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Automated, High-Throughput Assays for Evaluation of Human Pancreatic Islet Function

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Abstract

An important challenge in pancreatic islet transplantation in association with type 1 diabetes is to define automatic high-throughput assays for evaluation of human islet function. The physiological techniques presently used are amenable to small-scale experimental samples and produce descriptive results. The postgenomic era provides an opportunity to analyze biological processes on a larger scale, but the transition to high-throughput technologies is still a challenge. As a first step to implement high-throughput assays for the study of human islet function, we have developed two methodologies: multiple automated perifusion to determine islet hormone secretion and high-throughput kinetic imaging to examine islet cellular responses. Both technologies use fully automated devices that allow performing simultaneous experiments on multiple islet preparations. Our results illustrate that these technologies can be applied to study the functional status and explore the pharmacological profiles of islet cells. These methodologies will enable functional characterization of human islet preparations before transplantation and thereby provide the basis for the establishment of predictive tests for β-cell potency.

Keywords

High-throughput assay; Pancreatic islets; Human islet function; Type 1 diabetes

INTRODUCTION

The endocrine cells of the islets of Langerhans in the human pancreas strictly maintain blood glucose levels at about 5 mM. Long-lasting increases (hyperglycemia) and decreases (hypoglycemia) in blood glucose concentration can be fatal and are efficiently prevented by the secretion of the islet hormones insulin and glucagon, respectively. Death or malfunction of the insulin-secreting β-cells leads to diabetes mellitus. It is important to study human islets because relatively little is known about their function in normal physiology and during the progression to diabetes. Additionally, because islet transplantation is emerging as a new

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therapy to cure type 1 diabetes, examining functional parameters in vitro may predict islet potency after transplantation.

Numerous assays are being developed to optimize and assure quality of cells that are going to be used for transplantation (2,4,7,10,14). Assessment of human islet preparations before transplantation is required to characterize islet cell viability and potency. We have recently developed a method to assess β-cell content and viability in human islets that predicts functional performance of islet preparations (8). By using laser scanning cytometry, we showed that we could examine the viability of large numbers of cells in a short time. We expect that the availability of novel sophisticated techniques (6) will improve our ability to discriminate islet preparations that are suitable for transplantation from those that should be discarded [for a discussion see (16)].

The most powerful assays focus on the physiological processes of living cells. While several steps of the stimulus–secretion coupling in islet cells have been studied using conventional methods, the transition to quantitative and high-throughput technologies has not been made. For instance, cellular imaging of cytoplasmic-free Ca^{2+} concentration, $[Ca^{2+}]_i$, in islet cells is so far amenable only to small samples. Hormone secretion is generally examined by using static incubation of islets, in which secretory responses are likely to be blunted by the low temporal resolution of the technique and/or by desensitization due to unspecific accumulation of hormones. Dynamic changes in hormone secretion have been determined using the islet perifusion technique but, as far as we know, this has been conducted on single samples only. As such, these techniques are not suited to study the complexity of biological processes in islet cells on a large scale in order to establish whether an islet preparation is healthy, or to determine whether intervention and changes in culture conditions improve islet health prior to transplantation. Given the heterogeneity of islet cells and the variability between islets, reliable data about the functional status of an islet preparation can only be obtained with high-throughput assays.

Here we report two novel approaches that overcome many of the limitations of conventional physiological techniques. First, we have adapted the perifusion technique to allow simultaneous measurements of hormone release from multiple islet samples in parallel. We have built an automated perifusion device that simultaneously collects the perifusates of up to eight islet samples with a temporal resolution of <1 min (Biorep® Perifusion V2.0.0). The perifusates are then examined using Luminex assays to determine the content of up to 100 analytes in a single reaction (xMAP technology). Second, we have used a kinetic imaging system, the BD Pathway™ 855 Bioimager, to examine islet cell populations with fluorescence-based high content kinetic assays. Our results validate the application of these systems to study islet health, pharmacological profiles of islet cells, and species differences in islet cell function.

MATERIALS AND METHODS

Islet Isolation and Culture

Human pancreata were obtained from multiorgan cadaveric donors ($n = 8$). Monkey islets were isolated from cynomolgus monkeys (*Macacca fascicularis*; $n = 3$) >4 years of age at the time of pancreas procurement. The pig pancreata ($n = 3$) were procured from the local slaughterhouse. Mice (C57Bl/6; $n = 3$) were killed by exposure to a rising concentration of CO_2 followed by cervical dislocation. All experimental protocols using mice were approved by the University of Miami Care and Use Committee.

Islets from human pancreata were isolated at the cGMP Human Islet Cell Processing Facility of the Cell Transplant Center at the Miller School of Medicine, University of Miami. The

glands were cold preserved in University of Wisconsin solution (12). Islets were isolated using a modification of the automated method (17) applying seven different lots of the enzyme Liberase HI (Roche, Indianapolis, IN) and a standard purification step, as described previously (17). Monkey, pig, rat, and mouse islet isolations were performed using modifications of the automated method for human islet isolation (17), adapted for isolation of monkey islets (11) and mouse islets (3). Human islets were dissociated into single cells using enzyme-free cell dissociation buffer (Invitrogen, Grand Island, NY). Islets and islets cells from all three species were cultured identically (37°C, 5% CO_2) in CMRL medium-1066 (Invitrogen), niacinamide (10 mM, Sigma), ITS (BD Biosciences, San Jose, CA), Zn_2SO_4 (15 μM, Sigma), glutamax (Invitrogen), HEPES (25 mM, Sigma), fetal bovine serum (10%, Invitrogen), and penicillin-streptomycin (Invitrogen).

