Li-Cor Biosciences). The membranes were washed with TBST and then imaged using the Li-Cor Odyssey infrared imaging system. Blots were quantified using Li-Cor Odyssey application software (version 3.0).

**RNA Isolation, cDNA Synthesis, and Real-time Quantitative PCR**—Cells or human islets were harvested in TRI reagent® solution, and RNA was extracted according to the instructions of the manufacturer (Applied Biosystems). cDNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems). SYBR Green Supermix with ROX was purchased from Bio-Rad. GAPDH was used as an internal expression control. The primers were as follows: GAPDH, 5'-CTGGAGAAAACCTGCAAAGTA-3' (forward) and 5'-TGTGTCGTGTAAGCCGTATCCA-3' (reverse); actin, 5'-AGGTCATCACATTGCAACAGA-3' (forward) and 5'-CCTTCTACGTATGGAATTGATGTAG-3' (reverse); M3R (human) muscarinic receptor, 5'-ATTAGACACTTGTGTTCATTGTTAGT-3' (forward) and 5'-CCAGGCCACGAGAAACCTTA-3' (reverse); M1R muscarinic receptor, 5'-CCCTGGACAGGTTGCTTCATC-3' (forward) and 5'-AGCAGACCTGGACGTTCAGCAG-3' (reverse); mouse M3R muscarinic receptor, 5'-ACAGGCACTTGAGACCGCC-3' (forward) and 5'-AAAGCAACACTTCAAGAGGAGTC-3' (reverse); M5R muscarinic receptor, 5'-GGGCAAGAGGAGGGGCTAC-3' (forward) and 5'-CCGGGGTCGGTGACATCGT-3' (reverse); Gαq, 5'-GCCGACCCCTTCCTCCTCCTGC-3' (forward) and 5'-CCCCCTACATCGACGATTCACTGTA-3' (reverse); RGS4, 5'-TCTGGCGGTCCTCCATGCTATG-3' (forward) and 5'-TCTGGCTTTACCACTGGCAAGTTG-3' (reverse); and TRPC6, 5'-GGGAGACAGCCGCTACCCGCA-3' (forward) and 5'-AATCCTGCTCGCCGGCTAGG-3' (reverse). Validated T1R3 primers were purchased from Bio-Rad (unique assay no. qmnuCED004159).

**Statistical Analysis**—Results were expressed as means ± S.E. determined from three independent experiments. Statistical significance was calculated using Student's t test.
RESULTS

Carbachol-induced Changes in Ca\(^{2+}\)_i and M3R Expression Are Enhanced in MIN6 Cells after Depletion of T1R3—To determine the basis for the increased carbachol-induced ERK1/2 phosphorylation in MIN6 cells with reduced T1R3 expression (12), we first examined the effect of carbachol on Ca\(^{2+}\)_i after loading cells with the ratiometric calcium indicator Fura-2. Stimulation of cells with carbachol produced a bi-phasic rise in Ca\(^{2+}\)_i, composed of a rapid and transient peak followed by a sustained plateau phase (20, 21). Compared with the control, we observed a larger rise in peak Ca\(^{2+}\)_i in MIN6 cells with depleted T1R3 as well as a faster decline during the second phase of the carbachol response (Fig. 1, A and B).

One possible reason for enhanced carbachol-induced changes in Ca\(^{2+}\)_i in MIN6 T1R3 knockdown cells could be increased expression of mAChRs. Therefore, we performed real-time quantitative PCR to compare changes in expression of the Gq-coupled mAChRs, M1, M3, and M5, in MIN6 control and T1R3 knockdown cells. There was 2.35 ± 0.06-fold increase in M3R mRNA in the T1R3 knockdown cells compared with the control, whereas the expression of M1 and M5 receptors did not change significantly (Fig. 1C). The increase in M3R mRNA in T1R3 knockdown cells was mirrored by an increase in M3R protein expression that was found by immunoblotting lysates from control and T1R3 knockdown cells by Western blotting (B). ERK1/2 were blotted in the same lysates as the loading control. Blots were quantified using Li-Cor Odyssey application software. Means ± S.E. from three independent experiments are shown. *, p < 0.02, paired Student’s t test.

