Muscarinic Stimulation of Pancreatic Insulin and Glucagon Release Is Abolished in M_3 Muscarinic Acetylcholine Receptor−Deficient Mice

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Pancreatic muscarinic acetylcholine receptors play an important role in stimulating insulin and glucagon secretion from islet cells. To study the potential role of the M_3 muscarinic receptor subtype in cholinergic stimulation of insulin release, we initially examined the effect of the muscarinic agonist, oxotremorine-M (Oxo-M), on insulin secretion from isolated pancreatic islets prepared from wild-type (WT) and M_3 receptor−deficient mice ($M3^{+/-}$ and $M3^{-/-}$ mice). At a stimulatory glucose level (16.7 mmol/l), Oxo-M strongly potentiated insulin output from islets of WT mice. Strikingly, this effect was completely abolished in islets from $M3^{-/-}$ mice and significantly reduced in islets from $M3^{+/-}$ mice. Additional in vitro studies showed that Oxo-M−mediated glucagon release was also virtually abolished in islets from $M3^{-/-}$ mice. Consistent with the in vitro data, in vivo studies showed that $M3^{-/-}$ mice displayed reduced serum insulin and plasma glucagon levels and a significantly blunted increase in serum insulin after an oral glucose load. Despite the observed impairments in insulin release, $M3^{-/-}$ mice showed significantly reduced blood glucose levels and even improved glucose tolerance, probably due to the reduction in plasma glucagon levels and the fact that $M3^{-/-}$ mice are hypophagic and lean. These findings provide important new insights into the metabolic roles of the M_3 muscarinic receptor subtype. *Diabetes* 53:1714–1720, 2004

A key feature of type 2 diabetes is that glucose fails to stimulate adequate release of insulin from pancreatic β-cells (1,2). Characteristically, the β-cell eventually fails to compensate for the gradually developing increase in insulin resistance, resulting in overt hyperglycemia. Sulfonylureas and related compounds stimulate insulin release in the absence of high glucose levels (3). Antidiabetic drugs that can potentiate insulin release in a glucose-dependent fashion would therefore be highly desirable (3).

Glucose-dependent insulin secretion is modulated by several hormones and neurotransmitters, among which acetylcholine plays a prominent role (reviewed in 4–6). Acetylcholine is released from intrapancreatic parasympathetic (vagal) nerve endings during the preabsorptive and, most likely, the absorptive phase of feeding (5,6). The acetylcholine-mediated preabsorptive phase of insulin secretion, although relatively small compared with the total release of insulin after a meal, seems to be of particular importance for maintaining normal glucose tolerance (5,6). A considerable body of evidence also suggests that increased vagal/cholinergic activity may be involved in enhanced insulin secretion in certain animal models of obesity (7–12).

An important feature of the cholinergic regulation of pancreatic insulin release is that acetylcholine stimulates insulin release in a strictly glucose-dependent manner, becoming more and more effective as the plasma glucose concentration increases (5,6). This concept is supported by a large number of in vivo (13–15) and in vitro (16–21) functional studies.

The acetylcholine/vagus effects on pancreatic insulin release are mediated by activation of muscarinic acetylcholine receptors located on the pancreatic β-cells (4–6). Molecular cloning studies have revealed the existence of five molecularly distinct muscarinic receptor subtypes (M_1−M_5) (22). Receptor localization studies suggest that multiple muscarinic receptors (M_1, M_3, M_4, and M_5) are expressed in pancreatic islets/β-cells or β-cell−derived tumor cell lines (23–25). However, the M_3 muscarinic receptor appears to be the predominant subtype expressed by pancreatic β-cells (4–6,23–25). Interestingly, previous studies suggest that acetylcholine can also stimulate the secretion of glucagon by acting on muscarinic receptors located on pancreatic α-cells (5,6,26–28).

To better understand the physiological roles of the M_3 muscarinic receptor, we recently used gene-targeting technology to generate M_3 muscarinic receptor−deficient mice ($M3^{-/-}$ mice) (29). In an initial study, we reported that $M3^{-/-}$ mice show a pronounced reduction in body weight associated with hypophagia and a significant decrease in serum leptin and insulin levels (29).

To study the potential role of M_3 muscarinic receptors in β- and α-cell function, we carried out systematic in vitro

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insulin and glucagon release studies using isolated pancreatic islets prepared from wild-type (WT), $M3^{+/-}$, and $M3^{-/-}$ mice. To examine whether the deficits observed in the in vitro hormone release studies were correlated with changes in blood glucose, insulin, and glucagon levels and altered glucose tolerance, we carried out additional in vivo experiments using WT, $M3^{+/-}$, and $M3^{-/-}$ mice.

Our results demonstrated in an unambiguous fashion that muscarinic stimulation of pancreatic insulin and glucagon release is mediated by the M_3 muscarinic receptor subtype. These deficits were accompanied by pronounced changes in plasma insulin and glucagon levels in vivo. Our findings highlight the usefulness of gene-targeting technology in shedding light on the metabolic roles of individual members of the muscarinic receptor family.

RESEARCH DESIGN AND METHODS

M_3 muscarinic receptor−deficient mice were generated as previously described (29). All mice used for the present study (WT, $M3^{+/-}$, and $M3^{-/-}$ mice) were littermates generated by intermating heterozygous M_3 receptor mutant mice ($M3^{+/-}$ mice; genetic background: 129SvEv/C57BL/6J). Unless indicated otherwise, all experiments were carried out with adult male mice ages 3–7 months at the time of testing.

