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The GluCre-ROSA26EYFP mouse: A new model for easy identification of living pancreatic α-cells

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Abstract **The control of glucagon secretion by pancreatic α-cells is poorly understood, largely because of the difficulty to recognize living α-cells. We describe a new mouse model, referred to as GluCre-ROSA26EYFP (or GYY), allowing easy α-cell identification because of specific expression of EYFP. GYY mice displayed normal glycemic control during a fasting/refeeding test or intraperitoneal insulin injection. Glucagon secretion by isolated islets was normally inhibited by glucose and stimulated by adrenaline. $[Ca^{2+}]_c$ responses to arginine, adrenaline, diazoxide and tolbutamide, were similar in GYY and control mice. Hence, this new mouse model is a reliable and powerful tool to specifically study α-cells.**

Keywords: Glucagon; Transgenic mouse model; EYFP; ROSA26 loci; Cre/loxP; Free cytosolic calcium concentration

1. Introduction

Insulin and glucagon, secreted by pancreatic islet β- and α-cells, respectively, play a major but opposite role in glucose homeostasis: insulin decreases blood glucose whereas glucagon increases it. Alterations in β- and α-cell functions are implicated in the pathogenesis of the chronic hyperglycemia of diabetes and in certain forms of hypoglycemia [1–4].

Whereas the mechanisms regulating insulin secretion are relatively well established, those controlling glucagon secretion are still very controversial [5]. Elucidating α-cell pathophysiology requires measurements of various parameters at the cellular level, such as the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) which has been shown to serve as a triggering signal for exocytosis of glucagon-containing granules [6,7]. However, these experiments are difficult to perform because α-cells represent only ~20% of islet cells and, in situ, are almost indistinguishable from the most abundant β-cells.

Several methods have been developed to purify or identify α-cells [8–15], but none of them allows easy, reliable, and direct identification of living α-cells. To overcome this limitation, we have developed a new transgenic mouse model, referred to as GluCre-ROSA26EYFP or GYY mice, expressing the enhanced yellow fluorescent protein (EYFP) specifically in α-cells. These mice were obtained by crossing two strains: ROSA26-EYFP [16] and glucagon-Cre mice [17]. ROSA26-EYFP mice are reporter mice that have a sequence loxP-STOP-loxP-EYFP in the locus ROSA26 of their genome. EYFP is not expressed unless the STOP sequence is excised by a Cre recombinase, which recognizes loxP sites on both sides of the STOP sequence. Glucagon-Cre mice express Cre recombinase specifically in α-cells because expression of the enzyme is under control of the glucagon promoter. Hence, crossing ROSA26-EYFP mice with glucagon-Cre mice yielded a new strain of mice with EYFP expression restricted to α-cells. Comparison with two strains of control mice showed that the fluorescent α-cells of the GYY mouse model displayed normal characteristics and can thus reliably be used to study α-cell physiology.

2. Materials and methods

2.1. Generation and detection of hybrid transgenic mice

Glu-Cre and RIP-Cre mice were generated as previously described [17]. Homozygous ROSA26-EYFP (R26YY) mice were kindly given by Dr. S. Srinivas (London, UK) [16]. Hybrid heterozygous GluCreR26Y+ (GY+) mice were generated by crossing R26YY with Glu-Cre mice. GluCreR26YY (GYY) mice were generated by crossing GY+ mice together. The presence of Cre and ROSA26-EYFP transgenes was assessed by PCR genotyping as previously described [17,18]. The same strategy was followed to obtain RIPCreR26YY (RIPYY) mice. NMRI and C57BL/6J mice were used as controls as indicated. All experiments were performed with 4–8-month-old mice of both genders. The study was approved by and conducted following the guidelines of the Commission d'Ethique d'Experimentation Animale of the University of Louvain School of Medicine.

