The G Protein-coupled Receptor P2Y\textsubscript{14} Influences Insulin Release and Smooth Muscle Function in Mice\textsuperscript{[a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z,aa,ab,ac,ad,ae,af,ag,ah,ai,aj,ak,al,am,an,ao,ap,aq,ar,as,at,au,av,aw,ax,ay,az,ba,bc,bd,be,bf}]

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Background: The relevance of the widely expressed GPCR P2Y\textsubscript{14} is only partially understood.

Results: Analysis of P2Y\textsubscript{14}-KO mice revealed decreased gastrointestinal emptying, reduced glucose tolerance, and insulin release.

Conclusion: P2Y\textsubscript{14} function is required for proper intestine emptying and adequate glucose response.

Significance: P2Y\textsubscript{14} plays a role in smooth muscle function and maintaining energy homeostasis by influencing insulin release.

UDP sugars were identified as extracellular signaling molecules, assigning a new function to these compounds in addition to their well defined role in intracellular substrate metabolism and storage. Previously regarded as an orphan receptor, the G protein-coupled receptor P2Y\textsubscript{14} (GPR105) was found to bind extracellular UDP and UDP sugars. Little is known about the physiological functions of this G protein-coupled receptor. To study its physiological role, we used a gene-deficient mouse strain expressing the bacterial LacZ reporter gene to monitor the physiological expression pattern of P2Y\textsubscript{14}. We found that P2Y\textsubscript{14} is mainly expressed in pancreas and salivary glands and in subpopulations of smooth muscle cells of the gastrointestinal tract, blood vessels, lung, and uterus. Among other phenotypical differences, knock-out mice showed a significantly impaired glucose tolerance following oral and intraperitoneal glucose application. An unchanged insulin tolerance suggested altered pancreatic islet function. Transcriptome analysis of pancreatic islets showed that P2Y\textsubscript{14} deficiency significantly changed expression of components involved in insulin secretion. Insulin secretion tests revealed a reduced insulin release from P2Y\textsubscript{14}-deficient islets, highlighting P2Y\textsubscript{14} as a new modulator of proper insulin secretion.

Although the physiological function is known for many members of the family of GPCR, functional ligands have not been identified yet for more than 100 so-called orphan receptors. In the past years, academic research groups and pharmaceutical companies have engaged in large scale deorphanization efforts for these receptors employing ligand screening studies and analyses of receptor-deficient mouse models (1). One of these studies revealed P2Y\textsubscript{14} (formerly named GPR105) as a receptor that is activated by UDP-glucose and closely related sugars (2, 3). This was an interesting finding because UDP-glucose is a pivotal metabolite in intracellular glucose storage pathways including glycogen biosynthesis. In more recent studies, UDP alone was described as a ligand for P2Y\textsubscript{14} (4, 5); however, another group tested the agonistic activity of UDP but found no activation of P2Y\textsubscript{14} (2). P2Y\textsubscript{14} belongs to the P2Y\textsubscript{12}-like receptor group within the family of rhodopsin-like receptors. The P2Y\textsubscript{12}-like receptor group comprises the ADP receptors P2Y\textsubscript{12}, P2Y\textsubscript{13}, and P2Y\textsubscript{14} and the receptors GPR34, GPR82, GPR87, and GPR171 (6). P2Y\textsubscript{14} mRNA-expression analysis showed a widespread expression pattern including high expression levels in placenta, adipose tissue, stomach, and intestine and moderate levels in spleen, lung, heart, and different brain regions (3, 7). P2Y\textsubscript{14} was also detected in immune cells like B- and T-lymphocytes, neutrophils, dendritic cells, astrocytes, and microglia (7–11), suggesting a physiological function in immune response. Indeed, expression of P2Y\textsubscript{14} was up-regulated in response to inflammatory injury (12), and similar results were shown by in vivo treatment with lipopolysaccharide for rat brain and spleen (7, 13). However, because the strongest expression was found in the gastrointestinal (GI) system, we explored a possible role for P2Y\textsubscript{14} in the regulation of nutrient uptake and energy metabolism.

**EXPERIMENTAL PROCEDURES**

\textit{Cell Culture, Transfection, and Functional Assays—For yeast experiments, the haploid Saccharomyces\textit{cerevisiae} yeast strain MPY578t5 (provided by Dr. Mark Pausch) was used for the expression of receptor constructs. Cells were transfected with plasmid DNA using electroporation as described (14). For
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![Diagram](image)

**FIGURE 1. Generation of a P2Y\textsubscript{14}-deficient mouse strain and P2Y\textsubscript{14} qPCR design.** P2Y\textsubscript{14}-deficient mice were generated at Takeda Ltd. The coding sequence was partly replaced by an Encephalomyocarditis virus-internal ribosome entry site (EMCV-IRES) β-galactosidase cassette followed by a loxP-flanked neomycin selection cassette. We removed neomycin cassette by breeding with Ella-Cre mice, and successful removal was verified by PCR. For qPCR, a primer pair matching to exon 1 and 2 was designed. Genotyping of mice was performed as described under “Experimental Procedures” with the primers indicated: forward (forw), reverse (rev), and β-galactosidase sense (β-gal s).

expression in mammalian cells, COS-7 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator. Transient transfection experiments of COS-7 cells with receptor constructs for cAMP inhibition measurements and for direct cAMP measurements (co-transfected with the chimeric G protein G\textsubscript{oi155}) were essentially performed as described previously (15). For measurements of dynamic mass redistribution (16) HEK293 cells were grown in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator and transiently transfected with the receptor constructs and split onto fibronectin-coated 384-well plates 1 day prior to the assay.