Mice were housed four to five per cage in a room with a 12-h light/dark cycle (lights on at 6:00 A.M.) and given ad libitum access to food and water. All manipulations were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland.

RT-PCR analysis. Total RNA was extracted from mouse pancreatic islets and total brain using the total RNA isolation kit from Invitrogen. Extracted RNA samples were treated with 4 units of RNAse-free DNase (Ambion) at 37°C for 1 h to remove residual genomic DNA. The RNA was then reversed transcribed with an oligo-dT_{16} primer and MuLV RT using the GeneAmp RNA PCR kit, as described by the manufacturer (Applied Biosystems). The reverse transcription step was omitted in control samples to test for the presence of contaminating genomic DNA. The reverse-transcribed products were screened for the presence of M_1−M_5 cDNA by PCR, using the GeneAmp RNA PCR kit (Applied Biosystems) and an Eppendorf Mastercycler thermal cycler (40 cycles of 94°C at 1 min, 56°C at 2 min, and 72°C at 3 min). PCRs were carried out in a final volume of 50 μl containing 10 μl of the RT reaction product (corresponding to ~0.5–1 μg RNA), 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 1 mmol/l of each dNTP, 100 ng each of the sense and corresponding antisense primers, and 1.25 units of AmpliTaq DNA polymerase. The identity of the PCR products was confirmed by restriction analysis (data not shown). The RT-PCR products were separated on 1.5% agarose gels.

The sizes of the expected RT-PCR products were M_1 (497 bp), M_2 (480 bp), M_3 (498 bp), M_4 (474 bp), and M_5 (485 bp). Subtype-specific primers were designed based on the mouse M_1−M_5 muscarinic receptor sequences (GenBank data accession numbers: M_1, J04192; M_2, AF264049; M_3, F264050; M_4, X63473; and M_5, AF264051). The following primers were used: *1*) M_1: 5′-TCTGCTCATCAGCTTTGACCG-3′ (forward), 5′-CATCCTCTTCCTCTTCT TCTTTCC-3′ (reverse); *2*) M_2: 5′-TGTCAGCAATGCCTCCGTTATG-3′ (forward), 5′-GCCTTGCCATTCTGGATCTTG-3′ (reverse); *3*) M_3: 5′-GGTGTGAT GATTGGTCTGGCTTG-3′ (forward), 5′-GGAAGCAGAGTTTTCCAGGGAG-3′ (reverse); *4*) M_4: 5′-TCAAGAGCCCTCTGATGAAGCC-3′ (forward), 5′-AGATT GTCCGAGTCACTTTGCG-3′ (reverse); and *5*) M_5: 5′-GCTGACCTCCAAGGTT CCGATTC-3′ (forward), 5′-CCGTCAGCTTTTACCACCAAT C-3′ (reverse).

Islet isolation. Mice were killed by cervical dislocation, and their abdominal wall was opened. The pancreas was distended by injecting 3–6 ml of dissociation solution (Hanks' balanced salt solution containing 0.23 mg/ml liberase enzyme; Roche Diagnostics) via the common bile duct. The organ was then removed from the abdomen and incubated with an additional 1.5 ml of dissociation solution for 23 min at 37°C. Islets of Langerhans were then hand picked from the digested pancreatic tissue using a dissection microscope.

In vitro insulin and glucagon secretion studies. Insulin and glucagon secretion were measured after static incubation of pancreatic islets (23) prepared from WT and M_3 receptor mutant mice. Islets were preincubated for 60 min in a modified Krebs solution containing (in mmol/l) 5.6 glucose, 120 NaCl, 5 KCl, 1.1 $MgCl_2$, 2.6 $CaCl_2$, 25 $NaHCO_3$, and 0.5% BSA. The solution was equilibrated with 95% O_2/5% CO_2 and maintained at 37°C at a pH of 7.4 throughout the experiments. Batches of 10–12 islets were then transferred into tubes containing the same solution supplemented with various amounts of glucose and oxotremorine-M (Oxo-M; Sigma). The tubes were incubated for 1 h in a chamber maintained at 37°C and equilibrated with 95% O_2/5% CO_2. The medium was then withdrawn and frozen for the analysis of insulin and glucagon concentrations. The remaining islet insulin and glucagon content was extracted by sonication in acid/ethanol (30). The amount of insulin or glucagon secreted during the incubation period was normalized to the total insulin or glucagon content of the islets in each tube. Insulin concentrations were determined by enzyme-linked immunosorbent assay (ELISA; Alpco). Glucagon concentrations were measured via radioimmunoassay (RIA; Linco).

Oral glucose tolerance test. After an overnight (8–10 h) fast, mice received an oral load of glucose (2 mg/g body wt) via oral gavage. Blood samples were collected via retro-orbital sinus puncture before (t = 0 min) and 15, 30, 60, and 120 min after glucose administration. Blood glucose levels were determined using an automated blood glucose reader (Glucometer Elite Sensor; Bayer).

At the same time points, blood collected via the retro-orbital sinus puncture was transferred into serum separator tubes (0.5 ml, Capojet; Terumo Medical). Serum was obtained by centrifugation (10 min at 4°C) at 1,200*g* using an Eppendorf bench top centrifuge. Serum insulin concentrations were determined via ELISA (Alpco) using mouse insulin as a standard.

