The aim of this study was to define when an insulin-positive cell becomes functional in vivo and starts to exocytose insulin in a regulated nutrient-dependent manner. Insulin-positive cells appear in embryonic life (midgestation) and complete their maturation, presumably around birth. In order to work with embryonic and newborn endocrine pancreas, we used organotypic slices. The mouse embryonic pancreas slices show high basal insulin release that is not further elevated by high glucose levels. Despite the presence of functional voltage-activated ion channels, the cells are not electrically active in the presence of secretagogues. At birth, the high basal insulin release drops and, after postnatal day 2, the insulin-positive cells show both adult-like bursting electrical activity and hormone release induced by high glucose levels. These properties allowed us to define them as beta cells. Despite the apparent stability of the transcription factor profile reported in insulin-positive cells during late-embryonic life, functional beta cells appear only 2 days after birth.

Key words: insulin release; embryo; newborn; beta-cell maturation; developing pancreas
detailed functional analysis of the development of excitability and secretory competence in the mouse is lacking.

In this study, we used organotypic cultured slices from mouse pancreas, a novel preparation, to study the functional development of pancreatic beta cells. The main reason for using this approach is the high yield of endocrine tissue from young animals when the pancreatic tissue is scarce and fragile. In fact, the insulin-positive cell clusters appear as small elongated shapes close to the ducts in late-embryonic life. We found that embryonic beta cells have a higher basal insulin secretion rate but very little glucose responsiveness. This fact can be at least explained by its depolarized membrane potential. At postnatal day 2, the basal insulin secretion is significantly reduced to the adult level, coinciding with the gradually hyperpolarized membrane potentials. This change is primarily a result of delayed expression of ATP-sensitive potassium channels (K<sub>ATP</sub>).

**Materials and Methods**

**Experimental Animals**

Naval Medical Research Institute (NMRI) mice were provided by Göttingen University (ZTE) and used between embryonic day 17 (E17) and postnatal day 7 (P7). The adults (pregnant) were killed by cervical dislocation and decapitation and embryos and newborns by decapitation. All animal procedures were conducted according to the National Institutes of Health guidelines and approved by the local animal care authorities. Blood samples were collected immediately after decapitation from the carotid artery to measure glucose (One-Touch Ultra, LifeScan, Milpitas, CA) and to extract plasma for insulin measurement (EDTA-prepared microtubes, Sarstedt, Nuernbrecht, Germany; ultrasensitive mouse insulin ELISA, Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions.

**Slices**

Whole pancreas slices were prepared as previously reported. Briefly, low-melting point agarose (1.9% in extracellular solution at 37°C; Cambrex Corporation, East Rutherford, NJ) was injected under the organ capsule to stabilize the tissue. Blown-up pancreas was embedded in the same agarose and cut into 100-μm thick slices, using a vibratome (VT1000S, Leica Microsystems AG, Wetzlar, Germany) in ice-cold solution bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. To wash out the pharmacologically active compounds released by the cutting procedure, the slices were stored in cold, bubbled, extracellular solution for at least 1 h and then used for experiments. Between E17 and P3 both the animals and pancreata increased their weight more than 300% and, accordingly, the number of slices prepared from each animal increased from more than 10 to about 20, with five to 15 insulin-positive cell clusters or islets each. This gave us the possibility of using tissue from the same animal for electrophysiological hormone-release and immunostaining experiments.

**Immunostaining**

The slices were used fresh or after overnight culture in a humid incubator (37°C with 5% CO<sub>2</sub>). For overnight culture, slices were placed in 24-well plates, free floating in about 1 mL of incubation medium. Both fresh and cultured slices were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h. The slices were then incubated in the blocking solution (reducing unspecific binding and increasing the penetration of antibodies) for 1 h at room temperature or overnight at 4°C with two primary antibodies: anti-insulin (prepared in mouse, 1:1000 dilution, Sigma, St Louis, MO) and anti-S100 (in rabbit, 1:200, DAKO, Carpinteria, CA). After washing, the slices were incubated with secondary antibodies anti-mouse Alexa 488 and anti-rabbit Alexa 647 (in goat, 1:500; Molecular Probes, Carlsbad, CA) for 1 h in blocking
solution. All the incubations were at room temperature unless otherwise indicated. Finally, slices were mounted using a solution of 95% glycerol in PBS, and fluorescence immunostaining was acquired using a confocal microscope (Leica TCS SP2 AOBS; Leica Microsystems, Mannheim, Germany) using 488 nm (Ar) and 633 nm (He-Ne) lasers for sequential excitation. The images were processed by Leica confocal software.