Dynamic mass redistribution measurements were performed with the Epic system (Corning, NY).

For all transfection experiments the mouse and human P2Y\textsubscript{14} coding sequence attached to an N-terminal hemagglutinin epitope and a C-terminal FLAG epitope was cloned into the mammalian expression vector pCDps. Additionally, for yeast expression in mammalian cells, COS-7 cells were grown in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator. Transient transfection experiments of COS-7 cells with receptor constructs for cAMP inhibition measurements and for direct cAMP measurements (co-transfected with the chimeric G protein G\textsubscript{oi155}) were essentially performed as described previously (15). For measurements of dynamic mass redistribution (16) HEK293 cells were grown in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator and transiently transfected with the receptor constructs and split onto fibronectin-coated 384-well plates 1 day prior to the assay.

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For all transfection experiments the mouse and human P2Y\textsubscript{14} coding sequence attached to an N-terminal hemagglutinin epitope and a C-terminal FLAG epitope was cloned into the mammalian expression vector pCDps. Additionally, for yeast experiments the mouse receptor was introduced into the yeast vector p416GPD. All control receptor plasmids were cloned with the described strategy.

**Generation of P2Y\textsubscript{14}-deficient Mice—** P2Y\textsubscript{14}-KO mice were generated at Takeda Ltd. (Cambridge, UK) by targeted disruption of the mouse P2Y\textsubscript{14} locus and ES cell blastocyst injection. Thus, most of the coding region of P2Y\textsubscript{14} was exchanged by a β-galactosidase cassette harboring an internal ribosome entry site followed by a loxP-flanked neomycin cassette (Fig. 1). After removal of the neomycin cassette, the resulting KO animals were bred with 129S6 animals, and all studies were done on a mixed C57BL/6 × 129S6 background with a predominance of 129S6. For experiments, WT and KO littermates from intercrossed WT and KO parents were used. Genotyping of mice was carried out by PCR with the following primers: β-galactosidase sense (5’-AGAAGGACATGGCTGATATCGA-3’), forward (5’-AGCTGCGGAGAGAGAGCACCTCTGCT-3’), and reverse (5’-GGTTTTGGAAACCTCTAGGTCATTCT-3’) in two separate PCRs (Fig. 1): forward/reverse to amplify WT allele (180 bp) and β-galactosidase sense/reverse to amplify KO allele (400 bp). The PCR conditions were 95 °C for 3 min followed by 35 cycles with 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min and a final amplification step of 72 °C for 10 min.

All mice were maintained in a specific pathogen-free barrier facility on a 12-h light/12-h dark cycle with ad libitum access to water and food. Experiments were performed according to the accepted standards of animal care and were approved by the respective regional government agency of the State of Saxony, Germany (T27/11; T46/12; T07/13).

**Quantitative Expression Analysis—** Several tissues were removed from three WT and three KO mice, and RNA was isolated using TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions. RNA quantity was measured with a spectrophotometer (Nanodrop ND 1000; NanoDrop Products, Wilmington, DE), and RNA quality was controlled by gel electrophoresis. For quantitative real time PCR (qPCR), 1 μg of total RNA was reversely transcribed (Superscript II RT; Invitrogen) using oligo(dT) primers. qPCR was performed by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer’s instructions. Primers were designed for the intron-flanking exons 1 and 2 sequences (Fig. 1) to exclude genomic contaminations (sense, 5’-GAAGGCA-GACGTGAAAGAGTT-3’; antisense, 5’-CAGGAATCT-AAGGCAAGCCT-3’) resulting in a 156-bp product. qPCR was performed with the MX 3000P instrument (Agilent Technologies GmbH, Boeblingen, Germany) using the following protocol: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A product dissociation curve was recorded to verify the presence of a single amplicon. Threshold cycle (Ct) values were determined during the exponential
increase of the product in the PCR. After normalization to the housekeeping gene β2-microglobulin calculated ΔCt values were used to determine the relative expression of P2Y14.

LacZ Reporter Gene Assay—Tissue samples of several organs were prepared from WT and KO mice, covered in Tissue-Tek® (Sakura, Torrance, CA) and snap frozen in liquid nitrogen. The organs were cryosectioned (10 μm) and fixed in acetone-methanol (1:1) solution. The sections were incubated with X-Gal staining solution at 30 °C overnight to detect the expression of the LacZ reporter gene via β-galactosidase activity.