In a separate experiment, we also determined plasma glucagon levels at defined time points after oral administration of glucose (2 mg/g) to mice that had been fasted for 8–10 h overnight. Plasma glucagon concentrations were measured via RIA (Linco).

Determination of pancreatic insulin content. Mice were killed by cervical dislocation, and whole pancreata were dissected and immediately frozen in liquid nitrogen. Protein extracts were prepared using the acid-ethanol method (26). In brief, pancreata weighing 200–300 mg were homogenized in 1 ml acid-ethanol (95% ethanol and 10.2 N HCl at a ratio of 50:1) by an Ultrasonic Homogenizer (Biologic) for 2 min applying 20 pulses. After an overnight incubation at 4°C, the extracts were centrifuged at 650*g* for 30 min at 4°C. Insulin concentrations in pancreatic extracts were measured via ELISA (Alpco), following the manufacturer's instructions. The protein content of the pancreatic extracts was determined using the Bio-Rad protein assay kit.

Statistics. Data are expressed as means ± SE for the indicated number of observations. P values were calculated using one-way ANOVA followed by appropriate post hoc tests (Fisher's least significant differences method for islet insulin and glucagon measurements and Bonferroni method for all other studies).

RESULTS

RT-PCR analysis of muscarinic receptor expression in mouse pancreatic islets. To examine which muscarinic receptor subtypes are expressed in pancreatic islets of the mouse, we subjected total RNA prepared from WT mouse pancreatic islets to RT-PCR amplification using M_1−M_5 mouse muscarinic receptor−specific primers. Because all five muscarinic receptors are known to be expressed in the brain (31), mouse brain total RNA served as a positive control. As expected, all five muscarinic receptors were found to be expressed in the WT mouse brain (Fig. 1*A*). In contrast, only M_1 and M_3 receptor cDNA could be detected in samples from WT mouse islets (Fig. 1*B*). Figure 1*C* shows the absence of M_3 receptor transcripts in pancreatic islets prepared from $M3^{-/-}$ mice.

In vitro insulin release studies. To study the potential role of the M_3 muscarinic receptor subtype in augmenting pancreatic insulin release, we carried out a series of in vitro insulin release studies using isolated islets prepared from WT, $M3^{+/-}$, and $M3^{-/-}$ mice. Oxo-M, a non–subtype-selective muscarinic agonist, was used as a hydrolytically stable muscarinic stimulant throughout all experiments.

In the absence of Oxo-M, static incubation of pancreatic islets from WT mice with a basal concentration of glucose (5.6 mmol/l) led to the release of only very small amounts of insulin (Fig. 2). The magnitude of this basal secretory response was not significantly affected by the absence of M_3 receptors or the presence of Oxo-M (0.5 or 20 μmol/l) (Fig. 2).

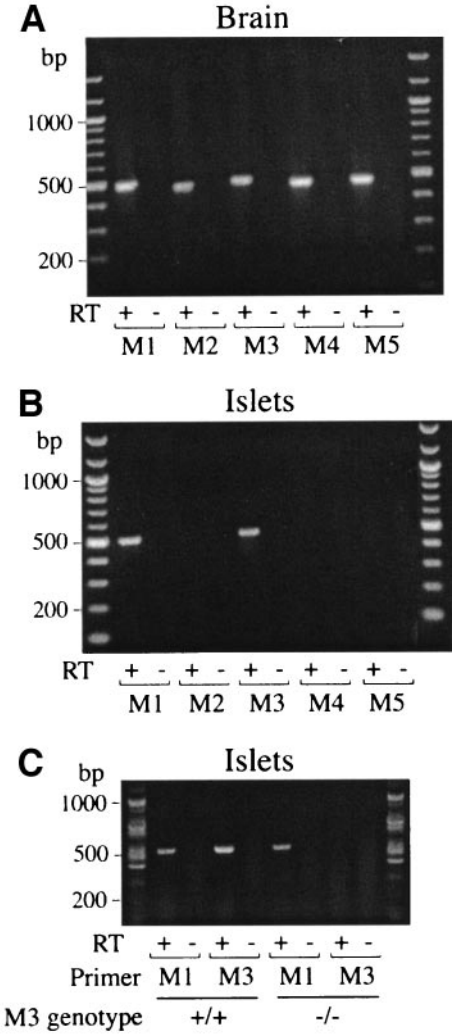

FIG. 1. RT-PCR analysis of M_1–M_5 muscarinic receptor expression in mouse pancreatic islets and brain. Representative 1.5% agarose gels (stained with ethidium bromide) are shown. Primers specific for the individual mouse muscarinic receptors were used to amplify cDNA prepared from mouse pancreatic islets and brain total RNA. *A*: In WT mice brain, as expected, all five muscarinic receptor subtypes were found to be expressed (positive control). *B*: In WT mice pancreatic islets, only M_1 and M_3 muscarinic receptor mRNA could be detected. *C*: In islets from $M3^{-/-}$ mice, M_3 receptor transcripts were not detected. Control samples that had not been treated with RT did not give any detectable RT-PCR products, confirming the absence of contaminating genomic DNA. Three separate experiments gave similar results. Marker DNA: 100-bp DNA ladder (Biolabs).