**Transmission Electron Microscopy**

For the electron microscopy, the tissue was embedded in epoxy resin (Agar Scientific Ltd., Essex, UK). The tissue was then dissected and fixed in 2.45% glutaraldehyde and 2.45% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at room temperature for 3 h and at 4°C overnight. Small fragments of tissue were washed in 0.1 mol/L sodium cacodylate buffer at room temperature for 3 h and postfixed in 2% OsO4 at room temperature for 2 h. Then, the tissue was washed in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at room temperature for 3 h and dehydrated using a graded ethanol series and embedded in epoxy resin. The samples were sectioned on an Ultracut E Ultramikrotom (Reichert, Depew, NY). Semithin sections were stained with 0.5% toluidine blue in aqueous solution. For electron microscopy, ultrathin sections (70 nm) were obtained and transferred to copper grids (300 mesh). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 902 transmission electron microscope.

**Insulin Release**

After a washing step, the slices were incubated at 37°C in culture medium for at least 10 min. Single slices were incubated sequentially in low (5 mmol/L) and high (15 mmol/L) glucose in 0.2 mL solution in a humid incubator (37°C, 5% CO2) and the media prewarmed. After 30 min of incubation a sample of medium was collected to measure the insulin released and the remaining medium was discarded. Each slice was then homogenized and the insulin content extracted by sonication in acid ethanol (25 min, 200 μL) to normalize the release to the hormone cellular content in the slice.

**Electrophysiology**

The slices were superfused (1.5 mL/min) with warm, bubbled, extracellular solution (35°C; 95% O2, 5% CO2; pH 7.4) in a recording chamber (400 μL). A platinum frame with nylon fibers kept the slices flat on the bottom of the recording chamber. An upright microscope (Eclipse E600FN, Nikon, Tokyo, Japan) with a 60× water objective was used. Borosilicate capillaries (GC150F-15; Harvard Apparatus Ltd, Edenbridge, UK) were pulsed to resistances of 3–6 MΩ by a horizontal puller (P-97 Sutter Instruments, Novato, CA).

Recordings were performed in whole-cell configuration with a standard patch-clamp amplifier (SWAM IIC, Celica, Ljubljana, Slovenia; or EPC9/10, HEKA Electronik, Lambrecht, Germany). Data were acquired with WinWCP 3.2.9 software (John Dempster, University of Strathclyde, Glasgow, UK) via an A/D converter (PCI-6035E, National Instruments, Austin, TX). The same software was used to analyze data and export them to SigmaPlot (version 7.0, SPSS Inc., Chicago, IL). Putative beta cells were identified by the characteristic voltage-activated Na+ current inactivation properties.9,12 ATP concentration inside the pipette was 0, 2, or 5 mmol/L as indicated.

**Solutions and Chemicals**

Extracellular solution (in mmol/L) was as follows: NaCl 125, KCl 2.5, MgCl2 1, CaCl2 2 or 10, NaH2PO4 1.25, NaHCO3 26, Na-pyruvate 2, ascorbic acid 0.25, myo-inositol 3, lactic acid 6, and glucose between 3 and 15, pH 7.4 (with carbogen at 35°C). K-intracellular (in mmol/L) solution was as follows: KCl 150, HEPES 10, MgCl2 2, EGTA 0.05, Na2-ATP as indicated, pH 7.2 (with KOH). Cs-intracellular
Figure 1. Relation between insulin-positive cells and ducts in postnatal day 0 (P0) mice. 