2.2. In vivo analyses

Blood samples were collected from the tail vein. Blood glucose was measured with a portable glucometer (Bayer, New York, NY). Plasma glucagon was determined using the glucagon radioimmunoassay kit from Linco (St. Charles, MO). For the insulin tolerance test, mice were injected intraperitoneally with insulin (0.75 IU/kg, Actrapid®, Novo-Nordisk, Bagsvaerd, Denmark) after 6 h of fast.

2.3. Preparations, solutions and drugs

Islets were obtained by collagenase digestion of the pancreas, and when necessary, dispersed into single cells [19]. Intact islets were cultured overnight and islet cells were cultured for 1–4 days in RPMI

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Abbreviations: $[Ca^{2+}]_c$, free cytosolic calcium concentration; EYFP, enhanced yellow fluorescent protein; FACS, fluorescence activated cell sorting; K_{ATP} channel, ATP-sensitive K^+ channel

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1640 medium containing 7 mM glucose and 10% heat-inactivated fetal calf serum. No deterioration of the viability of EYFP-expressing cells was observed during cell culture since the cells were similarly responsive to all tested agents after 1 or 4 days of culture.

The medium used for all experiments contained (in mM): 120 NaCl, 4.8 KCl, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 1 mg/ml BSA and various agents as indicated. It was gassed with O_2:CO_2 (94:6%) to maintain a pH of 7.4. The 2.5 mM amino acid mixture used in some experiments contained (in mM): 0.5 alanine, 0.5 leucine, 0.75 glutamine and 0.75 lysine.

Diazoxide was a gift from Schering-Plough (Brussels, Belgium). Adrenaline was obtained from Denolin (Brussels, Belgium) and amino acids from Merck (Darmstadt, Germany). All other chemicals were from Sigma (St. Louis, MO).

2.4. Insulin and glucagon secretion experiments

For static determination of insulin and glucagon secretion, overnight cultured islets were preincubated for 60 min in the presence of 0.5 mM glucose. Batches of 7 islets were then incubated at 37 °C for 60 min in the presence of the indicated compounds. For perifusion experiments, batches of 200 overnight cultured islets were transferred into perifusion chambers [20]. Islets were then perifused at 37 °C, at a flow rate of 0.5 ml/min, with test solutions as described in the legends. Insulin [20] and glucagon (Linco) were measured by radioimmunoassay.

2.5. $[Ca^{2+}]_c$ measurements

Cells were loaded at 37 °C for 2 h with 2 μM fura PE3-AM (MoBiTec, Göttingen, Germany) in RPMI culture medium. $[Ca^{2+}]_c$ was measured at 37 °C by dual wavelength (340 and 380 nm) excitation microspectrofluorimetry as previously described [21]. EYFP expression did not interfere with fura PE3 fluorescence. α-Cells from control NMRI mice were identified by their typical $[Ca^{2+}]_c$ response to adrenaline at the end of the experiments [22,23].

2.6. Immunostaining

To assess the distribution of α-cells in islets from GYY mice, the whole pancreas was fixed in Bouin Allen's fluid for 24 h, embedded in paraffin, and processed for immunohistochemistry with a rabbit polyclonal anti-porcine glucagon antibody (NovoCastra, Newcastle, UK) as previously described (peroxidase-diaminobenzidine technique) [24].

To verify the cell specificity of EYFP expression in RIPYY and GYY islet cells, cultured cells were fixed for 3 h with 4% paraformaldehyde, which preserves endogenous EYFP fluorescence (excitation: 490 nm, emission: 535 nm). The cells were then immunostained for insulin, glucagon or somatostatin with a mouse monoclonal anti-porcine insulin antibody (Chemicon, Temecula, CA), a rabbit polyclonal anti-porcine glucagon antibody (NovoCastra) or a rabbit polyclonal anti-porcine somatostatin antibody (gift from W. Gepts, Free University of Brussels, Belgium), respectively. To permit simultaneous observation of EYFP, insulin, glucagon or somatostatin was detected by the red fluorescence of Alexa Red 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa Red 594-conjugated goat anti-rabbit IgG (Molecular Probes), respectively.