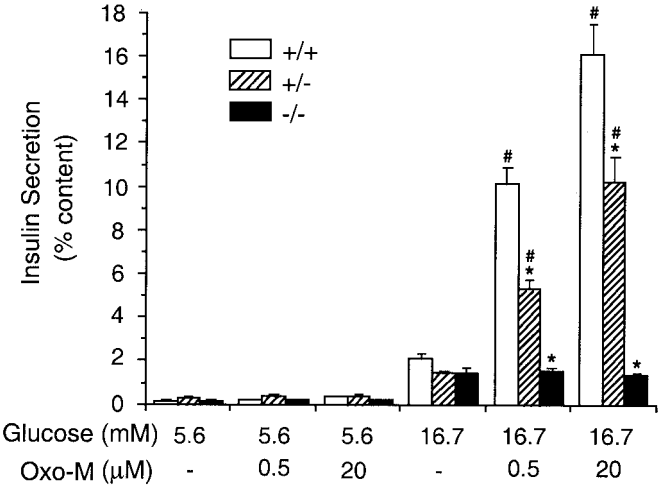

FIG. 2. Muscarinic agonist–mediated potentiation of glucose-dependent insulin release in WT and M_3 muscarinic receptor mutant mice. Isolated pancreatic islets from mice of the indicated M_3 receptor genotypes were incubated for 1 h in Krebs solution containing the indicated glucose concentrations, either in the absence or presence of the muscarinic agonist Oxo-M (0.5 or 20 μmol/l). The amount of insulin secreted into the medium during the 1-h incubation period was normalized to the total insulin content of the islets in each tube. Data are means ± SE (n = 4–10). *P < 0.01 vs. corresponding WT value; #P < 0.01 vs. corresponding control (16.7 mmol/l glucose, no Oxo-M) value.

In contrast, incubation of pancreatic islets from WT and M_3 receptor mutant mice with a high concentration of glucose (16.7 mmol/l) led to a significant increase (~10- to 15-fold above basal levels determined in the presence of 5.6 mmol/l glucose) in insulin release (Fig. 2). In islets from WT mice (16.7 mmol/l glucose), the addition of Oxo-M (0.5 or 20 μmol/l) led to a pronounced potentiation of glucose-dependent insulin release (Fig. 2). Strikingly, the Oxo-M–mediated potentiation of glucose-dependent insulin release was totally abolished in islets prepared from $M3^{-/-}$ mice and significantly reduced (by ~30–50%) in islets from $M3^{+/-}$ mice (Fig. 2).

Insulin content in the whole pancreas. Total pancreatic insulin content did not differ significantly between 6-month-old WT and M_3 receptor mutant mice (276 ± 40 [WT] vs. 228 ± 15 [$M3^{+/-}$] vs. 341 ± 36 [$M3^{-/-}$] pg insulin/μg pancreatic protein; n = 6). Moreover, mean wet weights of the pancreata (expressed as a percentage of total body weight) did not differ significantly among the three genotypes (0.92 ± 0.06 [WT] vs. 0.80 ± 0.05 [$M3^{+/-}$] vs. 0.95 ± 0.08% [$M3^{-/-}$]; n = 6). Preliminary immunohistochemical studies showed that the number and size distribution of pancreatic islets were similar in WT and M_3 receptor mutant mice (data not shown).

Blood glucose and serum insulin levels. To examine whether M_3 receptor mutant mice showed altered blood glucose and insulin levels, we continuously monitored these parameters in freely fed WT, $M3^{+/-}$, and $M3^{-/-}$ mice over a 6-month period. As shown in Fig. 3*A*, $M3^{+/-}$ mice showed blood glucose levels that, at most time points, were not significantly different from the corresponding levels obtained with the WT control mice. In contrast, $M3^{-/-}$ mice showed a significant reduction in blood glucose levels (by ~20–50%) starting at age 3 months (Fig. 3*A*). This hypoglycemia persisted in $M3^{-/-}$ mice that were >1 year old (data not shown). The serum insulin levels of 1-month-old WT, $M3^{+/-}$, and $M3^{-/-}$ mice did not differ significantly among the three genotypes (Fig. 3*B*). In contrast, 2- to 6-month-old $M3^{+/-}$ and $M3^{-/-}$ mice showed significant reductions (by approximately two- to sixfold) in serum insulin levels as compared with their WT littermates (Fig. 3*B*). As we reported previously (29), $M3^{-/-}$ mice showed pronounced reductions in body weight (by ~25%) throughout the 6-month observation period (Fig. 3*C*). This difference in body weight persisted throughout the entire life of the $M3^{-/-}$ mice (data not shown). In contrast, $M3^{+/-}$ mice showed body weights that were similar to those of their WT littermates (Fig. 3*C*).

Oral glucose tolerance test. To determine whether the lack of M_3 receptors was associated with changes in glucose tolerance in vivo, WT, $M3^{+/-}$, and $M3^{-/-}$ mice were subjected to an oral glucose tolerance test (OGTT; 2 mg glucose/g body wt). As shown in Fig. 4*A*, $M3^{+/-}$ mice