Dynamic Measurements of Insulin and Glucagon Secretion

A high-capacity, automated perifusion system (Bio rep® Perifusion V2.0.0) was developed to dynamically measure hormone secretion from pancreatic islets (Fig. 1). A low pulsatility peristaltic pump pushed HEPES-buffered solution (mM: 125 NaCl, 5.9 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, 0.1% BSA, pH 7.4; 100 μl/min) through a sample container harboring 100 pancreatic islets immobilized in Bio-Gel P-4 Gel (BioRad, Hercules, CA). Except where otherwise noted, glucose concentration was adjusted to 3 mM for all experiments. Stimuli were applied with the perifusion solution. The perifusate was collected in an automatic fraction collector designed for a multiwell plate format. The sample containers harboring the islets and the perifusion solutions were kept at 37°C in a built-in temperature controlled chamber, and the perifusate in the collecting plate was kept at <4°C, to preserve the integrity of the analytes in the perifusate. Perifusates were collected every minute.

Determination of Hormone Release

Hormone release in the perifusate was determined with the human, rat, or mouse Endocrine LINCOplex Kit following the manufacturer's instructions (Linco Research, St. Charles, MO). The biomolecular assays were performed on a Bio-Plex protein array system (Bio-Rad). This system is a flow-based dual laser system that simultaneously identifies and quantifies up to 100 different analytes in a single assay.

Determinaton of $[Ca^{2+}]_i$

Kinetic imaging of cytoplasmic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed on a BD Pathway™ 855 Bioimager (5). Dispersed islet cells were plated at a density of 20,000 cells per well in black-sided 96-well plates coated with poly-D-lysin, and cultured overnight in CMRL medium.

For imaging, islet cells were immersed in HEPES-buffered solution (mM: 125 NaCl, 5.9 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, 0.1% BSA, pH 7.4). Glucose was added at a basal concentration of 3 mM. Islet cells were incubated in Fura-2 AM (2 μM; 30 min). Cell plates were washed, leaving a residual volume of 100 μl, and transferred to the BD Pathway™ 855 Bioimager for kinetic imaging. The atmosphere in the BD Pathway™ 855 Bioimager was kept at 37°C and 5% CO_2. Stimuli were prepared at 5× the final concentration such that addition of 25 μl would yield the desired concentration. Stimuli were applied with an automated integrated disposable tip liquid handling. Islet cells loaded with Fura-2 were excited alternatively at 340 and 380 nm and images were acquired. Islet cells were automatically identified and numbered as individual regions of interest (ROIs) by the segmentation menu of the BD Pathway™ 855 Bio-imager. Changes in the 340/380 fluorescence emission ratio over time were analyzed in the ROIs. Peak changes in the fluorescence ratio constituted the response amplitude.

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Immunostaining

After kinetic imaging, the islet cells were fixed in 4% paraformaldehyde (10 min). After several rinses with OptiMax Wash Buffer (Biogenex, San Ramon, CA), cells were incubated in Universal Blocker Reagent (5–10 min; Biogenex), rinsed again in OptiMax Wash Buffer, and incubated in Protein Block (20 min; Bio-genex). Thereafter, cells were incubated overnight with anti-insulin (1:500, Accurate Chemical & Scientific Corp., NY) or anti-glucagon (1:5000; Sigma) antibodies. Immunostaining was visualized using Alexa Fluor conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR). Cell nuclei were stained with DAPI (Molecular Probes). The cell plate was placed back in the BD Pathway™ 855 Bioimager bioimager and images were acquired and compared to those obtained during the kinetic imaging. It was always possible to correlate the cells from the kinetic and immunostaining experiments. As a negative control, we substituted the primary antibody with the serum of the animal used to raise this antibody. No staining was observed under these conditions. Digital images were compiled using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose CA). Only brightness and contrast were adjusted.

RESULTS

High-throughput assays require automation and the precise coordination of various steps in an integrated workflow (15). Our experiments comprised three independent steps: sample preparation, data acquisition, and data analysis (Fig. 1). Cultured islets were processed in parallel for perifusion assays and kinetic imaging. For perifusion assays, intact islets were loaded in a sample container (see Material and Methods). For kinetic imaging, islets were dissociated into single cells and plated in 96-well plates. Loaded sample containers and imaging plates were placed in fully automated systems for data acquisition. High-throughput kinetic imaging of islet cell responses was performed on an automated fluorescence-based image acquisition system, the BD Pathway™ 855 Bioimager (BD Biosciences). Image aquisition and data analysis was done using BD Atto-vision™ (Version 1,5,2,3; BD Biosciences). Perifusion assays were conducted on an automated perifusion device (Biorep® Perifusion V2.0.0) designed and built by the Diabetes Research Institute, University of Miami and Biorep® Technologies, Inc. (Miami, FL). Up to eight sample containers with islets were placed in a built-in temperature-controlled chamber, and solutions flowing through the sample containers (perifusates) were collected in 96-well plates moved by a built-in robotic arm. The movements of the robotic arm could be adjusted to change the time resolution of the experiment. Perifusates were processed for detection of three hormones in multiple, simultaneous assays using Luminex technology (Luminex® Corporation, Austin, TX). Quantitative data of the hormone concentrations were plotted as a function of time to obtain a dynamic profile of the secretory responses.

To validate these approaches, we damaged whole islets or dispersed islet cells and determined whether changes in islet physiology could be detected. Islets were treated for 2 h with different concentrations of staurosporine (10–10,000 nM), a potent, cell-permeable protein kinase C inhibitor that induces cell death. The different experimental groups were examined in parallel for their capability to secrete insulin and glucagon using the perifusion assay. Incubation with staurosporine strongly reduced the insulin responses to high glucose concentration (11 mM) and the glucagon responses to kainate (100 μM) in a concentration-dependent fashion (Fig. 2B, C). We further incubated islets under anoxic conditions and found that insulin responses to stimulation with high glucose (11 mM) and KCl depolarization were decreased (Fig. 2D–F). The insulin response to glucose became smaller as the exposure to anoxia was increased. After very long anoxia, insulin responses to KCl depolarization also became smaller.