(A) Confocal image of double staining of 100-μm thick pancreas slice from P0 with anti-insulin (green) and anti-S-100 (Schwann cells and ducts, red) antibodies. Many S-100 positive cells are evident in the lower part of the picture and can be identified as Schwann cells (arrowheads) along fibers, around the ganglion (arrows), and the islets (asterisks). The solitary insulin-positive cell in the ganglion seems to be part of a neuroinsular complex. The islet in the upper left area of the picture is located at the bifurcation of a duct and is magnified and rendered in three dimension in (B). The anatomical relation between ducts and beta cells appears strict at P0 with numerous, isolated, insulin-positive cells along the ducts, the putative source of endocrine precursors. At higher magnification (C) the two cell populations appear mixed and focal insulin accumulations in the cytoplasm are visible. (D) Insulin-positive cell (green) embedded in the pancreatic duct (S-100, red). (E) Transmission electronmicrography showing the dilatations of the extracellular space and absence of docked insulin granules in P0 beta cells. Scale bars: (A) 250 μm; (B) 100 μm; (C) 50 μm; (D) 25 μm; (E) 500 nm.

Statistics

Statistics are given as means ± SEM. The statistical significance for the comparison was assessed using paired or unpaired Student’s t-test.

Results

Morphology

We first used immunostaining to determine the morphological development of pancreatic beta cells in organotypic slices. Double staining of anti-insulin and anti-S-100 antibodies during the first postnatal days showed the presence of many insulin-positive cells associated with ducts in the terminal region of the pancreatic ductal system (Fig. 1B). In embryonic and newborn pancreata, Schwann cells were found around islets, ganglia, and neuronal fibers (Fig. 1A). Unexpectedly ductal structures with typical morphology were also stained.

As already reported, the insulin signal distribution in embryo and P0 beta cells is condensed to a perinuclear area (Fig. 1C).
More rarely, the anti-GluT2 (Fig. 1D) stained apical parts of the ductal tree, which harbored a small number of isolated insulin-positive cells, confirming the ongoing neogenesis of endocrine cells from precursors during the first few postnatal days.\(^\text{13,14}\) Larger dilatations of extracellular space observed between endocrine cells at P0 may represent disconnections from cell replication (Fig. 1E; Susan Bonner-Weir, Joslin Diabetes Center, personal communication).

**Insulin versus Glucose Levels**

The plasma glucose and insulin levels are shown in Figure 2A. Before birth (E17–18) the plasma level of insulin was high. In contrast, the plasma glucose level in embryos was relatively low, although it was largely controlled by the mother. The plasma glucose levels increased just before birth (E19), despite an elevated insulin level (Fig. 2A). After birth, the glucose level rose above 4 mmol/L (Fig. 2A) and then progressively reached a plateau after P2. In the same period, the insulin level decreased. This result indicated the establishment of an adult-like regulatory mechanism for insulin release.

**Hormone Release from Slices**

Insulin release was tested using static incubation (Fig. 2B) in basal (5 mmol/L, black hatched) and stimulatory (15 mmol/L, hatched) glucose concentrations. The basal insulin release was high in embryonic life and dropped after birth from 6.7 ± 0.9 to 2.6 ± 0.7% of its cellular content (E19 vs. P0). Despite this difference, the response to high glucose levels was negligible in both ages. This high basal release in the embryo was stable for hours and was inhibited when incubated at room temperature (data not shown).\(^\text{15,16}\) From P2 onward, 15 mmol/L glucose resulted in an additional insulin release of 2.3 ± 0.8% on top of its basal release (3.4 ± 0.5%).

**Electrical Activity**

Electrical activity was recorded in whole-cell mode in pancreas slices. Until P0 the cells were depolarized (−47 ± 5 mV, \(n = 13\)) and
application of the K<sub>ATP</sub> modulator, tolbutamide (100 μmol/L), induced only a mild depolarization (Fig. 3A). At P2, the cells showed more hyperpolarized potential (−83 ± 7 mV; n = 6) and the K<sub>ATP</sub> block induced a sustained depolarization and firing activity. However, only from P3 did the cells start to show adult-like bursting activity with plateau phases between clusters of Ca<sup>2+</sup> firing. The injection of a current (+10 pA steps) also showed absence of firing in insulin-positive cells in animals younger than P0; however, similar current injection induced action potential-like electrical activity in insulin-positive cells of older animals (>P2; Fig. 3B).