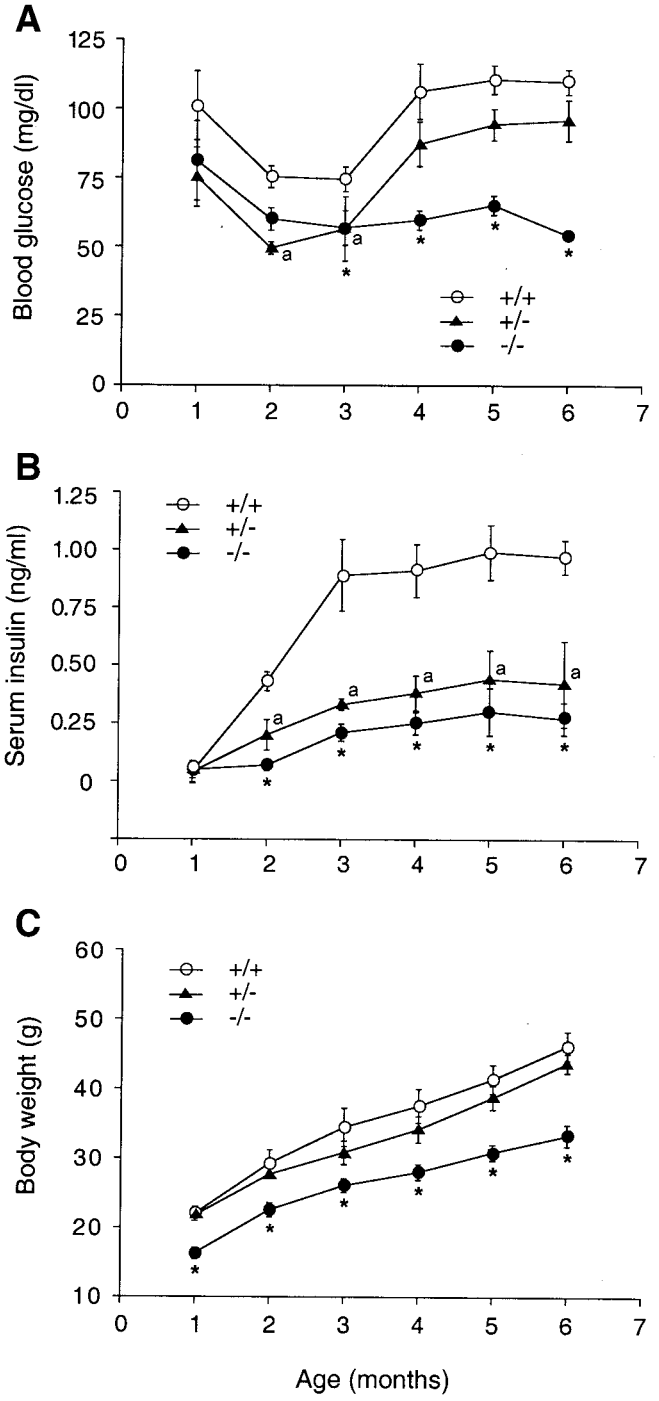

FIG. 3. Blood glucose and serum insulin levels of WT and M_3 muscarinic receptor mutant mice. Blood was collected via retro-orbital sinus puncture from freely fed male mice of the indicated M_3 receptor genotype that were ages 1–6 months. Blood glucose (*A*) and serum insulin levels (*B*) were determined at the indicated time points. *C*: Changes in body weight during the observation period. Data are means ± SE (n = 6 per group). *P < 0.05 for $M3^{-/-}$ vs. WT (t test); [a]P < 0.05 for $M3^{+/-}$ vs. WT (t test).

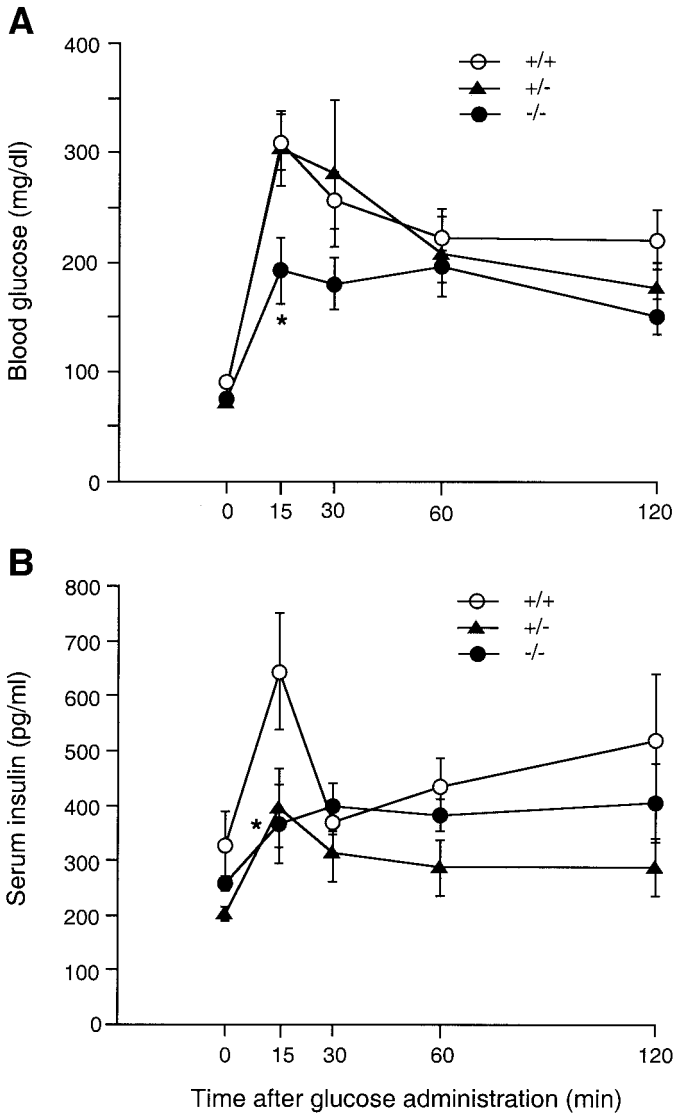

FIG. 4. OGTTs with WT and M_3 muscarinic receptor mutant mice. Fasted 6-month-old male mice of the indicated M_3 receptor genotype received an oral dose of glucose (2 mg/g). Blood glucose (*A*) and serum insulin (*B*) levels were determined at the indicated time points. Data are means ± SE (n = 6 per group). *P < 0.05 vs. corresponding WT value (t test).