Laboratory Chemistry/Histology/Behavioral Tests—Serum levels of electrolytes, metabolites, enzymes, and hormones were analyzed in 3-month-old WT and KO mice according to the guidelines of the German Society of Clinical Chemistry and Laboratory Medicine, using a Hitachi PPE-Modular analyzer (Roche). Histological slices (5 μm) were prepared from organs being fixed in 4% formaldehyde solution and embedded in paraffin wax. Slices were stained with H&E. Blood cell counting from EDTA blood samples was performed automatically (Scil-Vet abc; Scil Corporation, Viernheim, Germany). Open field and hot plate tests were performed as reported previously (17) using automated measuring technology (TSE Systems, Bad Homburg, Germany; Hot Plate 602001).

Gastrointestinal Transit and Contractility Studies—A gastrointestinal emptying test was performed by oral application of 200 μl of dye-labeled nonabsorbable liquid blue dextran (20 mg/ml) to mice. Feces were collected hourly for 8 h, vortexed with 300 μl of PBS, and centrifuged at 13,000 rpm for 10 min. The amount of hourly excreted blue dextran was measured photometrical at 620 nm in supernatants. Mice that showed no excretion for at least 2 h were excluded from the experiment. For contractility studies mice were sacrificed by cervical dislocation. The intestine from mice was harvested and placed in ice-cold Krebs-Ringer buffer (KRB). Equal 1-cm-long segments of intestine were placed in 50-ml organ baths (37 °C) containing ice-cold Krebs-Ringer buffer (KRB). Each 1-cm-long segment of intestine were placed in 50-ml organ baths (37 °C) containing KRB solution and continuously gassed with 95% O₂ and 5% CO₂. To record the contractile activity, each intestine segment was connected to an isometric force transducer (MLT0201 Force Transducer; ADInstruments GmbH, Spechbach, Germany). The basal tension was set to 10 mN. Several parameters like mean muscle tension, average tension maximum and minimum, average amplitude, rate of contractions, and the area under the curve and the effect of 100 μM UDP-glucose and 100 μM carbachol (positive control) were analyzed using Lab Chart software (ADInstruments). The data were normalized to recorded spontaneous activity and expressed as percentages.

Hemodynamic Parameters—Blood pressure and pulse were determined by noninvasive tail-cuff method using the BP-2000 blood pressure analysis system (Visitech Systems, Apex, NC). Following manufacturer’s instructions, mice were trained to the procedure each day for at least 5 days prior to the actual experiment. After the training period, 10 measurements per day of each mouse on 5 consecutive days were performed. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse were calculated from daily experiments for each mouse, and mean values from 10 animals/group were determined.

Airway Responsiveness—Lung resistance and dynamic compliance were measured by invasive plethysmography (emka TECHNOLOGIES, Paris, France) in response to inhaled UDP-glucose and methacholine (Sigma-Aldrich). Female mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine; Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline lung resistance, dynamic compliance, and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to 100 μM aerosolized UDP-glucose and increasing doses (10–80 mg/ml) of aerosolized methacholine (18).

Metabolic Studies—1-month-old mice were kept on standard (Sniff M-Z: 5% sugar, 4.5% raw fat, 34% starch, 22% raw protein) or western (Sniff EF R/M TD88137: 32.8% sugar, 21.2% raw fat, 14.5% starch, 17.1% raw protein) type diet (Sniff GmbH, Soest, Germany) for 3 months. During this period body-weight development was monitored weekly. For glucose tolerance tests mice on a standard or a western type diet had been fasted overnight (15 h), and blood glucose levels were measured before (0 min) and 20, 40, 60, and 120 min after oral application (80 mg) or intraperitoneal injection (2 mg/g of body weight) of glucose. For insulin tolerance tests, blood glucose levels were measured before (0 min) and 15, 30, and 60 min after intraperitoneal injection of human insulin (0.75 unit/kg of body weight). Blood glucose levels were determined by using an automated blood glucose meter (FreestyleLite; Abbott Diabetes Care Ltd., Oxon, UK). For insulin secretions tests, mice fasted overnight were injected intraperitoneal with glucose (2 mg/g of body weight) and blood samples were taken before (0 min) and 20, 40, and 60 min after injection. Serum insulin concentrations were measured using an ultra sensitive mouse insulin ELISA kit (Crystal Chem, Inc., Downers Grove, IL). All metabolic tests were performed with the same set of animals (1-week interval between the tests).

Morphometric Analysis—Pancreata from five WT and five KO mice fed with western type diet were fixed in 4% formaldehyde and embedded in paraffin wax. Two longitudinal sections (4 μm) were generated every 100 μm of tissue. Each time, the first section was stained with H&E, and the second was immuno stained for insulin with the avidin-biotin complex technique using anti-insulin monoclonal rabbit antibody (100 μg/ml; Cell Signaling, Millipore, Germany) and goat anti-rabbit secondary antibody (7.5 mg/ml; Vector Laboratories, Biozol Diagnostica, Germany). Both sections were photographed with a Zeiss Axio imager Z1 microscope (Carl Zeiss Jena GmbH, Jena, Germany). Ten H&E-stained sections per mouse were analyzed for number and area size of pancreatic islets using NIH Image) software. For determination of β-cell area per islet, the insulin-positive area of at least 50 islets per mouse was calculated using NIH Image) software and related to the total islet area of the investigated slides.