In addition to mAChRs, we also examined the expression of the regulator of G protein signaling 4 (RGS4) and transient receptor potential channel 6 (TRPC6). RGS proteins are GTPase-activating proteins that enhance G\(_\alpha\)-GTP hydrolysis, thereby decreasing the lifetime of active states of G protein subunits, and RGS4 is expressed in β cells (22). TRPC6 has been reported to be a receptor-operated cation channel that is activated upon GPCR stimulation and subsequent phospholipase C β activation (23, 24). Calcium entry occurring through plasma membrane channels as a result of GPCR activation independent of the state of Ca\(^{2+}\)_i stores is referred to as ROCE (25). It has been demonstrated that carbachol is capable of inducing
ROCE by activating and promoting cell surface expression of TRPC6 downstream of muscarinic receptor binding (26, 27). Expression of neither RGS4 nor TRPC6 was significantly different in T1R3 knockdown cells compared with the control, suggesting that the enhanced carbachol response was not a result of decreased RGS4 or increased TRPC6 expression (Fig. 1C).

**Carbachol-induced Changes in Ca^{2+} in MIN6 Cells with Suppressed T1R3 Expression Are Largely Dependent on Release of Ca^{2+} from Intracellular Stores** —The initial rapid rise in calcium observed upon carbachol stimulation in β cells has been shown to be due to the inositol 1,4,5-triphosphate-mediated release of calcium from ER stores, whereas the second phase is maintained by SOCE (21). SOCE refers to calcium influx that occurs through store-operated calcium channels (SOCCs) as a result of Ca^{2+} stores depletion (28). We investigated whether the contributions of Ca^{2+} stores or SOCE to carbachol-induced changes in Ca^{2+} differed in MIN6 cells with suppressed T1R3 expression compared with control cells. Cells were stimulated with carbachol in the absence of extracellular calcium to determine whether activation of M3R in the T1R3 knockdown cells induced a larger rise in Ca^{2+} as a result of the release of calcium from intracellular stores. We found that carbachol stimulated a larger rise in Ca^{2+} in the absence of extracellular calcium in the T1R3 knockdown cells compared with control cells (Fig. 2, A and B). This finding suggests that, in addition to elevated M3R, a larger release of calcium from intracellular stores may contribute to the enhanced first phase of the carbachol response in the MIN6 T1R3 knockdown cells.

We next determined whether the larger carbachol-induced release from intracellular stores was a consequence of more calcium stored in the ER of cells with reduced T1R3 by treating cells with thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, to deplete ER calcium stores (29). In the absence of extracellular calcium, thapsigargin induced a similar rise in Ca^{2+} in T1R3 knockdown and control cells (Fig. 2, C and D). When extracellular calcium was replenished, allowing SOCE to occur, calcium entry through SOCCs was similar in both T1R3 knockdown and control cells. We also explored ROCE, after calcium restoration and SOCE had occurred, by stimulating cells with carbachol. Despite little or no change in TRPC6 expression in MIN6 cells depleted of T1R3, ROCE was absent (Fig. 2, C and D), suggesting that another, as yet unidentified channel may contribute to ROCE in MIN6 cells.

Consistent with the larger release of calcium from intracellular stores induced by carbachol, a greater portion of the peak carbachol response was sensitive to inhibition by thapsigargin in the T1R3 knockdown cells (Fig. 3A). Thapsigargin blocked a greater portion of carbachol-induced changes in Ca^{2+}, at 2 min in the control cell line. It is possible that SOCE triggered by carbachol generates a smaller influx of Ca^{2+} through SOCCs in the T1R3 knockdown cells compared with control cells. This
might explain why the second phase of the calcium response declined at a faster rate and was less affected by thapsigargin in T1R3 knockdown cells. 2-APB, an inositol 1,4,5-triphosphate receptor antagonist, inhibited peak and 2 min carbachol-induced rises in Ca\(^{2+}\) to a similar degree in both control and T1R3 knockdown cells (Fig. 3, A and B). It is conceivable that the inhibitory effect of 2-APB on carbachol-stimulated changes in Ca\(^{2+}\) was due to its actions on SOCCs rather than through inhibition of inositol 1,4,5-triphosphate receptors (30). Using an ER-localized FRET sensor, it has been reported that, in MIN6 cells, carbachol-induced reductions in ER calcium were not inhibited by pretreatment with 2-APB despite lower overall Ca\(^{2+}\) (14). To determine whether or not 2-APB had an effect on SOCE or ROCE, MIN6 cells were pretreated with 50 \(\mu\)M 2-APB or 10 \(\mu\)M nifedipine for 30 min prior to stimulation. Unlike what was observed with 2-APB, nifedipine only had an effect on ROCE (Fig. 3, C and D). This is consistent with the data in Fig. 3, A and B, which demonstrated that nifedipine had a smaller effect on carbachol-induced changes in Ca\(^{2+}\) in the T1R3 knockdown cells in which ROCE was absent.