Figure 3. Electrical activity induced by application of tolbutamide. Three representative traces of the responses induced by tolbutamide (100 μmol/L, the arrows mark the start of application) in newborn beta cells at different ages. (A) At P0 (top trace), beta cells have a relatively depolarized resting membrane potential and tolbutamide only slightly depolarizes the membrane. After P1 (middle and bottom trace) the resting membrane potential was more hyperpolarized and the tolbutamide elicited much stronger depolarization, and from P3 onward, even bursting activity can be observed sometimes upon tolbutamide application. Right panels show expanded traces. (B) Electrical activity after the injection of different current pulses in E20 (left trace) and P3 (right trace) beta cells.

On pancreatic beta-cell membrane, the K<sub>ATP</sub> channel is the key molecule linking metabolism to the electrical activity. We used voltage ramps between −150 and −100 mV to estimate the K<sub>ATP</sub> conductance (Fig. 4AB). Immediately after establishment of the whole-cell recording, the K<sub>ATP</sub> currents developed within 2 min as a result of washing out of intracellular ATP (Fig. 4B). We found that the K<sub>ATP</sub> channels at P0 were closed under resting conditions (0.4 ± 0.1 nS) but opened after a few minutes of zero ATP dialysis (peak conductance 5.9 ± 1.1 nS; Fig. 4BC). In beta cells from P2–3 animals, more K<sub>ATP</sub> channels were opened under resting conditions (0.6 ± 0.1 nS, P < 0.05);
Figure 5. Development of Ca\(^{2+}\) currents in insulin-positive cells. (A) Two representative traces of Ca\(^{2+}\) currents induced in embryo (E, gray) and newborn (P0, black) applying a ramp from −110 to +60 mV with a slope of 0.5 mV/ms. Note that the P0 cell has not only a smaller current but also a larger surface (+0.7 pF) according to its increase in cell size. The dialysis effect on current amplitude is shown in the insert. (B) The Ca\(^{2+}\) current density is compared between different ages. Numbers on bars indicate the number of cells tested.

Figure 6. Increase in capacitance triggered by activation of voltage-activated Ca\(^{2+}\) channels. A train of depolarizations from −60 to +10 mV was applied (10 Hz, 40 ms per pulse). (A) Two representative traces in P0 (gray) and P3 (black) insulin-positive cells. (B) Statistically significant difference in capacitance change (P < 0.002) and responsiveness of the insulin-positive cells. At P0 12/21 cells responded to a depolarization train (>20 fF), while 12/14 cells responded at P2. The ATP concentration inside the pipette was 5 mmol/L to exclude any response limitation from ATP washout.

peak conductance after zero ATP washout was not significantly different from P0 (not shown).

Ca\(^{2+}\) Channels

The pancreatic beta cell has calcium channel-mediated action potentials.\(^{17}\) The difference in membrane activity between E19 and P2 could be partially a result of different calcium channel expression on the membrane. We used Cs\(^+\)-based solution to record the Ca\(^{2+}\) currents from both embryonic and newborn beta cells (Fig. 5A). We found the normalized Ca\(^{2+}\) peak current amplitudes were higher in embryonic cells and dropped significantly at birth (from \(-57.6 \pm 6.4\) to \(-30.6 \pm 4.4\) pA/pF, respectively; Fig. 5B).

In E20, a relatively rare condition (approximately 1%) of delayed birth from a small litter size (four or five pups vs. 14 and 18 pups) was observed; the weight of these embryos (2.09 ± 0.03 g) was comparable to P2 (2.03 ± 0.15 g) and was 58% higher than E19 (1.32 ± 0.05 g). Despite the increased body size, the insulin-positive cells maintained their embryonic features, suggesting a critical role for delivery or the start of independent life in triggering the functional maturation of beta cells (not shown).

Single Cell Exocytotic Activity

The application of a train of 100 depolarizing pulses from a holding potential at −60 mV to +10 mV (40-ms duration, 10 Hz) was used to open voltage-activated Ca\(^{2+}\) channels and test the secretory capacity in a single beta cell (Fig. 6A). This protocol was applied between 20 and 30 s after the establishment of the whole-cell patch-clamp mode. The number of responsive cells increased from 57 to 86% between E18–P0 and P2–P3, and the capacitance change amplitude increased from less than 100 fF to about 300 fF (Fig. 6B).
Discussion

Insulin-positive cells at birth are not responsive to stimulatory levels of glucose but show a high basal insulin release (Fig. 2). This observation can account for both low levels of insulin immunostaining close to the plasma membrane and the minuscule capacitance change induced by the depolarization train (Fig. 6). This suggests that insulin takes over the glycemic control only postnataally.