Detection of EYFP and Alexa Red 594-labelled insulin in GYY islets was performed with a Nikon microscope equipped with a confocal QLC100 spinning disk (Visitech International, Sunderland, UK).

2.7. Statistical analysis

Results are shown as representative traces or means ± S.E. for the indicated number of cells or batches of islets from at least three different cultures. The statistical significance of differences between means was assessed by unpaired Student's *t*-test, or analysis of variance followed by a Newman–Keuls test for multiple comparisons.

3. Results

3.1. Cell-specific expression of EYFP

To assess the cell specificity of EYFP expression, dispersed islet cells from GYY mice were processed for glucagon, insulin or somatostatin immunodetection. As illustrated in Fig. 1A–C, all EYFP-tagged cells were immunoreactive for glucagon. Counting EYFP-tagged and glucagon positive cells revealed that ~76% α-cells were fluorescent for EYFP (133/176 cells). Importantly, none of the fluorescent cells was immunostained for insulin (Fig. 1D–F, M) or somatostatin (Fig. 1G–I), confirming the specific expression of EYFP in α-cells from GYY mice. The cell specificity of EYFP expression was also verified in β-cells from RIPYY mice. EYFP fluorescence was observed only in β-cells (Fig. 1J–L), and ~80% β-cells were fluorescent

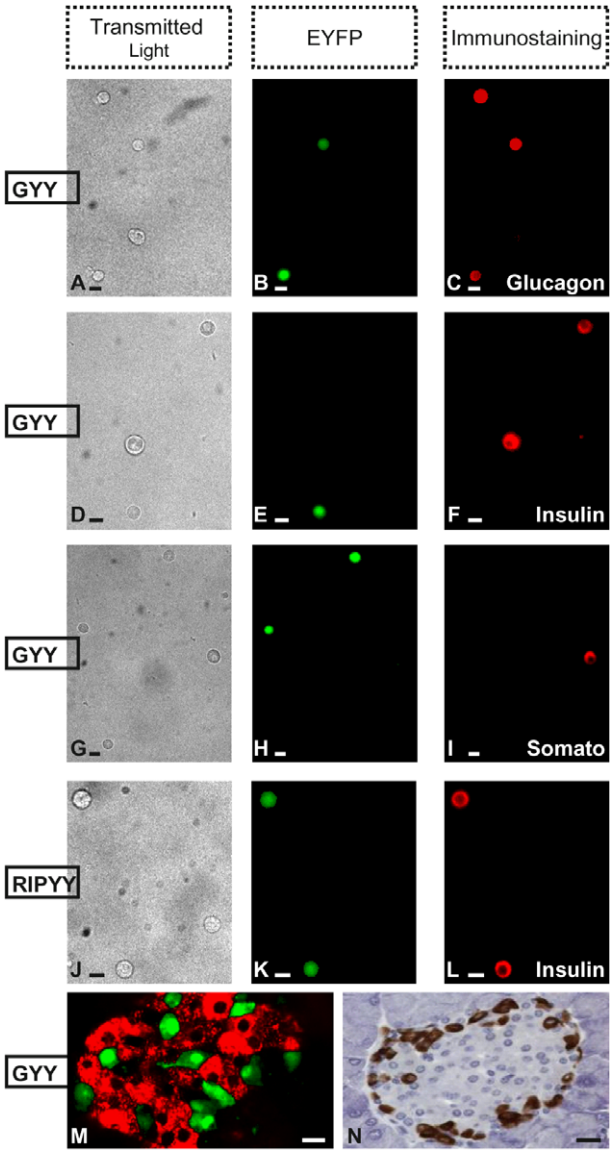

Fig. 1. EYFP is specifically expressed in α- and β-cells from GYY and RIPYY mice, respectively. (A–L): After immunodetection of glucagon (C), insulin (F, L), and somatostatin (I), islet cells were visualized in bright field (A, D, G, J), or in fluorescence with the EYFP filter set (B, E, H, K) or with the Alexa Red 594 filter set (C, F, I, L). A–C: Two out of three glucagon-labelled α-cells from GYY mice (C) are tagged with EYFP (B). D–F: The EYFP-tagged cell (E) from GYY mice is not labelled for insulin (F). G–I: The EYFP-tagged cells (H) from GYY mice are not labelled for somatostatin (I). J–L: Both EYFP-tagged cells (K) from RIPYY mice are labelled for insulin (L). M: Confocal section of a GYY islet revealing distinct distribution of EYFP (green cells) and insulin expressing cells (red cells). N: Anti-glucagon immunohistochemistry using DAB on a pancreatic section of GYY mice. Scale bars: A–M: 10 μm; N: 20 μm.