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We further exposed human islet cells to staurosporine and tested whether changes in $[Ca^{2+}]_i$ responses could be detected using the BD Pathway™ 855 Bioimager. Islet cells placed in 96-well plates were exposed to a gradient of staurosporine concentrations. Islet cells were loaded with the Ca^{2+} indicator Fura-2 and stimulated with high glucose (11 mM). Islet cells were classified by the Atto-vision™ software into three groups according to the amplitudes of their $[Ca^{2+}]_i$ responses to high glucose (Fig. 3A). The proportion of nonresponding cells increased in wells incubated with staurosporine (blue traces and bars in Fig. 3B, C). None of the human β-cells treated with the highest staurosporine concentration tested showed large responses (Fig. 3B, E). These effects were concentration dependent (Fig. 3C–E).

We used our functional approaches to obtain pharmacological profiles of human islet cells. We applied compounds that activate neurotransmitter receptors to test their effects in islet cells. We tested six agonists for ATP (purinergic) receptors and found that most of them elicited insulin responses in human islets (Fig. 4A). Two of the agonists stimulated somatostatin secretion. The agonists had little effects on glucagon secretion. When islets were incubated with agonists for glutamate receptors of the AMPA/kainate type, human islets responded with increased secretion of glucagon but not insulin (Fig. 4B). By using $[Ca^{2+}]_i$ imaging, we found that ATP and AMPA/kainate receptor agonists induced responses in islet cells (Fig. 5). Acetylcholine also induced large $[Ca^{2+}]_i$ responses. To establish which cell types were responsive, we performed immunofluorescence for the islet hormones insulin and glucagon after $[Ca^{2+}]_i$ imaging. We found that almost all cells sensitive to AMPA/kainate receptor agonists were also glucagon immunoreactive (Fig. 5). ATP receptor agonists elicited $[Ca^{2+}]_i$ responses in insulin-immunoreactive cells and also in unstained cells. Small responses were also seen in a fraction of glucagon-immunoreactive cells.

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We further used our assays to study species differences with regard to islet function. Insulin responses to high glucose (11 mM) were different in the species studied (Fig. 6A). Human and monkey islet responses showed an initial sharp peak followed by a sustained response that lasted for the duration of the stimulus application. In contrast, insulin secretion in mouse islets was only transiently increased by high glucose and returned to almost basal levels in the presence of the stimulus. As an additional validation of the perifusion assay, we determined whether insulin secretion was impaired in islets from donors with type 2 diabetes. We found that insulin responses to high glucose were smaller in islets from donors that had a longer history of type 2 diabetes, indicating that insulin secretion becomes gradually impaired with progression of the disease (Fig. 6B).

DISCUSSION

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It is now possible to reverse type 1 diabetes and restore glucose homeostasis by transplanting islets from deceased donors into individuals with the disease. This has opened new therapeutic avenues for the treatment of type 1 diabetes. In this context, it is particularly important to establish techniques that allow screening of drugs and compounds for effects on islet cell differentiation, islet cell signaling pathways, and islet cell pathology. Here we have shown how high-throughput assays can be implemented for the study of islet cell function. Our results illustrate that our approaches can reliably measure changes in the health status of human islet cells, determine pharmacological profiles of human islet cells, reveal species differences in islet function, and determine that function is impaired in islets from individuals with type 2 diabetes.

Given its major implications for normal glucose homeostasis, insulin secretion has been extensively studied. In most studies in the literature, insulin secretion has been measured using static incubations of islets. This method has drawbacks, such as a low temporal resolution and desensitization of secretory responses because secretory products accumulate

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around the endocrine cells. These experiments do not provide any temporal information and results may be misleading because the final state of the examined islets may be an indirect consequence of a series of sequential processes. Alternatively, dynamic hormone secretory responses have been measured with perifusion assays in which solutions flowing through the islet preparation (perifusates) are collected for analysis (9). However, these assays have been amenable only to small-scale experimental samples. Our perifusion approach overcomes most of these limitations. First, we have built a perifusion device that allows studying up to eight different islet samples simultaneously. Stimulus delivery and collection of the perifusate are fully automated. The samples and stimulating solutions are incubated in the same temperature-controlled chamber. The samples are collected in a multiwell format plate. At the end of the experiment, the collecting plate is exposed in a way that an external robot can transfer it to an automated analysis system. Second, when coupled to Luminex assays, perifusates can be examined for the content of up to 100 analytes. This means that hundreds of endpoints can be measured with a time resolution of less than 1 min. This flexible technology enables screening of multiple drugs and compounds for effects on insulin and glucagon secretion as well as on the release of other active substances such as cytokines. Given the large variability among preparations, using our device and approach, which can examine up to eight samples in parallel, is likely to be successful to get robust information about the secretory power of the actual islet preparation prior to transplantation.