exhibited a similar temporal pattern of changes in blood glucose levels as their WT littermates. In contrast, $M3^{-/-}$ mice showed a significant reduction in blood glucose levels compared with WT mice ($P < 0.05$ at 15 min after glucose administration), indicating that $M3^{-/-}$ mice cleared glucose more efficiently from the circulation than the WT control animals.

In WT mice, the increase in blood glucose levels was accompanied by a significant increase in serum insulin levels that reached peak values 15 min after glucose administration (Fig. 4*B*). Interestingly, the glucose-induced spike in serum insulin levels observed 15 min after glucose administration was significantly blunted in $M3^{-/-}$ mice ($P < 0.05$) (Fig. 4*B*). $M3^{+/-}$ mice also showed a reduction in serum insulin levels at this time point, which, however, failed to reach statistical significance (P = 0.08 vs. WT) (Fig. 4*B*).

In vitro glucagon release studies. The incubation of isolated pancreatic islets with muscarinic agonists not only facilitates insulin secretion but also stimulates the release of glucagon (5,6,26–28). To assess the potential role of the M_3 muscarinic receptor subtype in this activity, we carried out in vitro glucagon-release studies using isolated islets prepared from WT, $M3^{+/-}$, and $M3^{-/-}$ mice. In the absence of Oxo-M and in the presence of 16.7

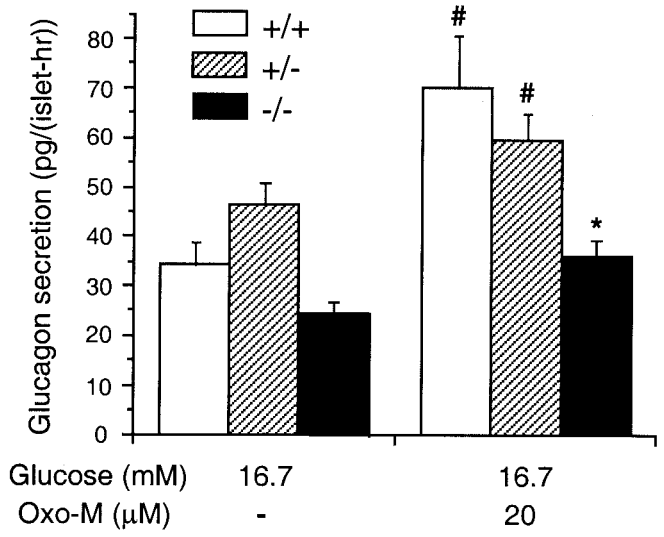

FIG. 5. Muscarinic agonist–mediated glucagon release in pancreatic islets from WT and M_3 muscarinic receptor mutant mice. Isolated pancreatic islets from mice of the indicated M_3 receptor genotypes were incubated for 1 h in Krebs solution containing 16.7 mmol/l glucose, either in the absence or presence of the muscarinic agonist, Oxo-M (20 μmol/l). The amount of glucagon secreted into the medium during the 1-h incubation period was normalized to the number of islets in each tube. Data are means ± SE (n = 4–8). *P < 0.01 vs. WT value; #P < 0.05 vs. corresponding control (no Oxo-M) value.

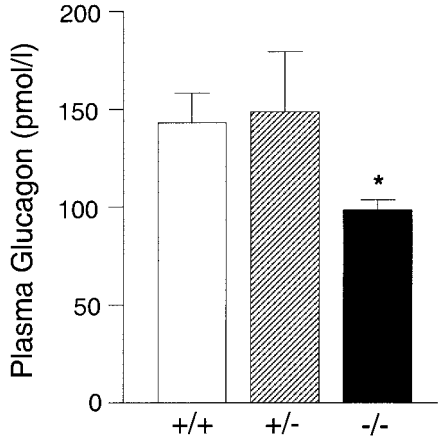

FIG. 6. Plasma glucagon levels in WT and M_3 muscarinic receptor mutant mice. Blood was collected via retro-orbital sinus puncture from freely fed 7-month-old male mice of the indicated M_3 receptor genotype. Data are means ± SE (n = 6–8 per group). *P < 0.05 vs. corresponding WT value (t test).

mmol/l glucose, the amount of glucagon released from $M3^{+/-}$ and $M3^{-/-}$ preparations did not differ significantly from the corresponding WT value.

The addition of Oxo-M (20 μmol/l) led to a significant increase ($P < 0.05$) in glucagon release from WT and $M3^{+/-}$ islets, as compared with the corresponding preparations that were not treated with Oxo-M (Fig. 5). In the presence of Oxo-M, $M3^{-/-}$ islets secreted significantly less ($P < 0.01$) glucagon than WT islets, clearly demonstrating a role for M_3 receptors in cholinergic control of glucagon secretion. Moreover, the amount of glucagon released by $M3^{-/-}$ islets in the presence of Oxo-M did not differ significantly ($P > 0.05$) from the amount released by $M3^{-/-}$ islets in the absence of Oxo-M.

