# Effect of an Acute Glucose Overload on Islet Cell Morphology and Secretory Function in the Toad 

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The aim of this work was to study the effect of induced hyperglycemia on islet cell mass and insulin secretion in normal toads. Immunolabeled $\boldsymbol{\beta}$ cell area, replication (bromodeoxyuridine) and apoptosis (propidium iodide) rate, islet neogenesis (cytokeratin), and insulin secretion in vitro were measured in adult male specimens of Bufo arenarum during and after interruption of the injection of either a $50 \%$ glucose solution ( $2 \mathrm{~g} / 100 \mathrm{~g}$ ) or its vehicle for 4 days. Glucose administration caused hyperglycemia ( $122.6 \pm 16.7$ and $508.3 \pm 115.9 \mathrm{mg} / \mathrm{dl}$ vs $23.5 \pm 1.26$ and $22.8 \pm 1.8 \mathrm{mg} / \mathrm{dl}$, at days 3 and 5 , respectively, P < 0.05 ) and a significant decrease in the number of islets/ $\mathrm{mm}^{2}$ (day 3: $9.7 \pm 0.9$ vs $3.3 \pm 0.4, \mathrm{P}<0.05$; day $5: 9.4 \pm$ 0.8 vs $7.4 \pm 0.6$; day $9: 9.6 \pm 0.9$ vs $6.2 \pm 0.4, P<0.05)$ and in the percentage of immunolabeled $\beta$ cell area (day $3: 2.07 \pm 0.2$ vs $0.5 \pm 0.1 \%, P<0.05$; day $5: 1.8 \pm 0.1$ vs $0.6 \pm 0.1 \%$; day $9: 1.7 \pm 0.1$ vs $0.7 \pm 0.1 \%, P<0.05)$. Glucose-injected animals had a simultaneous significantly higher percentage of BrdU-labeled $\beta$ cells (day 3: $0.46 \pm 0.02$ vs $0.23 \pm 0.03 \%$; day $5: 0.54 \pm 0.13$ vs $0.22 \pm 0.02 \%$; day 9: $0.61 \pm 0.0$ vs $0.27 \pm 0.05 \%, \mathrm{P}<$ 0.05 ) and cytokeratin-labeled endocrine cells (day 3: $0.21 \pm 0.06$ vs $0.01 \pm 0.00 \%$; day $5: 0.17 \pm 0.06$ vs $0.01 \pm 0.01 \%$; day 9: $1.25 \pm 0.2$ vs $0.01 \pm 0.008 \%, \mathrm{P}<$ 0.05 ) and a higher rate of apoptotic $\beta$ cells (day 3: $0.14 \pm$ 0.04 vs $0.05 \pm 0.02 \%$; day $5: 0.4 \pm 0.06$ vs $0.05 \pm 0.2$, $P<0.05$; day 9: $0.47 \pm 0.04$ vs $0.06 \pm 0.03, \mathrm{P}<0.05$ ). C omparable amounts of insulin were secreted in vitro by both groups in response to $\mathbf{2 ~ m M}$ glucose, whereas there was a significantly reduced response to 8 mM glucose in treated animals (day 3: $73 \pm 12$ vs $165 \pm 20 \%$; day 5 :
$74 \pm 11$ vs $204 \pm 18 \%, \mathrm{P}<0.05)$. This decreased response to high glucose reverted to normal after removal of the glucose injection. These results show for the first time that short-term hyperglycemia triggers marked morphological and transient secretory changes in the toad pancreas similar in part to those elicited in the pancreas of several mammals. As with other results previously reported, these results support the usefulness of the toad as an alternative easily handled model to study the growth and secretory function of the endocrine pancreas.

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Key W ords: Bufo arenarum; hyperglycemia; islet function; insulin secretion; $\boldsymbol{\beta}$ cell replication rate; islet neogenesis; cytokeratin; apoptosis.

> An adequate insulin secretory response to a peripheral demand is essential to keep plasma glucose levels within the narrow normal range. An insufficient insulin output in the face of an increased demand causes the disruption of glucose homeostasis that results in diabetes.
> $\beta$ Cells can cope with an increased demand of insulin by enhancing their secretory capacity and/or expanding their mass. New $\beta$ cells can be formed by either the mitotic division of preexisting $\beta$ cells (replication) or the differentiation from a developmental precursor or stem cell (neogenesis) (Bonner-Weir, 1994; Bouwens and Klöppel, 1996; Leahy, 1996).

> Hyperglycemia induced by a great variety of processes, such as partial pancreatectomy (Cardezza and Rodríguez, 1949; Lee et al., 1989), infusion of glucose
solutions (Bonner-Weir et al., 1989), and dietary manipulation (Lombardo et al., 1983; Luo et al., 1998), stimulate $\beta$ cell growth (Swenne, 1982; Bonner-Weir et al., 1989; Bonner-Weir and Smith, 1994). Understanding of the precise mechanisms underlying these changes becomes of great interest, especially within the context of the prevention (Pan et al., 1997) and treatment (Salmerón et al., 1997) of type 2 diabetes. Whereas all studies reporting the effect of sustained hyperglycemia have been made in mammals, little is know about its effect on the islets of nonmammalian species such as amphibians. Such information is important because of the similar acute effect of glucose on islet morphology and function of rats and toads (Luna et al., 1999), the normal low range of serum glucose in amphibians, five times lower than in mammals (Francini and Gagliardino, 1995), and the lethal effect of glucose administration observed in toads for a rather short period (von Lawzewitsch, 1962).

The aim of this study was to provide information on the effect of sustained short-term hyperglycemia on islet cell growth and insulin secretion in normal toad islets. To this end, normal toads were injected daily with glucose-sufficient to produce marked hypergly-cemia-for 4 days, measuring islet morphology, $\beta$ cell replication rate, islet neogenesis, $\beta$ cell death (apoptosis), and insulin secretion in vitro during and after interruption of this treatment.