Studies of complex biological processes require the use of high-throughput approaches. However, neither of the existent, successfully applied techniques—protein analysis by mass spectrometry or transcription profiling by DNA microarrays—provide information about functional cellular processes. In contrast, fluorescence-based imaging assays examine such processes in the physiological context of intact living cells (15). These assays, however, are technically challenging and few applications of high-throughput microscopy have been reported (1,13). Here we have introduced a new imaging platform using the BD Pathway™ 855 Bioimager and have demonstrated that it can be implemented to study islet cell signaling and pathology. We have been able to obtain a preliminary pharmacological profile of islet cells that has revealed that kainate is a specific stimulus for α-cells. Thus, kainate can be used in future high-throughput assays to identify human α-cells. By screening many drugs and compounds for their effects on $[Ca^{2+}]_i$ in islet cells, we will be able to identify additional cell-specific functional markers. More importantly, the systematic and quantitative functional characterization of islet cells will provide a biophysical fingerprint that will help us to understand the complex phenotype of endocrine cells of the islet. Such information could provide clues as to how signaling pathways in islet cells change during the ontogeny of diabetes.

Our high-throughput assays can further be used as diagnostic tools to test the efficacy of islets in the context of islet transplantation as a therapy. Islets from different islet preparations could be screened in large scale for their secretory and cellular responses to glucose and other stimuli. The temporal pattern as well as the magnitude of the responses could be indicators of islet health. Alternatively, the analyses could be focused on particular signaling pathways that are selectively impaired in unhealthy islets, which could serve as biomarkers (e.g., voltage-gated Ca^{2+} channels). These functional parameters can then be validated as predictors of islet potency by correlating them with transplantation outcome. Another application of our high-throughput assays is as screening tools to test the effects of compounds on islet cell biology. Compounds could be tested together with well-characterized molecules in islet cells loaded with various fluorescent probes that cover a range of cellular phenotypes (e.g., mitochondrial membrane potential, $[Ca^{2+}]_i$, and intracellular pH) and then be classified based on the biological outcome and mechanism of action. In addition, based on the high degree of reproducibility, our high-throughput assays

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can successfully be applied for research purposes, specifically defining species differences with regard to islets cell signal transduction.

The greatest challenges in using high-throughput approaches are likely not to be biological, but computational. A major issue is the analysis of massive amounts of data, and for many applications, the required software is still being developed. In our studies, for instance, acquisition of data was much faster than data processing. Nevertheless, experiments that were typically carried out manually for weeks were done in a matter of days or hours. Our results give a glimpse of the potential of high-throughput approaches. The flexibility and power of these approaches will make them key technologies for studying the complex biology of the islet endocrine cells.

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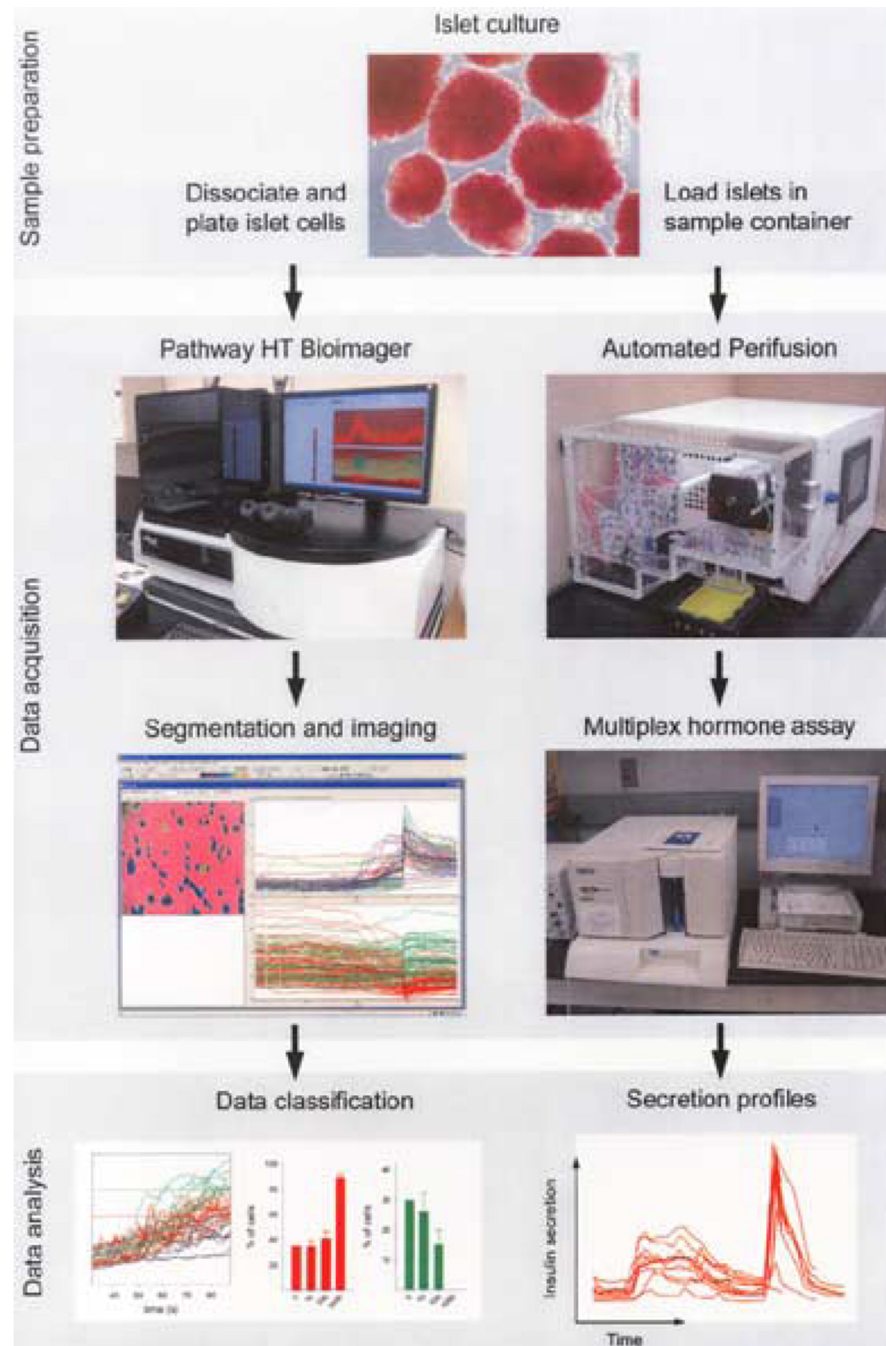

Figure 1.