**Carbachol-induced Changes in Ca\(^{2+}\) and ERK1/2 Phosphorylation Are Enhanced in MIN6 Cells Deprived of Amino Acids**—Because we observed these changes in carbachol signaling in MIN6 cells in which T1R3 expression was stably suppressed and because we have shown previously that the T1R1/T1R3 complex is an early sensor of amino acids, we hypothesized that depriving cells of amino acids may mimic some of the altered carbachol signaling observed in T1R3 knockdown cells. Therefore, we performed experiments in which MIN6 cells were incubated in KRBH with 10% dia-
Carbachol-stimulated ERK1/2 phosphorylation and changes in Ca\(^{2+}\) are enhanced in MIN6 cells in reduced amino acids. A, MIN6 cells were in either 1.0× (control) or 0.1× (reduced) amino acids for 16 h. Cells were then stimulated with 100 μM carbachol for the indicated times. Lysates (20 μg of protein) were analyzed by Western blotting (IB) to assess pERK1/2 and total ERK1/2. Blots are representative of three independent experiments. B, blots were quantified using Li-Cor Odyssey application software. Data are mean pERK1/2/ERK1/2 ratio ± S.E. from three independent experiments. *, p = 0.016; **, p = 0.002; paired Student’s t test. C, MIN6 cells as in A were loaded with Fura-2/AM and stimulated with 100 μM carbachol. Data are normalized mean 340/380 values ± S.E. from three independent experiments, each in triplicate. D, bar graph of mean peak and 2-min 340/380 values ± S.E. from data in C. *, p = 0.018; paired Student’s t test. RFU, relative fluorescent units.

Because M3 receptor expression increased in T1R3 knockdown cells, we wanted to determine whether amino acid deprivation also affected M3R expression. As shown in Fig. 5, A and B, M3R mRNA and protein were increased in MIN6 cells deprived of amino acids compared with non-deprived cells (1.89 ± 0.19- and 1.51 ± 0.13-fold, respectively). To determine whether this was a general response to nutrient deficiency, we examined effects of lowering the glucose concentration from 25 to 2 mM on M3R expression. In this case, we found that M3R expression was reduced, not increased (Fig. 5C), indicating a differential responsiveness of M3R expression to amino acids and glucose.

We verified, in human islets, that amino acid deprivation affected M3R expression. As shown in Fig. 5D, M3R expression was enhanced 1.5-fold in islets of higher purity (90 and 80%). In islets of lower purity (70%), no change was observed. The fold increase of M3R expression in deprived islets was less than that in deprived MIN6 cells (Fig. 5A, ~1.9-fold). This difference may be due to varied responses among other cell types in islets, in contrast to the relative homogeneity of the MIN6 cell line.

Carbachol-stimulated Mobilization of Calcium from Intracellular Stores Is Unaltered in MIN6 Cells Deprived of Amino Acids—We further investigated whether reduced amino acids could mimic T1R3 knockout in MIN6 cells by determining the ability of carbachol to induce the release of calcium from intracellular stores under these conditions. In contrast to the significantly enhanced ability of carbachol to stimulate the release of calcium from stores in T1R3 knockdown cells (Fig. 2, A and B), there was no difference in the release from stores in amino acid-deprived cells. Indeed, a larger rise in Ca\(^{2+}\) induced by carbachol was significantly higher in deprived cells, the second phase of the response assessed at 2 min did not differ (Fig. 4, C and D).
comparing cells with normal and low amino acids (Fig. 6, A and B).

We also investigated whether depletion of intracellular calcium stores changed SOCE or ROCE in MIN6 cells deprived of amino acids to compare with changes in MIN6 T1R3 knockdown and control cells (Fig. 2, C and D). Contrary to what was observed in MIN6 cells with depleted T1R3, Ca^{2+} i resulting from store depletion, SOCE, or ROCE was higher in amino acid-deprived cells compared with non-deprived cells (Fig. 6, C and D). It is possible that, in amino acid-deprived cells, larger amounts of calcium are stored in the ER because of reduced activation of T1R3 in the absence of amino acids. The larger release of calcium from stores induced by thapsigargin could cause a greater influx of calcium through SOCCs, consistent with enhanced SOCE in deprived cells (Fig. 6, C and D). The altered ROCE observed in deprived cells could not be associated with an increase in TRPC6 mRNA, again suggesting the involvement of another channel in ROCE in MIN6 cells (Figs. 5 and 6, C and D). Together, these findings indicate that, although M3R expression is up-regulated by lowering amino acids in the medium, leading to a larger rise in carbachol-induced changes in Ca^{2+} i and ERK1/2 phosphorylation, the effects on intracellular stores, SOCE, and ROCE that were observed in MIN6 T1R3 knockdown cells were not mimicked. Finally, because we showed that fasting increased the expression of T1R3 in mouse tissues (12), we wondered whether amino acid deficiency also had an effect on T1R3 under these conditions. We found that expression of T1R3 mRNA was increased by incubation in 0.1× amino acids (Fig. 6E).