Isolation of Pancreatic Islets and Insulin Secretion Experiments—Pancreatic islets were isolated as previously described (19–21). Briefly, mice were sacrificed by cervical dislocation, and the pancreas was inflated by intraductal injection of ice-cold DMEM containing 0.2 mg/ml (1.08 Wünsch Units) Liberase TL research grade (Roche). Distended pancreas was
digested by shaking in a 37 °C water bath for 17 min. Isolated islets were washed and picked under a stereomicroscope and incubated overnight at 37 °C and 5% CO₂ in RPMI 1640 medium containing fetal bovine serum for insulin secretion studies. For dynamic insulin secretion studies, groups of 50 pancreatic islets were placed in a perfusion chamber and continuously perfused with KRB for 30 min (flow, 1 ml/min). After this equilibration phase, islets were perfused for 30 min with low glucose (2.8 mM) to determine the basal insulin release and then challenged for 30 min with 16.7 mM glucose plus 100 μM UDP-glucose. Samples of perfusate were collected every minute, and insulin concentration was determined at indicated time points with an insulin AlphaLISA kit (PerkinElmer Life Sciences). For static incubation studies, groups of five islets were placed in a 96-well plate. After a 30-min equilibration period in KRB solution containing 2.8 mM glucose, pancreatic islets were stimulated with KRB solution containing either 2.8 or 16.7 mM glucose or 100 μM UDP-glucose in the presence of 16.7 mM glucose for 30 min. The supernatants were then collected for measurement of secreted insulin, and islets were lysed in acid-ethanol to extract the residual islet insulin (19, 20).

**RESULTS AND DISCUSSION**

Although widely expressed, previous studies on P2Y₁₄ mainly focused on immune cells and its relevance in inflammatory responses (7–13). Initial evidence indicates a role for P2Y₁₄ in UDP-glucose-mediated chemotaxis of neutrophils and bone marrow cells, mast cell degranulation, and proliferation of T cells (9, 10, 27, 28). However, studies suggest that UDP-glucose can mediate cellular effects via P2Y₁₄-dependent and -independent pathways (29–31). To identify phenotypes related to the lack of P2Y₁₄ function, this GPCR gene was removed by targeted deletion and replacement with a LacZ reporter gene. The P2Y₁₄-deficient offspring was vital and fertile. This is consistent with a very recent study investigating P2Y₁₄ relevance in embryonic lethality after radiation (32). Except for a significantly higher spleen weight (supplemental Table S1) and slightly increased blood pressure (supplemental Table S1), no obvious differences in genotype distribution, breeding, growth, gross morphology, histology, laboratory chemistry, and behavior were detected on primary inspection compared with WT mice (supplemental Table S1).

**P2Y₁₄ Is Widely Expressed in Mouse Tissues**—We next took advantage of the introduced LacZ reporter construct and monitored β-galactosidase activity in P2Y₁₄-deficient mouse tissues. Whereas WT control tissues showed no specific staining, β-galactosidase-positive staining was found in uterus, bronchioles, blood vessels, pancreas, salivary glands (Figs. 2A and 3), and immune cells. Interestingly, staining in the GI tract was positive only in a subpopulation of smooth muscle cells (Fig. 2, B and C). In all parts of the GI tract, smooth muscle cells of the thin layer of the muscularis mucosae were β-galactosidase-positive (Fig. 2, B and C). From the ileum to the rectum, a subpopulation of smooth muscle cells within the circular smooth muscle layer showed positive staining (Fig. 2, B and C). In the rectum, the longitudinal smooth muscle layer also displayed intensive β-galactosidase activity (Fig. 2, B and C). Co-staining of c-Kit, which is a marker of Cajal cells and mast cells in the GI tract (33, 34), revealed no obvious overlap with the β-galactosidase-positive structures (data not shown). In contrast the β-galactosidase staining was partly reminiscent of the reported divergent reactivity of intestinal smooth muscle cells with characteristic smooth muscle cell markers (i.e. α-smooth muscle actin and γ-smooth muscle actin) (35). To our best knowledge, there is no other smooth muscle subtype that may correspond to the cells marked by the P2Y₁₄ reporter construct.

Further assessment of the LacZ expression in pancreatic tissue showed β-galactosidase-positive staining in exocrine and endocrine glandular structures (Fig. 3). Interestingly, cells next to the pancreatic acini (Fig. 3, A–C) and myoepithelial cells of the salivary glands (Fig. 24) were stained. Investigations addressing the specific functions of the myoepithelial cells are ongoing.

One should keep in mind that P2Y₁₄ promoter-driven β-galactosidase expression was monitored in KO mice and may reflect transcript over- or even nonphysiological expression because of P2Y₁₄ deficiency. To address this issue, we measured expression of the 5′-UTR region of WT and KO P2Y₁₄ gene transcripts, which were structurally similar in both WT and KO animals, by qPCR in a number of tissues (Fig. 4). As presented in...
Fig. 4B, ΔCt values of transcript expression showed a high correlation between WT and KO in all tissues ($r^2 = 0.9058$). This almost excluded compensatory or reactive LacZ expression effects following P2Y$_{14}$ deficiency in KO tissues. Both data sets revealed that P2Y$_{14}$ is widely expressed in mouse tissues with high expression levels in pancreas, salivary glands, brain, lung, and parts of the gastrointestinal tract and lowest expression in liver (Fig. 4). This is in accord with previous findings in human and rat tissues (3, 7, 8) but now adds that distinct smooth muscle cells and immune cells are the cellular basis of broad expression levels previously found in qPCR and Western blot analyses.