Diagram showing the workflow using high-throughput kinetic imaging (left) to examine islet cellular responses and the multiple automated perifusion assay (right) to determine islet hormone secretion. The workflow consisted of sample preparation, data acquisition, and data analysis.

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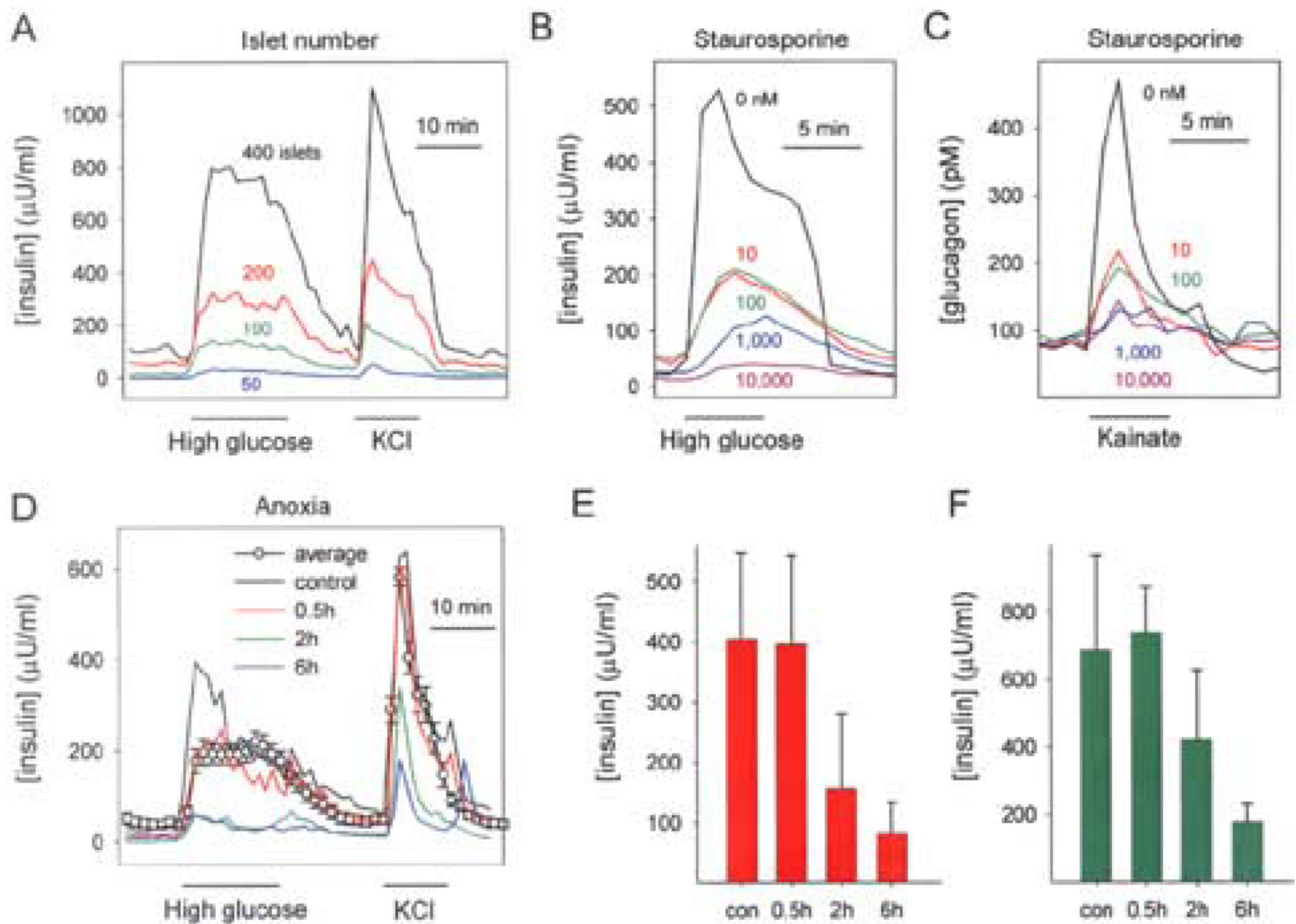

Figure 2.

Validation of the multiple automated perifusion assay to study human islet function. (A) Titration of human islet number showed that insulin secretion levels increased proportionally to the mass of islet tissue in the sample containers. The levels of basal insulin secretion as well as responses induced by high glucose (11 mM) and KCl (30 mM) depolarization correlated with islet number. (B) Cell injury induced by staurosporine (10–10,000 nM) concentration-dependently impaired insulin responses to high glucose (11 mM). (C) Treatment with staurosporine also impaired glucagon responses to kainate (100 μM) in a concentration-dependent manner. (D) Anoxia decreased the insulin responses to high glucose (11 mM) and to KCl (30 mM) depolarization. Empty symbols show the average ± SEM of 23 human islet preparations for comparison. The control trace (black) shows that the examined preparation had a robust response to high glucose that was gradually reduced as anoxia times increased (0.5, 2, and 6 h). (E, F) Quantification of the results shown in (D) shows that insulin responses to high glucose (E) and KCl depolarization (F) were sensitive to anoxia.