for EYFP (69/86 cells). The differential EYFP fluorescence intensity between isolated cells does not result from cell damage but rather reflects a true differential expression of EYFP because a similar heterogeneous fluorescence intensity between EYFP-expressing cells within intact islets was observed in confocal microscopy (not shown).

3.2. Characteristics of GYY mice and islets

Immunodetection of glucagon on pancreatic sections from GYY mice showed typical distribution of α-cells at the periphery of the islets (Fig. 1N). Physiological parameters of GYY mice were normal compared to C57BL/6J control mice. There were no differences in body weight and plasma glucagon levels between both strains of mice (Fig. 2A and B). Blood glucose concentrations were similar in fed GYY and C57BL/6J mice, and the responses to fasting and refeeding were also comparable (Fig. 2C). Thus, blood glucose decreased by ~25% after a 24 h fast and increased similarly after refeeding in both groups. GYY and C57BL/6J mice also displayed similar responses to intraperitoneal insulin administration. The blood glucose concentration dropped by ~40% 60 min after insulin injection, and the rate of recovery was similar in both types of mice (Fig. 2D).

We then verified that expression of Cre recombinase and EYFP did not impair α- and β-cell function. In the presence of 20 mM arginine, insulin and glucagon secretion were similar in islets from GYY and C57BL/6J control mice (Fig. 2E and F). Increasing the glucose concentration from 0.5 to 15 mM stimulated insulin secretion and inhibited glucagon secretion to a similar extent in both types of islets.

We also compared the insulin and glucagon responses of islets from GYY mice to those of islets from NMRI mice, an outbred strain unrelated to C57BL/6J mice. Isolated islets were perifused in the presence of a 2.5 mM mixture of amino acid to promote glucagon secretion. In the presence of low glucose (0.5 mM), the rate of insulin secretion by islets from GYY and control NMRI mice was low and stable. Increasing the glucose concentration to 15 mM elicited a similar biphasic insulin secretion in both types of islets, which was reversed upon lowering the glucose concentration back to 0.5 mM (Fig. 3A). Subsequent addition of 10 μM adrenaline had no effect on basal insulin secretion. Glucagon secretion was steadily elevated in the presence of 0.5 mM glucose. It displayed similar biphasic changes in islets from GYY and NMRI mice challenged by 15 mM glucose: an initial short-lived increase followed by a strong, sustained and reversible inhibition (Fig. 3B). Adrenaline markedly stimulated glucagon secretion. The glucagon content of both types of islets was similar (577 ± 18 and 588 ± 47 pg/islet in NMRI and GYY mice, respectively).

3.3. $[Ca^{2+}]_c$ in cells expressing Cre recombinase and EYFP

To verify that expression of Cre recombinase and EYFP does not affect $[Ca^{2+}]_c$ changes, we first tested whether glucose and drugs acting on ATP-sensitive K^+ (K_{ATP}) channels induce normal $[Ca^{2+}]_c$ responses in EYFP-expressing β-cells. $[Ca^{2+}]_c$