Plasma glucagon levels in vivo. We next measured plasma glucagon levels in freely fed WT, $M3^{+/-}$, and $M3^{-/-}$ mice. Whereas $M3^{+/-}$ mice showed similar plasma glucagon levels as WT mice, $M3^{-/-}$ mice displayed significantly reduced plasma glucagon levels (~30–35% reduction vs. WT mice; $P < 0.05$) (Fig. 6). Similar findings were obtained when plasma glucagon levels were determined in fasted mice (116 ± 16 [WT] vs. 114 ± 22 [$M3^{+/-}$] vs. 52 ± 10 [$M3^{-/-}$] pmol/l; n = 6–8). Oral administration of glucose (2 mg/g) to these fasted animals resulted in decreased plasma glucagon levels in WT, $M3^{+/-}$, and $M3^{-/-}$ mice, measured 15 or 30 min after glucose administration. However, these decreases did not reach statistical significance ($P > 0.05$) for any of the three genotypes (data not shown).

DISCUSSION

The stimulatory effects of acetylcholine on pancreatic insulin release are known to be mediated by activation of muscarinic receptors (4–6). RT-PCR analysis showed that mouse pancreatic islets express M_1 and M_3 muscarinic receptors (Fig. 1). Similar findings were obtained with rat pancreatic islets (23,25). Whereas previous studies (24,25) reported the expression of additional muscarinic receptor subtypes (M_4 or M_5) in islet preparations or in β-cell–derived cell lines, we did not detect M_2, M_4, or M_5 receptor mRNA in mouse pancreatic islets. Because the majority of the cells in rat or mouse islets represent β-cells, it is highly likely that both M_1 and M_3 receptors are expressed by the insulin-secreting β-cells.

To better understand the role of the muscarinic cholinergic system in β-cell function, it is essential to identify the muscarinic receptor subtype(s) mediating stimulation of insulin release. Classic pharmacological studies using different "subtype-preferring" muscarinic antagonists have suggested that the M_3 receptor subtype (previously also referred to as "glandular M_2 receptor subtype") (32) plays a key role in the control of insulin secretion (26,27,32). However, the proper interpretation of these experiments is complicated by the limited subtype selectivity of the muscarinic antagonists used in these studies. For example, virtually all antagonists that have a high affinity for M_3 receptors (e.g., 4-DAMP, derivatives of sila-hexocyclium) also exhibit a high affinity for M_1 and M_5 receptors (33,34). Moreover, it is especially difficult to predict the simultaneous involvement of two or more muscarinic receptor subtypes (e.g., M_1 and M_3 receptors) in a specific functional response by using the currently available subtype-preferring muscarinic antagonists.

To circumvent these difficulties, we carried out in vitro insulin release studies using pancreatic islets prepared from mutant mice in which the M_3 muscarinic receptor gene had been inactivated by gene-targeting techniques (29). Consistent with previously published results (16–21), the muscarinic agonist, Oxo-M, had little effect on basal insulin release measured in the presence of a low concentration of glucose (5.6 mmol/l), using isolated islets from WT, $M3^{+/-}$, and $M3^{-/-}$ mice (Fig. 2). On the other hand, in the presence of a stimulatory glucose concentration (16.7 mmol/l), the addition of Oxo-M to islets prepared from WT mice resulted in a pronounced potentiation of insulin output (Fig. 2). Strikingly, this insulinotropic activity of Oxo-M was completely abolished in islets prepared from $M3^{-/-}$ mice (Fig. 2). Pancreata from $M3^{-/-}$ mice con-

tained normal amounts of total insulin, and preliminary studies showed that the number and size of pancreatic islets were similar in WT and $M3^{-/-}$ mice (data not shown). These observations demonstrated in an unambiguous fashion that muscarinic receptor–mediated stimulation of pancreatic insulin release is mediated by the M_3 receptor subtype and that M_1 or other muscarinic receptor subtypes do not contribute to this response to a significant extent. The potential functional role of islet M_1 receptors remains to be elucidated.

Interestingly, heterozygous M_3 receptor mutant mice ($M3^{+/-}$ mice), in which the density of M_3 receptors is reduced by ~50% (29), also showed significant impairments in muscarinic agonist–mediated potentiation of glucose-dependent insulin release (reduction in maximum secretory responses by ~30–50%). This observation indicated that >50% of the islet M_3 muscarinic receptors must be occupied to achieve maximum insulinotropic activity, at least in the mouse model. In contrast to many other muscarinic responses, M_3 muscarinic receptor–mediated augmentation of insulin release is therefore characterized by a very low degree of receptor reserve.

The M_3 muscarinic receptor, like the M_1 and M_5 receptor subtypes, is known to selectively couple to G proteins of the G_q family (22,33). Consistent with this finding, muscarinic stimulation of pancreatic β-cells leads to a series of biochemical events that are usually associated with the activation of G_q-type G proteins, including the activation of phospholipase C, protein kinase C, and phospholipase A2 (4–6). Stimulation of these signaling cascades eventually results in elevated intracellular calcium levels and an increase in the efficiency of calcium-dependent exocytosis of insulin-containing storage vesicles (4–6). These latter two mechanisms are predicted to be primarily responsible for the insulinotropic effects of M_3 receptor activation.