**P2Y$_{14}$ Deficiency Alters Smooth Muscle Functions in GI Tract and Airways**—To study the consequences of P2Y$_{14}$ deficiency in the different smooth muscle containing organs, we tested several GI tract and bronchial tract functions. A previous study in rat revealed high P2Y$_{14}$ mRNA levels and found UDP-glucose-dependent increase in the baseline muscle tension of the forestomach. However, comparison of WT and P2Y$_{14}$-deficient mice revealed no differences in gastric emptying (30).
Because we found no effect of UDP-glucose (up to 1 mM) on basal and carbachol-stimulated and electric-stimulated contraction of isolated small intestine and colon (data not shown), we speculated that the contribution of P2Y14 is more distinct and may present its significance only when the entire GI passage is observed. Thus, a dye-labeled dextran was applied to mice orally, and the excretion in feces was measured hourly. In comparison with WT animals, the expulsion of blue dextran was significantly delayed in KO mice (Fig. 5). Interestingly, more KO animals (five KO versus one WT) had to be excluded from analysis according to our exclusion criterion (see “Experimental Procedures”).

P2Y14 reporter gene expression was also found in smooth muscle cells of bronchioles (Fig. 2A). To dissect its function in the respiratory tract, invasive plethysmography tests were performed. Although UDP-glucose had no effect on respiratory functions tested, KO mice showed significantly higher dynamic compliance in response to increasing inhaled doses of methacholine (Fig. 6).

One should note the expression of P2Y14 in mainly venous blood vessels (Fig. 2A) and significant increases in blood pressure and heart rate of P2Y14-deficient mice (supplemental Table S1). There is some evidence that P2Y14 may have vasoconstrictile function on arteries (36), but P2Y14 expression in smooth muscle cells in arteries is controversially discussed (37, 38). We found some β-galactosidase positive arteries only in lung and CNS (circulus arteriosus Willisi) (data not shown). Future studies will address whether there is a physiological link between P2Y14 expression in the vascular system and hemodynamic functions. In sum, we show that P2Y14 is highly

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**FIGURE 4.** P2Y14 transcript and P2Y14 promoter-driven LacZ gene expression in mouse tissues. Total RNA was extracted from several tissues from WT and P2Y14-KO mice (n = 3), and mRNA levels of either P2Y14 or LacZ transcripts were determined by SYBR Green qPCR analysis. Primers were designed for the intron-flanking exons 1 and 2 (see Fig. 1). Expression data are shown as ΔCt values normalized to the housekeeping gene β2-microglobulin. A, qPCR analysis shows a widespread receptor distribution with high expression levels in pancreas, salivary glands, brain, and parts of the gastrointestinal tract and lowest expression in liver. The data are shown as means ± S.E. B, expression of bacterial LacZ reporter gene in KO mice correlates (r² = 0.9058) with the expression of P2Y14 in WT mice. The dashed line indicates the 95% confidence interval.

**FIGURE 5.** P2Y14-deficient mice showed a delayed gastrointestinal emptying. A dye-labeled dextran was applied to mice orally, and the excretion in feces was measured hourly. The excretion of blue dextran is shown relative to the total excreted blue dextran after 8 h. Five KO mice but only one WT animal had to be excluded from analysis according to our exclusion criterion (see “Experimental Procedures”). Student’s t test.

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expressed in a distinct subset of smooth muscle cells. P2Y14 is expressed on those cells modulate contractile function of the GI and the bronchial tracts.

**P2Y14 Deficiency Results in Reduced Glucose Tolerance**—Our phenotype screen also included basic metabolic tests. Following oral glucose application, KO mice showed significantly higher transient blood glucose levels (Fig. 7A). To differentiate whether the enteral glucose resorption was increased in KO mice because of the prolonged gastrointestinal emptying (see above), intraperitoneal glucose tolerance tests were performed. Blood glucose levels were also increased in KO mice following intraperitoneal glucose injection (Fig. 7B) excluding differences in enteral glucose resorption. To study whether high caloric nutrition can aggravate the phenotype found in KO mice, 4-week-old mice were kept on a western type diet for 3 months. WT and KO mice gained significantly body weight under the western type diet. However, although there were no alterations in body weight development between WT and KO mice kept on standard diet, KO mice fed the western diet gained significantly less weight (KO: 178 ± 15% versus WT: 196 ± 23%, p < 0.05).

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**FIGURE 6. Airway responsiveness is reduced in P2Y14-deficient mice.** A and B, lung resistance (A) and dynamic compliance (B) were measured by invasive plethysmography (Emka Technologies) in response to inhaled UDP-glucose and increasing doses of methacholine. The data are shown as means ± S.D. The baseline values (set to 100%) were: lung resistance, 1.28 ± 0.41 mm Hg × s/ml for WT mice and 1.11 ± 0.34 mm Hg × s/ml for KO mice, dynamic compliance 0.84 ± 0.56 ml/mm Hg for WT, and 0.72 ± 0.43 ml/mm Hg for KO mice. *, p < 0.05; Student’s t test. For statistic analysis of the entire methacholine test, a paired two-tailed t test (marked by a long line) was performed (p = 0.0105 for dynamic compliance).