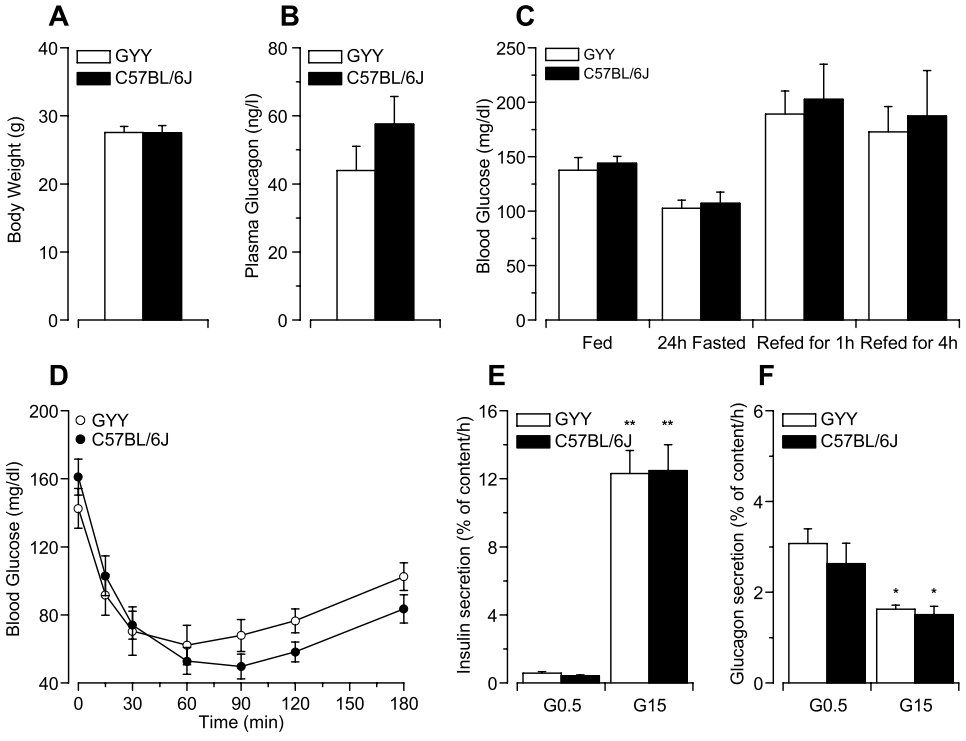

Fig. 2. Similarity of *in vivo* (A–D) and in vitro (E–F) biological parameters between GYY (open columns/open circles) and C57BL/6J control mice (filled columns/filled circles). A–B: Body-weight ($n = 14$) and plasma glucagon levels in the fed state (GYY $n = 9$; C57BL/6J $n = 8$). Values are means ± S.E. C: Fasting-refeeding test: blood glucose in the fed state, after a 24 h-fast, and 1 h and 4 h after ad libitum refeeding. Values are means ± S.E. for 7 mice. D: Insulin tolerance test: 0.75 IU/kg insulin was injected intra-peritoneally at $t = 0$ and blood glucose was measured at the indicated times. Values are means ± S.E. for 8 mice. E–F: Effect of 15 mM glucose (G15) on insulin and glucagon secretion from islets incubated in the presence of 20 mM arginine. Values are means ± S.E. for 12 batches of islets. $^*P < 0.05$ and $^{**}P < 0.01$ versus 0.5 mM glucose in the same strain of mice.