Our findings suggest that drugs that can selectively activate M_3 muscarinic receptors may be of potential therapeutic benefit in the treatment of type 2 diabetes. Such agents appear particularly attractive as they would exert their full insulinotropic effects only in the presence of stimulatory concentrations of glucose, an effect not seen with other antidiabetic drugs such as the commonly used sulfonylureas (3). Unfortunately, muscarinic agonists that can activate M_3 muscarinic receptors (or other muscarinic receptor subtypes) with a high degree of subtype selectivity are not available at present (22,33). The development of such agents therefore represents an important goal for medicinal chemists.

Consistent with the results of the in vitro insulin release studies, $M3^{+/-}$ and $M3^{-/-}$ mice showed reduced serum insulin levels in vivo (by approximately two- to sixfold, as compared with their WT littermates) (Fig. 3*B*). Moreover, $M3^{-/-}$ mice displayed a significant decrease in serum insulin levels 15 min after an oral glucose load (2 mg/g) (Fig. 4*B*). However, the $M3^{+/-}$ and $M3^{-/-}$ mice did not develop hyperglycemia or impaired glucose tolerance in vivo. In fact, $M3^{-/-}$ mice showed significantly reduced blood glucose levels (Fig. 3*A*) and even displayed a significant increase in glucose tolerance in the OGTT (Fig. 4*A*), indicative of an increase in insulin sensitivity. Consistent with this observation, $M3^{-/-}$ mice showed a more pronounced and prolonged hypoglycemia response than WT control mice in an insulin tolerance test (29).

We also demonstrated previously that the mass of peripheral fat deposits is significantly reduced in $M3^{-/-}$ mice and that this phenotype was linked to a reduction in food intake (29). It is well known that lean animals (individuals) generally exhibit an increase in insulin sensitivity (35). Muscarinic receptors located in insulin-sensitive tissues, such as liver, skeletal muscle, and fat, do not seem to play a significant role in regulating glucose utilization or other metabolic functions in these tissues (36). It is therefore likely that the increased insulin sensitivity displayed by the $M3^{-/-}$ mice in vivo was primarily due to the reduction in body fat mass (29). This observation may explain why the lack of cholinergic stimulation of insulin release observed with islets from $M3^{-/-}$ mice in vitro did not result in hyperglycemia and/or impaired glucose tolerance in vivo. The increase in insulin sensitivity displayed by the $M3^{-/-}$ mice may represent another major factor contributing to the hypoinsulinemia associated with the lack of M_3 receptors.

Interestingly, although the $M3^{-/-}$ mice were able to clear glucose from the circulation more efficiently than WT mice (OGTT), glucose clearance was unaltered in $M3^{+/-}$ mice (Fig. 4*A*). In contrast to $M3^{-/-}$ mice, which showed a significantly reduced body weight, the body weight of $M3^{+/-}$ mice did not differ significantly from that of their WT littermates (Fig. 3*C*). These findings therefore support the concept that the increased insulin sensitivity displayed by the $M3^{-/-}$ mice is caused primarily by the reduction in body weight/body fat mass (29).

In a recent study (29), we provided evidence that the absence of hypothalamic M_3 receptors may be responsible, at least partially, for the hypophagia displayed by the $M3^{-/-}$ mice. This hypophagia phenotype may represent a major factor contributing to the reduction in body weight and blood glucose levels associated with the absence of M_3 receptors (29).

The activation of vagal nerves not only promotes insulin release but also stimulates the release of glucagon from pancreatic α-cells (5,6,28). In vitro and in vivo experiments suggest that this activity is mediated by muscarinic receptors predicted to be located on pancreatic α-cells (5,6,26–28). In the present study, we demonstrated that the muscarinic agonist, Oxo-M, failed to stimulate the release of glucagon from isolated islets prepared from M_3 receptor–deficient mice ($M3^{-/-}$ mice) (Fig. 5), indicating that this response is mediated by the M_3 receptor subtype in WT animals. In agreement with this finding, previous in vitro and in vivo studies using muscarinic antagonists of limited receptor subtype selectivity have also suggested that the M_3 subtype plays a key role in muscarinic receptor–mediated glucagon release (26,27).

Consistent with the in vitro glucagon release data, we found that plasma glucagon levels were significantly decreased (by ~30–50%) in both fed and fasted $M3^{-/-}$ mice. Glucagon counteracts the hypoglycemic effects of insulin, primarily by stimulating hepatic glucose production. It is therefore likely that the reduced glucagon plasma levels of the $M3^{-/-}$ mice contribute to the hypoglycemia displayed by these mutant animals.

Because hypoglycemia usually triggers an increase in

circulating glucagon levels, it is possible that our findings underestimated the importance of pancreatic M_3 receptors in mediating glucagon secretion. Moreover, because hypoglycemia normally leads to reduced insulin secretion, the reduced blood glucose levels displayed by the $M3^{-/-}$ mice may also contribute to the hypoinsulinemia associated with the lack of M_3 receptors.

In conclusion, we have demonstrated that muscarinic stimulation of pancreatic insulin and glucagon release is mediated by the M_3 muscarinic receptor subtype. Consistent with this observation, M_3 receptor−deficient mice showed pronounced reductions in plasma insulin and glucagon levels. Given the lack of muscarinic ligands that can selectively block or inhibit specific muscarinic receptor subtypes with a high degree of selectivity, these findings highlight the usefulness of muscarinic receptor mutant mice as a novel tool for dissecting the metabolic roles of the M_1−M_5 muscarinic receptors.

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