**FIGURE 7. P2Y14-deficient mice showed reduced glucose tolerance.** Glucose tolerance tests were performed either by oral application (A and C) of 80 mg of glucose or by intraperitoneal (B and D) glucose injection (2 mg/g of body weight) to overnight fasted male mice kept under standard (A and B) or western (C and D) type diet. Blood glucose levels are shown as means ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001; Student’s t test.
Next, we performed insulin tolerance tests to evaluate whether the higher glucose levels observed in KO mice in the oral glucose tolerance test were because of altered insulin sensitivity. There were no differences in the decline of blood glucose levels following intraperitoneal insulin injection between WT and KO mice (Fig. 8A). Similar results were found for mice kept on the western type diet (Fig. 8B), although both genotypes showed lower insulin sensitivity as a result of higher body weight caused by high caloric nutrition. Because insulin sensitivity did not account for altered oral glucose tolerance test in KO mice, we analyzed insulin levels in WT and KO mice during intraperitoneal glucose tolerance tests. On chow diet, KO mice (16 weeks old) tended to have lower serum insulin levels after intraperitoneal glucose application (Fig. 8C). Interestingly, after 12 weeks of western type diet, glucose-stimulated insulin secretion of KO mice was significantly lower 40 and 60 min after intraperitoneal glucose injection compared with WT mice (Fig. 8D).

In a recent paper, Xu et al. (39) showed improved glucose tolerance in P2Y14-deficient mice fed with a high fat diet because of an elevated insulin sensitivity, e.g. of the liver. This is in contrast to our data where insulin sensitivity of KO and WT mice was not different (Fig. 8A and B). It was argued that reduced macrophage infiltration and hepatic inflammation account for improved hepatic insulin sensitivity (39). As in our study (Fig. 8C and D), Xu et al. (39) found reduced serum insulin levels after glucose administration in KO mice. This important finding remained unappreciated in their study, and other pathophysiological mechanisms were not considered. In a more reasonable scenario, lower insulin levels result in a reduced hepatic steatosis, as found in Ref. 39 under a high fat diet. In fact, the differential expression of genes involved in lipid and glycogen metabolism in liver, as found in Ref. 39, can be interpreted as a consequence of lower insulin levels. It is well known that insulin stimulates the expression of lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase and inhibits the transcription of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (40). Thus, reduced insulin secretion may also result in reduced weight gain following high caloric nutrition, as seen in our P2Y14-deficient mice kept under western type diet. The hepatic inflammation is most likely secondary to the alimentary hepatic steatosis found in Ref. 39. Because the western type diet we used does not significantly differ from the high fat diet (see “Experimental Procedures”) Xu et al. (39) fed the mice (26.3% carbohydrates, 34.9% fat, 26.2% protein), we further focused on the reason for the lower blood insulin levels that were seen already in animals fed with standard chow.

Components Involved in Insulin Release Are Differentially Expressed in P2Y14-deficient Islets—Our functional data pointed toward a lower pancreatic insulin secretion after intraperitoneal glucose administration. High expression of P2Y14 was noticed in the exocrine pancreas (Figs. 3 and 4) but also in pancreatic islets (Fig. 3D). We first performed morphometric analyses in H&E-stained and insulin immunohistochemistry-stained slices in P2Y14-KO mice. Neither the number nor the size of the pancreatic islets differed between WT and KO mice (Fig. 9, A and C). Also, the insulin immunopositive areas were
similar in both genotypes, suggesting no gross morphological abnormalities in islet development (Fig. 9, B and D). Because P2Y14-deficient islets did not show obvious morphological differences, we screened for those at a molecular level. Thus, mRNA expression in WT and KO pancreatic islets was analyzed in a transcriptome-wide approach using RNA sequencing. Approximately 13 million reads/animal were analyzed. As expected, insulin, glucagon, somatostatin, and SUR1/Kir6.2 transcripts were highly expressed in islets of both WT and KO mice. In line with the data from immunohistochemistry, there was no difference in insulin mRNA expression levels between WT and KO (supplemental Table S2). The expression of P2Y14 gene was significantly reduced in KO mice (supplemental Table S2), but one should note that detected P2Y14 transcripts in the KO islets represented mRNA sequences of the 5′-UTR region and not of the coding region (Fig. 1). In line with the data from immunohistochemistry, there was no difference in insulin mRNA expression levels between WT and KO (supplemental Table S2). The expression of P2Y14 gene was significantly reduced in KO mice (supplemental Table S2), but one should note that detected P2Y14 transcripts in the KO islets represented mRNA sequences of the 5′-UTR region and not of the coding region (Fig. 1). In line with our RNASeq data, qPCR analysis (ΔCt = 7.64 ± 0.55), β-galactosidase staining of pancreatic tissue (Fig. 3), and other studies (41) also clearly demonstrate P2Y14 expression in pancreatic islets.