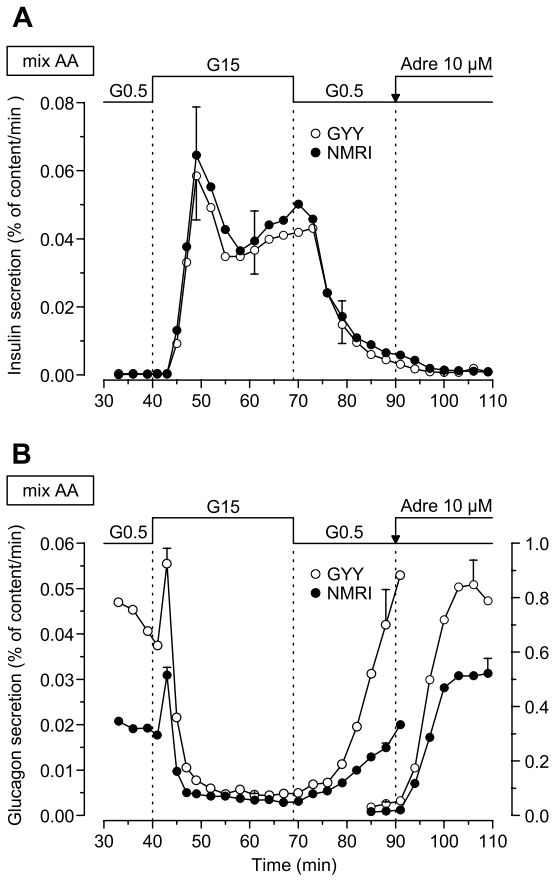

Fig. 3. Similarity of insulin (A) and glucagon (B) secretion between perifused islets from GYY and NMRI mice. Islets were first perifused in the presence of a 2.5 mM amino acid mixture (mix AA) and 0.5 mM glucose (G). The glucose concentration was then changed between 0.5 and 15 mM, and 10 μM adrenaline (Adre) was added when indicated. In B, the scale on the left applies to glucagon secretion between 30 and 90 min, whereas the scale on the right applies to secretion between 85 and 110 min. Traces are means ± S.E. for four experiments with islets from different preparations.

was low and stable in the presence of 3 mM glucose, and stimulation with 15 mM glucose elicited a transient drop in $[Ca^{2+}]_c$ followed by an increase with large oscillations. These oscillations were abolished by 50 μM diazoxide, an opener of K_{ATP} channels, and restored by addition of 500 μM tolbutamide, that closes the channels (Fig. 4A). These responses are thus similar to those previously reported for normal β-cells [25,26].

We then compared $[Ca^{2+}]_c$ responses in GYY α-cells and NMRI α-cells identified by their response to adrenaline [22,23]. In the presence of 0.5 mM glucose, $[Ca^{2+}]_c$ oscillated in ~30% GYY α-cells and in ~20% NMRI α-cells, and was low and stable in the others. This relatively low percentage of oscillating α-cells under low glucose conditions has been reported by others [23,27]. Arginine (10 mM), a well-established glucagon secretagogue, similarly increased $[Ca^{2+}]_c$ in both oscillating (not shown) and non-oscillating α-cells from GYY (Fig. 4B) and NMRI mice (Fig. 4C) [6]. Over 90% α-cells responded to arginine in GYY (94%) and NMRI (93%) mice (Fig. 4B and C). In the presence of a 2.5 mM mixture of amino acids and 0.5 mM glucose, α-cells from GYY mice displayed high and oscillating $[Ca^{2+}]_c$ (Fig. 4D; note that the oscillations