**DISCUSSION**

We explored the underlying mechanisms for the enhanced carbachol response in MIN6 cells following depletion of T1R3 and found that there was a significant increase in M3R expression in T1R3-depleted cells. A similar increase in M3R was also observed in amino acid-deprived cells. Thus, the increase in M3R is, at least in part, a response to amino acid deficiency. This change in M3R expression is not observed by reducing glucose. Given the remarkable sensitivity of cells to changes in glucose concentration, perhaps it is not so surprising that lowering glucose from 25 to 2 mM, a concentration often used as the control condition in experiments with these cells, caused a decrease in M3R expression. This result further emphasizes that the details of how isolated β cells or islets are handled ex vivo may have a large impact on signaling capability.

We evaluated the potential impact of other molecules known to impact β cell function through connections to GPCRs. RGS4 terminates signaling from M3Rs, thereby inhibiting their function in MIN6 cells and primary mouse islets (7). It seemed possible that RGS4 might have been suppressed in T1R3 knockdown cells to increase M3R function, but experimental findings did not support this idea. We also examined TRPC6 because a
microarray analysis suggested that it was up-regulated in T1R3 knockdown cells. Carbachol is capable of inducing ROCE by activating and promoting cell surface expression of TRPC6 downstream of muscarinic receptor binding (26, 27). Although the suspected change in its mRNA could not be validated, TRPC6 seemed a logical candidate and may be involved in some manner we did not detect (23, 24).

Differences in M3R signaling between shRNA depletion of T1R3 and amino acid limitation included effects on the kinetics of changes in intracellular free calcium. It is possible that differences in intracellular amino acid concentrations may have contributed to altered calcium signaling. Previously, we measured amino acid amounts in cells with T1R3 knocked down following amino acid withdrawal and subsequent amino acid repletion (12). Intracellular concentrations dropped rapidly when extracellular amino acids were removed. In contrast to amino acid withdrawal, T1R3 receptor knockdown did not reduce intracellular amino acid content. Branched side chain amino acids in particular were similar. Thus, intracellular amino acids may have led to some of the calcium responses that differed between receptor knockdown and amino acid-deprived cells. Finally, increased T1R3 expression in the amino acid-deprived cells may also have contributed to the differences noted.

Our findings suggest that multiple types of nutrient responses are linked with the M3R to support β cells during

**FIGURE 6.** Carbachol-stimulated mobilization of calcium from intracellular stores is unaltered in MIN6 cells deprived of amino acids. A, amino acid-deprived and control MIN6 cells were stimulated with 100 μM carbachol in calcium-free KRBH to assess release of calcium from intracellular stores. Data are normalized mean peak 340/380 values ± S.E. from three independent experiments, each in triplicate. B, mean peak 340/380 values ± S.E. from data in A. C, amino acid-deprived and control MIN6 cells loaded with Fura-2/AM were subjected to the calcium depletion/repletion protocol in Fig. 2C. Data are normalized mean 340/380 values ± S.E. from three independent experiments, each in triplicate. D, mean peak 340/380 values ± S.E. from data in C. *, p = 0.05; **, p = 0.046; ***, p = 0.048; paired Student’s t test. E, expression of T1R3 and GAPDH mRNA. Results are expressed as mean ± S.E. from three independent experiments. *, p = 0.001; paired Student’s t test. RFU, relative fluorescent units.
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nutrient stress. Beneficial effects of M3R activation in β cells have been deduced from defects in M3R knockout mice and were also revealed in studies of a designer Gq-coupled receptor engineered by incorporating mutations in M3R that rendered the receptor unresponsive to ACh but selectively sensitive to activation by the pharmacologically inert compound clozapine-N-oxide (13, 31–33). Chronic activation of the β cell Gq-coupled designer receptor in mice resulted in enhanced insulin release, decreased blood glucose concentrations, augmented β cell mass because of stimulation of β cell proliferation, increased insulin content, and amplified expression of several genes critical for β cell function (13, 33).

In addition to studies performed in mice, variations in the gene encoding M3R in humans are associated with a reduced acute insulin response and increased risk for early-onset type 2 diabetes (34). Type 2 diabetes is characterized by hyperglycemia resulting from the inability of β cells to secrete sufficient insulin to overcome peripheral insulin resistance (35). The increased demand on β cells to secrete insulin leads to β cell exhaustion, reduced β cell mass, and impaired insulin production (35, 36). The increase in M3R expression in amino acid-deprived cells provides independent support for the idea that modulating the expression of and/or signaling through β cell M3Rs enhances β cell function and protects against some types of nutrient stress.

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