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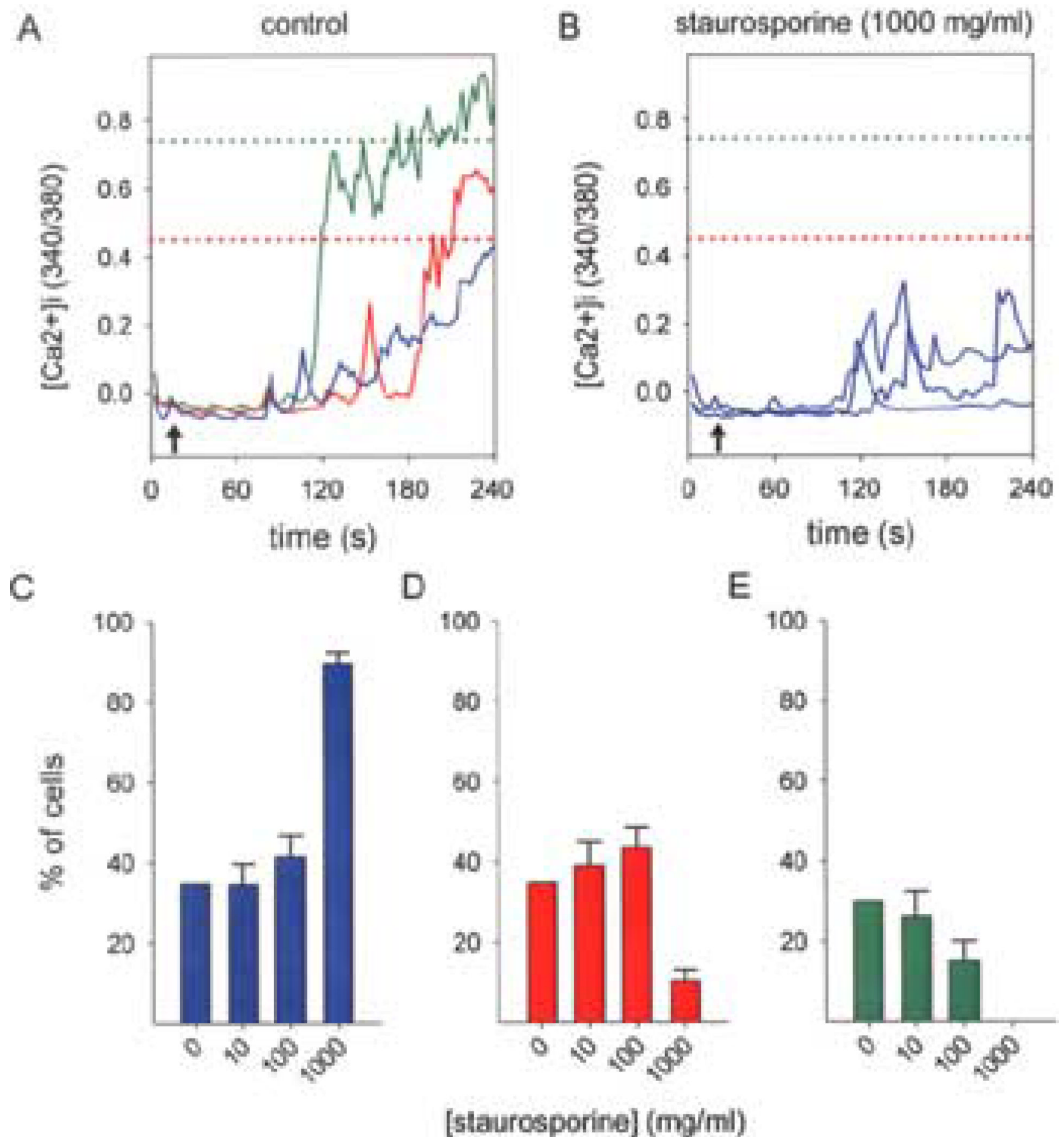

Figure 3.

Validation of the high-throughput kinetic imaging system to examine islet cellular responses. (A) Representative traces from individual human islet cells in a well of a 96-well plate showed a range of increases in $[Ca^{2+}]_i$, in response to high glucose (11 mM). Blue traces are from cells that the system did not include as responsive, red and green traces are from cells that were respectively considered responsive and highly responsive. (B) Representative traces from islet cells in a well that was treated with staurosporine (10,000 nM). (C–E) Treatment with staurosporine (10–10,000 nM) increased the number of nonresponsive cells (C) and diminished the number of responsive (D) and highly responsive

cells (E) in a concentration-dependent manner. Data other than control are pooled from at least 50 cells per well and three wells per condition.