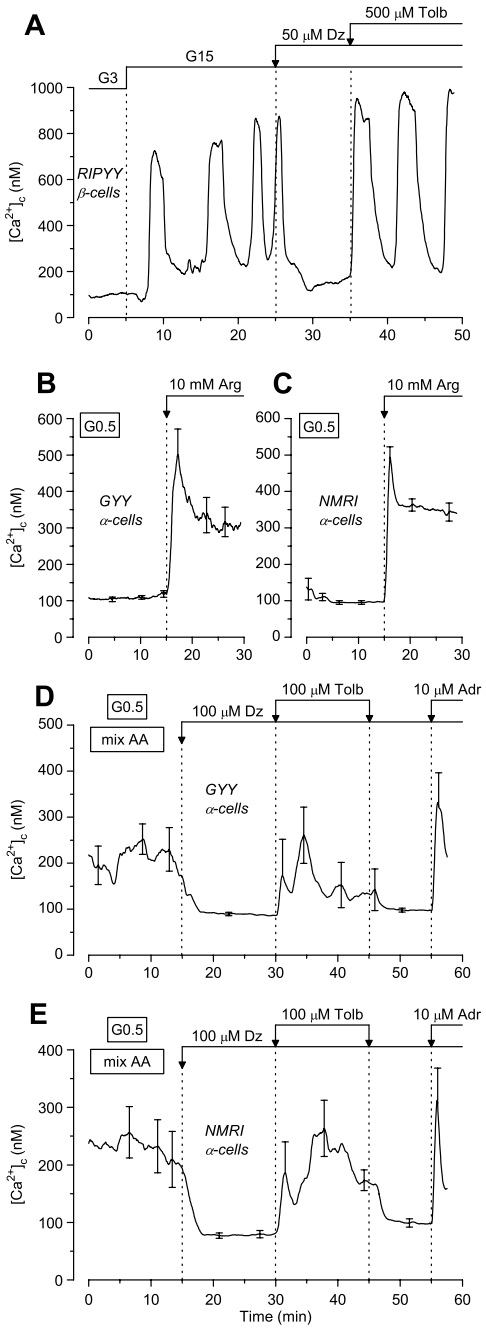

Fig. 4. EYFP and Cre expression do not alter α- or β-cell $[Ca^{2+}]_c$ responses. $[Ca^{2+}]_c$ was measured in single EYFP-tagged β-cells from RIPYY mice (A), single EYFP-tagged α-cells from GYY mice (B, D), and adrenaline-responsive cells (α-cells) from NMRI mice (C, E). A: The glucose (G) concentration was increased from 3 to 15 mM, and 50 μM diazoxide (Dz) and 500 μM tolbutamide (Tolb) were added to the perifusion medium as indicated. The trace is representative of results obtained in 5 β-cells. B–C: Experiments were performed in the presence of 0.5 mM glucose (G) throughout, and 10 mM arginine (Arg) was added as indicated. Traces are means ± S.E. of results obtained in 6 α-cells from GYY mice (B) and 11 adrenaline-responsive cells from NMRI mice (C). D–E: Experiments were performed in the presence of 0.5 mM glucose (G) and a 2.5 mM amino acid mixture (mix AA) throughout. 100 μM diazoxide (Dz), 100 μM tolbutamide (Tolb) and 10 μM adrenaline (Adr) were added as indicated. Traces are means ± S.E. of results obtained in 8 α-cells from GYY mice (D), and 9 adrenaline-responsive cells from NMRI mice (E).

are largely masked by the averaging). Diazoxide decreased $[Ca^{2+}]_c$ to basal levels. Subsequent addition of 100 μM tolbutamide reversed this effect triggering a reversible and rapid increase in $[Ca^{2+}]_c$ with oscillations or a stable elevation. Although diazoxide abolished $[Ca^{2+}]_c$ oscillations induced by the mixture of amino acids, it did not prevent the peak of $[Ca^{2+}]_c$ triggered by 10 μM adrenaline, supporting previous observations that adrenaline mobilizes Ca^{2+} from intracellular stores [12]. Similar results were obtained in α-cells from NMRI mice (Fig. 4E).

These results clearly show that neither Cre recombinase nor EYFP alters α- and β-cell function, and that GYY mice can reliably be used to identify and study living α-cells.

4. Discussion

This study describes the development of two new transgenic mouse models, referred to as GYY and RIPYY mice, specifically expressing the fluorescent EYFP protein in α- or β-cells, respectively. These mice were obtained by crossing the ROSA26-EYFP reporter mouse, that has in the locus ROSA26 of its genome a sequence loxP-STOP-loxP-EYFP [16] with either the Glu-Cre or the RIP-Cre mouse [17]. Other transgenic mouse models expressing a fluorescent protein in their β-cells have previously been reported [28,29] and are thus comparable to the presently described RIPYY mice. In contrast, the GYY mice are the first model with easily recognizable α-cells.