At birth, basal insulin release drops, and this event seems to be correlated to the drop in voltage-activated Ca\(^{2+}\) conductance. Two days after birth, the insulin and glucose levels are comparable with those in adult animals, suggesting that the glucose levels take over the control of insulin release. At this age, the cells appear similar to adult beta cells, considering both the secretory capacity and excitability. This last transition may be associated with an increase in resting K\(_{ATP}\) conductance that reduces the resting membrane potential.

The idea of nonmature hormonal release at birth is not surprising as insulin does not seem to be vital during the first days after birth. Recently, a number of knockout (KO) animals were produced eliminating genes involved in islet-cell differentiation. Some of these KOs, such as Nkx2.2, Nkx6.1, Pax4, and Arx-Pax4 almost completely lack insulin-producing cells, but despite this they survive through gestation and at birth are almost indistinguishable (or slightly smaller) from their wild-type and heterozygous littersmates. By the second day postpartum all of these KOs have severe problems related to glucose homeostasis. The phenotype of the aforementioned mutants and the high insulin plasma level observed and reported in wild-type embryos would suggest a predominant trophic and hemodynamic action for insulin during embryonic life.

The strict anatomical relationship between insulin-positive cells and ductal cells has already been shown with citokeratin-and GluT2. Using thicker slices allowed us to reconstruct larger portions of the tissue and obtain evidence that many insulin-positive cells are still not aggregated at birth and are putative precursor cells that are destined to migrate and/or aggregate into new islets (Fig. 1B).

In adults the K\(_{ATP}\) activity is known to be crucial to maintain the resting membrane potential, and the total K\(_{ATP}\) conductance was estimated to be about two times higher than in P0–P3. In beta cells, both perinatal and adult, dialysis with 2 mmol/L ATP slightly hyperpolarizes the cell, suggesting that, in P0, the resting ATP is higher than 2 mmol/L. Until P0, the resting K\(_{ATP}\) conductance is close to 6% of the increase significantly between nonresponsive P0 and mature-like beta cells at P2–3 (Fig. 5) suggests that the insensitivity of P0 to high glucose is not a result of Ca\(^{2+}\) channel properties and the redundant Ca\(^{2+}\) conductance in embryos is apparently causing the high basal release. It would be possible to speculate about the nonresponsiveness to high glucose levels in embryos, assuming saturation of the system already at basal glucose concentration (close to 7% in 30 min compared to less than 3% in adults). This would also explain the depletion of insulin-positive granules from the cell periphery (Fig. 1E). Furthermore, the lower response in capacitance suggests a rapid exhaustion of secretory vesicles in embryonic and P0 beta cells, reaching a plateau phase in a few seconds, blunting the glucose-stimulated insulin release.

S-100 has not yet been reported as a ductal marker in pancreas. This might stem from the fact that this feature seems to be absent in animals older than 3 weeks (data not shown). The high expression of this protein family in pancreatic ductal adenocarcinomas suggests a correlation between S-100 and the high proliferation rate present in embryo and newborn ducts as in tumors.
maximum; in P3 it reaches 12%, and in adults it was reported to be close to 20%.24 We do not have enough evidence to explain what causes the difference between these two different stages, but putative cytosolic factor(s) controlling ATP sensitivity may still be identified.

The knowledge on the maturation of functional beta cells in wild-type animals is vital for understanding the basic physiology of these important cells. This information is also key for evaluating the results of the numerous manipulations devoted to producing new or modified insulin-releasing cells that could improve cell replacement therapy in diabetic patients. The process of beta-cell neogenesis, naturally occurring between the late-embryonic and the early postnatal stages, appears to be a relevant model for studying regeneration. A detailed characterization of beta-cell physiology during the early postnatal period will also provide a solid base to evaluate the insulin-positive cells in many model mice with perinatal lethal phenotype (KO of Pax6, Pax4, Arx genes) and newly differentiated insulin-positive cells from embryonic25 or adult stem cells or from transdifferentiated cells both in vitro and in vivo. In addition, further studies are required to assess the epigenetic influence on the maturation of insulin-positive cells.

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Conflicts of Interest

The authors declare no conflicts of interest.

References