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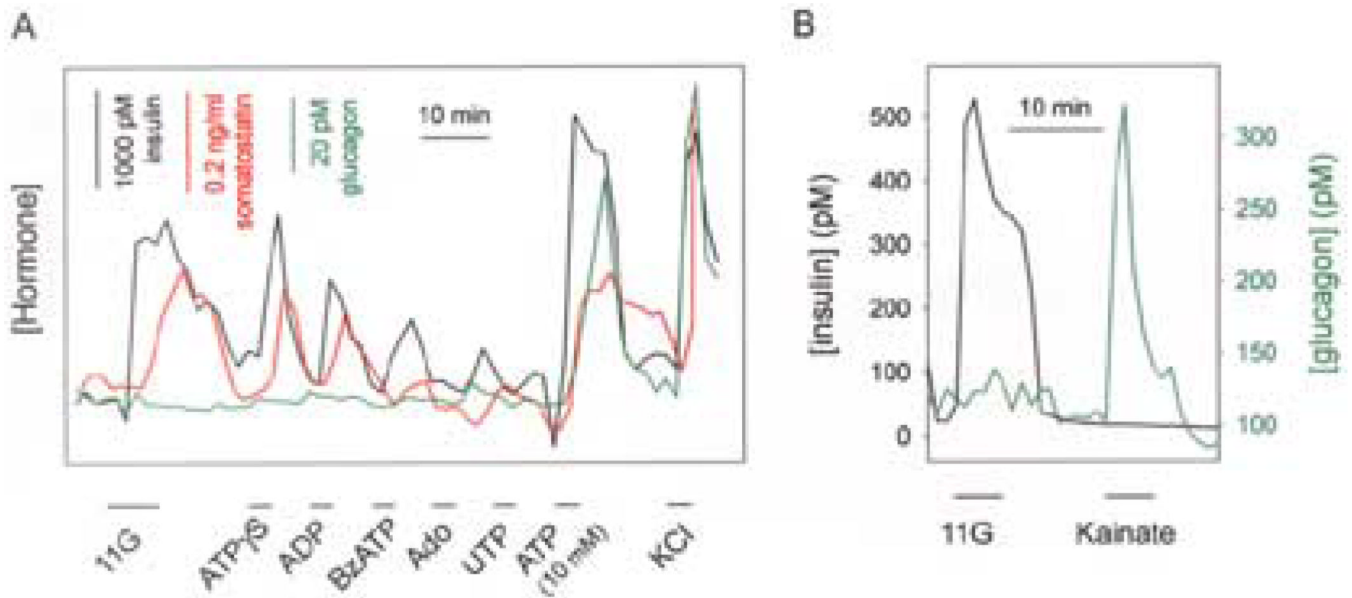

Figure 4.

Pharmacological profiling and phenotypic characterization of human islet cells by perifusion. (A) The ATP receptor agonists ATPγS, ADP, and BzATP increased insulin secretion at a nonstimulatory glucose concentration. ATPγS and ADP, but not BzATP, elicited somatostatin secretion. The agonists had little effect on glucagon secretion. Very high, unphysiological levels of ATP (10 mM) stimulated the secretion of all three tested hormones. (B) Kainate stimulated glucagon secretion but not insulin secretion.

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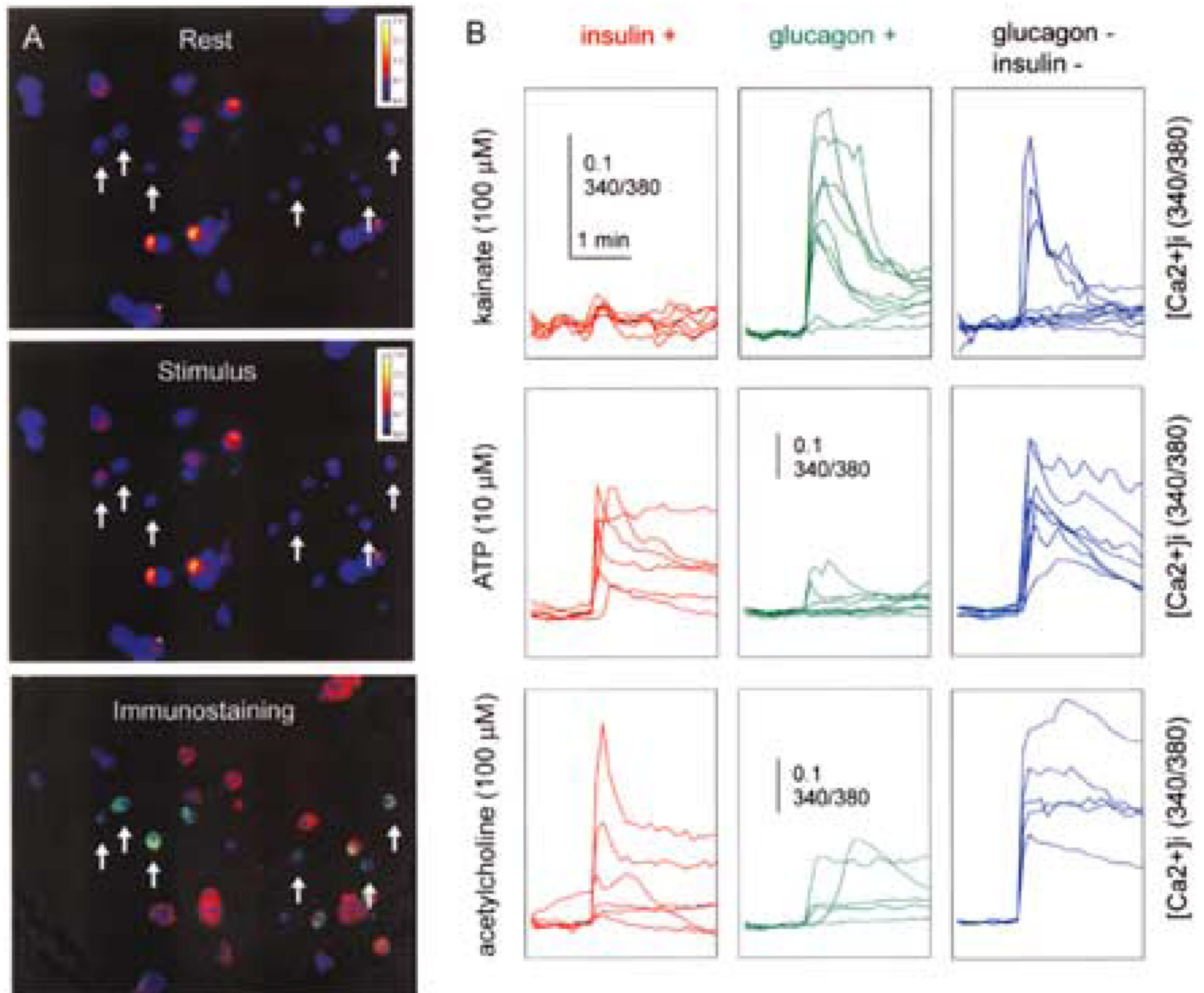

Figure 5.