815 genes were found differentially expressed between WT and KO mice (329 transcripts down-regulated, 486 transcripts up-regulated, p < 0.05, the complete data are provided in Gene Expression Omnibus under the accession number GSE59285). Functional enrichment analysis of differentially expressed genes disclosed several Gene Ontology categories related to protein synthesis, signaling pathways, and hormone secretion (supplemental Table S3). Because serum insulin levels were reduced in KO mice, we mainly focused on genes known to be related to glucose sensing, insulin exocytosis, and its modulation. Selected components involved in insulin release and their changes in transcript levels are schematically given in Fig. 10.

Glucose uptake through glucose transporter 2 is a first key step in regulation of insulin release (42) (Fig. 10). The expression of glucose transporter 2 (Slc2a2) was significantly down-regulated in KO mice and may indicate a possibly reduced glucose-sensing or -responding ability of the islets.

The major trigger of insulin vesicle exocytosis is an increase in intracellular Ca2+ (42), and cAMP and protein kinase A activity is known to amplify insulin exocytosis (43). In the classical model of GPCR-modulated insulin release, Gq (Ca2+) and Gs (cAMP) activating receptors promote insulin release, whereas Gi/Go protein-coupled receptors reduce insulin release by inhibiting adenylyl cyclases and reducing cAMP levels in

FIGURE 9. Normal architecture of pancreatic islets in P2Y14−/− mice. A, representative H&E-stained pancreatic section used for morphometric analyses. B, insulin immunohistochemistry of representative pancreatic islets. C, the number and area of islets in 10 serial slices of pancreatic tissue were determined using NIH ImageJ software. The number of islets was 141 ± 17 in WT and 157 ± 11 in KO mice. D, β-cell mass was determined as the ratio of insulin-positive area to total pancreatic islet area. The data are shown as means ± S.D.
β-cells. Indeed, several components involved in receptor- and ion channel-controlled intracellular Ca\(^{2+}\) levels were downregulated in islets of KO mice, among them the subunits CACNA1C and CACNA1H of the voltage-dependent Ca\(^{2+}\) channels, the endoplasmic reticulum inositol 1,4,5-trisphosphate receptor (ITPR1), cAMP-degrading phosphodiesterases PDE1C and PDE4D (44), several GPCR known to modulate insulin release such as the free fatty acid receptor FFAR1 (45), calcium-sensing receptor, GLP1 receptor, GPR119, and somatostatin types 1 and 2 receptors (and their agonist somatostatin) (41, 46). In sum, transcriptome data showed significant changes in relevant components of glucose-sensing, Ca\(^{2+}\)-triggered, and GPCR-modulated insulin release pathways in islets of P2Y\(_{14}\)-deficient mice, which suggested changes in islet function of KO animals.

**Reduced Insulin Release from Pancreatic Islets of P2Y\(_{14}\)-deficient Mice**—It is almost impossible to functionally analyze all components found to be differentially expressed between WT and KO at both their individual level and their signaling network level. Therefore, we studied insulin secretion as one integral islet function in perifused and static treated islets in vitro. Although intracellular insulin content was similar in lysed islets isolated from WT and KO mice (Fig. 11A), the insulin release following stimulation with low (2.8 mM) and high glucose (16.7 mM) during a 30-min incubation was significantly reduced in islets lacking P2Y\(_{14}\) (Fig. 11, B and C). To study the kinetics of insulin release, isolated islets were placed in a perfusion chamber and continuously perifused with KRB solution containing different glucose concentrations. The initial insulin release peak and the sustained insulin release were significantly reduced in KO islets stimulated with a high glucose concentration (16.7 mM) (Fig. 11D). These data clearly demonstrated that P2Y\(_{14}\) deficiency has a negative impact on glucose-dependent insulin release from isolated pancreatic islets and confirmed our in vivo findings.

Because P2Y\(_{14}\) couples to G\(_{i/o}\) proteins, inhibits adenylyl cyclases, and mobilizes intracellular Ca\(^{2+}\) (11, 28), most probably via release of G\(_{i}\)/G\(_{o}\) dimers from Gi proteins (47), one would expect that loss of this gene should rather improve glucose-mediated insulin release from β-cells. However, UDP-glucose alone was neither able to significantly release insulin nor significantly reduce glucose-induced insulin release from isolated islets (Fig. 11). Therefore, we re-evaluated the agonistic properties of UDP- and UDP-glucose at the mouse and human P2Y\(_{14}\) with different methods in vitro. Techniques using chimeric G proteins in yeast and mammalian heterologous expression systems showed high basal activity of P2Y\(_{14}\) and some minor UDP- and UDP-glucose-mediated responses (Fig. 12A and Table 1). However, UDP and UDP-glucose did not significantly activate P2Y\(_{14}\) in classic cAMP inhibition assay (Table 1) and label-free dynamic mass redistribution (Fig. 12B), whereas the G\(_{i}\)-coupled neuropeptide Y receptor Y2 showed robust agonist-mediated signals in all tests. At the current stage, UDP and UDP-glucose show weak agonistic activity at P2Y\(_{14}\) in functional assays using chimeric G proteins (2). Label-free and classic second messenger assays, as well as our in vivo data with

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**FIGURE 10.** Signaling pathways involved in insulin secretion from β-cells. Several components involved in insulin release from pancreatic islets are shown. P2Y\(_{14}\) deficiency led to increased (green) and decreased (red) transcription of several genes compared with islets from WT mice. Detailed data from RNASeq experiments are given in supplemental Tables S2 and S3. A list of all expressed transcripts and the raw sequencing data are deposited in Gene Expression Omnibus under the accession number GSE59285.