Two strategies are possible to generate transgenic mice expressing a fluorescent protein in α-cells specifically. The expression of a reporter protein could be directly controlled by the glucagon promoter, but this promoter has been reported to be weak, compared to, e.g. the insulin promoter [14,30]. The Cre/loxP strategy circumvents this difficulty and permits stronger expression of the reporter. Indeed, even if expression of the Cre recombinase driven by the glucagon promoter is weak, it is sufficient to allow excision of the loxP-flanked transcriptional STOP sequence and drive the expression of EYFP placed under control of the strong promoter present in the ROSA26 locus. Amplification of gene expression by the Cre/loxP strategy has previously been reported not to compromise the cellular specificity of expression [14,30]. However, EYFP expression was heterogeneous among α-cells, namely the fluorescence was weaker in some cells than others, and it was sometimes undetectable. We do not know whether the lack of detection reflects a limitation of our fluorescence system or corresponds to a true lack of EYFP expression. Similar observations have sometimes been reported in studies using the Cre/loxP strategy [31,32]. It is however not a major drawback since most (~80%) α-cells were labelled by EYFP.

One advantage of our mouse models over other EGFP-expressing models is that EYFP fluorescence does not interfere with the widely used fluorescent Ca^{2+} probes, fura-2 or fura-PE3. Models expressing EGFP impose the use of the less bright and less convenient fura-red [28].

To ascertain that GYY mice permit reliable investigation of α-cell physiology, we assessed whether their α-cells behaved normally, similarly to those of two mouse strains used as controls, i.e. C57BL/6J and NMRI mice. The GYY mice were phenotypically normal, the architecture of their islets was unaltered, and the insulin and glucagon secretory responses to arginine, glucose and adrenaline were normal. Interestingly, 15 mM glucose elicited a biphasic change in glucagon secretion, characterized by an initial, short lived stimulation occurring before insulin secretion increased and a subsequent robust and sustained inhibition. These observations are compatible with the hypothesis that glucose exerts both stimulatory and inhibitory effects on glucagon secretion, but whether these effects are direct or indirect, involving paracrine factors, is controversial (for review, see [5]), and beyond the scope of this study. The presence of this biphasic glucagon response in both NMRI and GYY islets further strengthens the validity of the GYY mouse model.

Since some cells did not express detectable EYFP levels, it could be argued that the normal glucagon secretion reflects the response of these cells rather than of EYFP-tagged cells. This is not the case for several reasons. First, glucose strongly (~90%) inhibited glucagon secretion in perifused islets from GYY mice. It is unlikely that this robust inhibition reflects the response of the ~20% non-EYFP tagged α-cells only. Second, the $[Ca^{2+}]_c$ responses of EYFP-tagged α-cells to adrenaline, arginine and K_{ATP} channel modulators were similar to those of NMRI α-cells and those reported by others [6,22,23]. Moreover, glucose and K_{ATP} channel modulators induced the expected $[Ca^{2+}]_c$ responses [25,26] in EYFP-tagged β-cells from RIPYY mice. All these results indicate that GYY mice can reliably be used to study α-cell physiology.

Specific expression of EYFP in α-cells should be useful for a wide variety of applications including islet cell sorting by fluorescence activated cell sorting (FACS). Thus, sorting α- from β-cells from non-transgenic animals by FACS usually relies on differences in autofluorescence and size between both cell types [8,33]. This method provides an enriched but not pure preparation of cells. The α-cell fraction is often contaminated by PP- and δ-cells also present in islets. EYFP-expressing α-cells from GYY mice should be sorted at almost 100% of purity. This will help to study the physiology and gene expression of pure α-cell populations. Developmental biology could also take advantage of the GYY mouse model. Pancreatic islet cell lineage and islet differentiation become increasingly well understood [34,35]. However, mobility of cells during development hampered the study of cell lineages and there is a strong necessity to develop *in vivo* labelling techniques to fully understand this process. Therefore, the new GYY mouse model could provide an interesting tool to spatially track the fate of α-cell progenitors. Another advantage of these transgenic mice is the rapid identification of α-cells before performing electrophysiological or imaging experiments. Indeed, progress in our understanding of the cellular mechanisms controlling glucagon secretion has been slowed down by the difficulty to recognize living α-cells. GYY mice should help to evaluate $[Ca^{2+}]_c$, metabolic and other changes in α-cells in situ and to address major controversial issues such as the mechanisms by which glucose and amino acids interact to control glucagon secretion.

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