Pharmacological profiling and phenotypic characterization of human islet cells with the high-throughput kinetic imaging system. (A) Two consecutive images, at rest and during stimulation, of a culture well plated with human islets cells loaded with the Ca^{2+} indicator Fura-2 show increases in $[Ca^{2+}]_i$ in response to kainate (50 μM). Fluorescence intensity is shown in pseudocolor scale. Arrows point at highly responsive cells. Bottom image shows the same well after immunostaining for insulin (red) and glucagon (green). The cells that were highly responsive to kainate were glucagon immunoreactive. (B) Traces of $[Ca^{2+}]_i$ responses from human islet cells in three wells exposed to either kainate (top), ATP (middle), or acetylcholine (bottom). Cells were identified with immunostaining after $[Ca^{2+}]_i$ imaging. Glucagon-immunoreactive cells (left column), but not insulin-immunoreactive cells (middle column), responded to kainate. ATP predominantly stimulated insulin-immunoreactive cells and unstained cells; only few glucagon-immunoreactive cells responded to ATP. Acetylcholine stimulated all cell types. Scales are the same for each row to allow response magnitudes to be compared.

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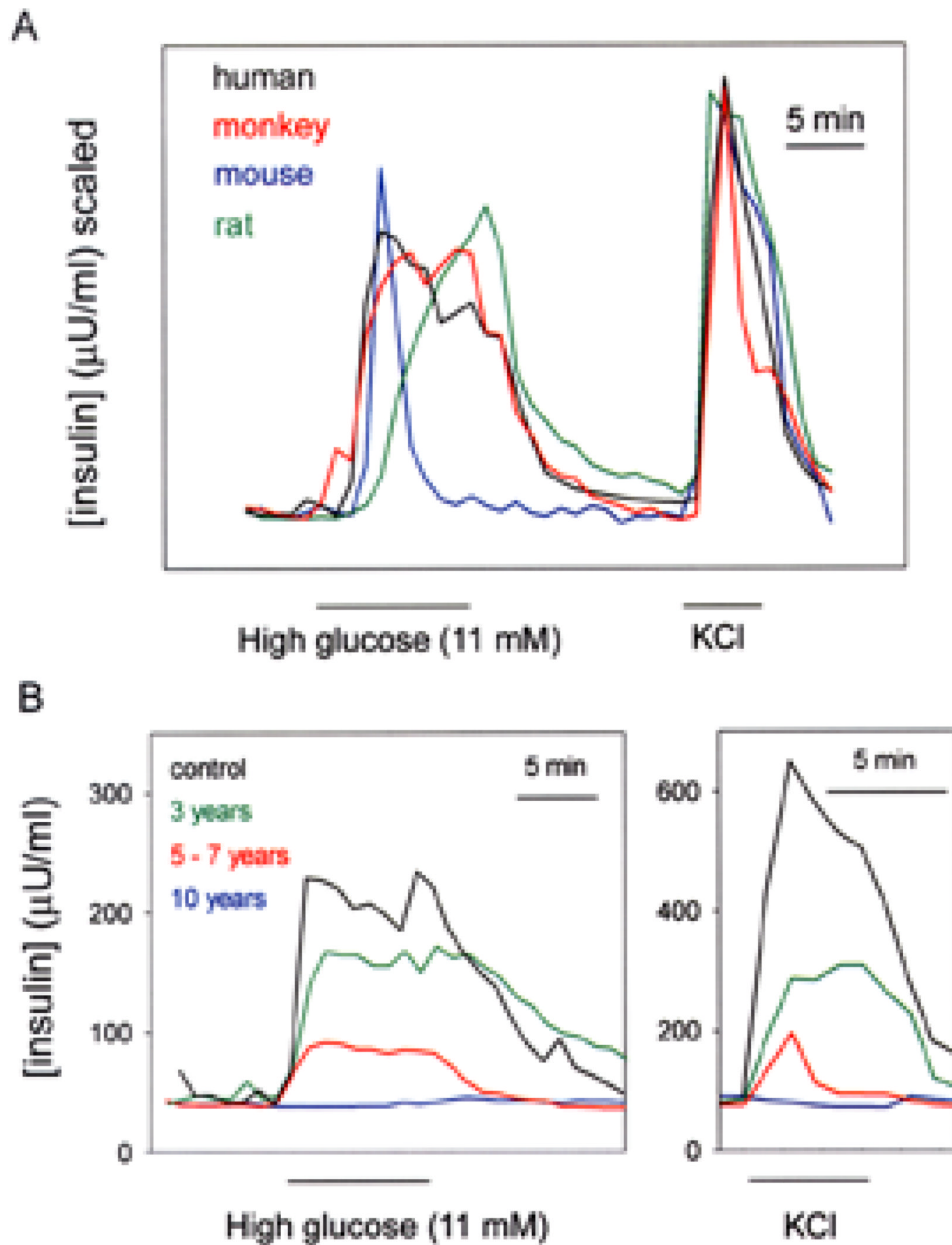

Figure 6.

The automated multiple perifusion assay revealed species differences in the insulin secretory profile and that this profile is altered in islets from individuals with type 2 diabetes. (A) Perifusion assays showed differences in insulin secretion in response to high glucose (11 mM) in four different species. (B) Perifusions of islets from individuals with type 2 diabetes showed that insulin secretion in response to high glucose (left) and KCl depolarization (right) decreased gradually with the progression of diabetes.