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knock-out mice, do not provide evidence for physiological activities of UDP and UDP-glucose at P2Y14. Further studies using clear-cut controls (e.g. gene-deficient animals) are required to finally prove or disprove UDP-Glc and UDP as physiological agonists for P2Y14.

The cell type within Langerhans islets, where a specific GPCR is expressed, determines its function on insulin exocytosis. Currently, the cell type(s) of pancreatic islets expressing P2Y14 is unknown. Further, the effect of a given GPCR on insulin release apparently not only depends on its G protein-coupling mode. Thus, Gs protein-coupled receptors such as the GnRH receptor inhibit insulin release, whereas the exclusively Gi protein-coupled cannabinoid receptor type 1 and the melanin-concentrating hormone receptor stimulate insulin release from islets (41). The most abundant somatostatin type 3 receptor (see supplemental Table S2), which is Gsto protein-coupled, has no significant effect on insulin release (41). As for several GPCR expressed in pancreatic islets (41), the specific signaling pathway by which P2Y14 modulates gene expression and functions of islets needs to be studied further.

Conclusion—Mice with P2Y14 deficiency present a rather mild phenotype under specific pathogen-free conditions. However, our studies revealed involvement of P2Y14 in GI tract emptying and glucose homeostasis regulation. The lack of P2Y14 function reduced intestine passage and glucose-stimulated insulin release from pancreatic islets in vitro and in vivo. It

FIGURE 11. Impaired insulin secretion of isolated pancreatic islets of P2Y14−/− mice. A–C, for cumulative insulin measurements, pancreatic islets were isolated from WT and KO male mice and incubated 30 min with KRB solution containing 2.8 mM glucose (Glc), 16.7 mM glucose, or 16.7 mM glucose plus 100 µM UDP-glucose (n = 5–7). Insulin levels of lysed islets (A), in the supernatants (B), and the ratio of both (C) are shown. D, for kinetic insulin measurements, pancreatic islets were isolated from WT and KO male mice and continuously perfused with KRB solution containing 2.8 mM glucose, 16.7 mM glucose, or 16.7 mM glucose plus 100 µM UDP-glucose. The insulin levels shown as means ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001; Student’s t test.
Reduced Insulin Release in P2Y14−/− Mice

![Image of a graph showing reduced insulin release in P2Y14−/− mice](image)

**TABLE 1**

Functional measurements of mouse and human P2Y14 receptors

| Transfected construct | Agonist | cAMP inhibition: FSK stimulation (n = 10) | cAMP: cotransfected Gαi3 (n = 4) |
|-----------------------|---------|----------------------------------------|---------------------------------|
| Mock                  | FSK/basal| 100 (7.78 ± 1.29)                     | 2.71 ± 1.42                     |
| Mouse P2Y14           | FSK/basal| 100 (8.96 ± 1.92)                     | 7.22 ± 1.99                     |
| Human P2Y14           | FSK/basal| 100 (8.73 ± 3.12)                     | 7.49 ± 3.00                     |
| Human M2R             | FSK/basal| 100 (11.54 ± 3.21)                    | 4.18 ± 0.98                     |

is of interest that several GPCR, e.g. GLP1 receptor, GPR39, M3 muscarinic receptor, and galanin receptor, modulate both GI motility and glucose-induced insulin secretion (48–51). For P2Y14, it is currently unclear whether this GPCR realizes its effect by acute activation of its signaling cascade or during islet ontogenesis. The gross transcriptome changes found in P2Y14−/− deficient islets may also suggest a more complex alteration of the expression profile, indicating a role of P2Y14 in subdifferentiation of islets cells. Indeed, some genes that are involved in differentiation and maturation of pancreatic islet cell types (e.g. MAFA, MAFB, REG2) (52–54) are differentially expressed in the islets isolated from P2Y14−/− deficient mice (Fig. 10). Although we did not find differences in gross islet morphology and islet size distribution (Fig. 9), there is evidence that pancreatic β-cells, for example, are not homogenous but rather present functionally different subpopulations with distinguishable functionality (55, 56). Thus, P2Y14-mediated signaling may be one signal that modulates distinct functionalities of islet cells. Because Langerhans islets derived from pancreatic ductal cells during ontogenesis and P2Y14 expression was morphologically also found in the exocrine pancreas (Fig. 3), more detailed studies should focus on the relevance of P2Y14 in pancreas development. Further studies with conditional β-cell-specific KO mice can help to differentiate between acute and adaptive effects of P2Y14 deficiency. Nevertheless, our findings identify P2Y14 as a novel modulator of insulin secretion.

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