## MATERIALS AND METHODS

## Animals

Adult male toads (Bufo arenarum), caught wild in the city of La Plata, Argentina, in spring ( $125 \pm 12 \mathrm{~g}$ body wt ), were kept at $25^{\circ} \mathrm{C}$ for at least 1 week in a suitable enclosure containing water and mealworms to standardize their metabolic state. The animals were then subjected to a $24-\mathrm{h}$ fasting period before every experiment.

## Experimental M odel U sed to Induce Hyperglycemia

Toads were injected daily for 4 consecutive days in the dorsal lymphatic sac with a $50 \%$ glucose solution (w/v) in Krebs-Ringer buffer (2 g glucose/100 g body
wt) (von Lawzewitsch, 1962). Control amphibians received an equal volume of vehicle. Animals were killed for sampling at the 3rd (two glucose injections), 5th (four glucose injections), and 9th (four glucose injections for 5 days) days of treatment.

## Glycemia

Serum glucose from each animal was measured at the time of killing by the glucose-oxidase GOD-PAP method (Roche Diagnostics, Mannheim, Germany).

## Quantitative Immunocytochemical Studies

Twenty four-hour-fasted toads were pithed by the severing of the spinal cord; the abdominal cavity was then opened and the entire pancreas excised and placed in a petri dish containing cold Krebs buffer for removal of fat and connective tissue. For immucytochemical studies, pancreata from three animals were then dissected apart to separate the free lobe (von Lawzewitsch, 1963). Only the free lobe was used because it has both the greatest islet mass and the most abundant population of non- $\beta$ cells (von Lawzewitsch, 1970; Francini et al., 1996). The pancreatic lobe was next resected, fixed in Bouin's solution, and embedded in paraffin. Serial sections thinner than $5 \mu \mathrm{~m}$ were finally obtained from three different levels of the blocks.

After deparaffinization and rehydration, the sections were stained by the immunoperoxidase method (Sternberger et al., 1970) for detection of $\beta$ cells using a guinea-pig-specific serum against insulin (final dilution 1:10,000). This antibody and those used throughout the study have been successfully tested by our group in previous studies to identify the islet hormones in the toad (Francini and Gagliardino, 1996; Luna et al., 1999). These sections were incubated overnight with the antibody and then counterstained with hematoxylin. Controls for serological immunospecificity were performed by preabsorbtion of the antiserum with an excess of insulin from nonimmune sera from guinea pigs. Both types of controls gave negative immunostaining in the experiments described.

For morphometric studies, an image analyzer utilizing Optimas software was used. In this method, the areas occupied by $\beta$ cells and the areas of the total pancreas-excluding connective tissue-were mea-
sured in the lobe. More than six endocrine cells forming a group of at least two cell types were considered as a pancreatic islet.

## $\boldsymbol{\beta}$ C ell Replication Rate

The $\beta$ cell replication rate was estimated in pancreatic sections from animals previously injected with bromodeoxyuridine (BrdU; Boehringer, Mannheim, Germany; $25 \mathrm{mg} / 100 \mathrm{~g}$ body $\mathrm{wt}, 1 \mathrm{~h}$ before killing) (Gratzner, 1982) by first incubation of the specimens with BrdU-specific antibodies (Cell Proliferation Kit; Amersham International, UK) and then incubation of the specimens with the insulin antibody on the same slide, quantifying the fraction of $\beta$ cells among the labeled nuclei. The replication rate is expressed as the percentage of BrdU-labeled cells among the total $\beta$ cells.

## Cytokeratin Expression

For cytokeratin immunostaining, deparaffinized sections were pretreated with 250 ml of antigen retrieval solution (Vector Lab., U.S.A.) in a microwave oven (500 W) for 10 min (Hazelbag et al., 1995; Madsen et al., 1997). A monoclonal antibody against human CK 19 (clone K4.62) from Sigma Immunochemicals (St. Louis, MO) was used. After various trials using several CK antibodies, antibody CK 19 was selected because it ensured the best data reproducibility and the strongest cross-reaction with the amphibian antigen, displaying an expression pattern compatible with that described previously in mammals. After deparaffinization and rehydration, the sections were treated with normal porcine serum to prevent nonspecific staining, while endogenous peroxidase was blocked by methanol-hydrogen peroxide. The streptavidinbiotin complex (ABC) technique (Sigma Chemical Co., St. Louis, MO) was then used, with peroxidase and carbazole used in the chromogenesis step. Primary antibodies (1:40 dilution) were incubated for 1 h . The secondary, biotinylated antibody was applied for 30 min, whereas the subsequent incubation with avidin was for 30 min (both reagents from Sigma Immunochemicals). The reaction with carbazole was run for $5-10 \mathrm{~min}$. Controls for serologic specificity were performed either by replacing the specific monoclonal antibody with normal mouse serum or by preabsorb-
ing a given antiserum with an excess of its corresponding antigen.

## $\beta$ C ell Death (Apoptosis)

For quantification of apoptotic $\beta$ cells, sections were both immunostained for insulin and stained with propidium iodide (Scaglia et al., 1997). This last compound is a fluorescent dye that binds to nucleic acids and allows the detection of condensed or fragmented nuclei characteristic of apoptotic cells. Serial sections thinner than $5 \mu \mathrm{~m}$ from the same blocks used for the above-mentioned detections were deparaffinized and rehydrated. Sections were washed three times each for 5 min in phosphate-buffered saline, pH 7.4 , and then incubated with propidium iodide (Sigma; $4 \mu \mathrm{~g} / \mathrm{ml}$ ) and RNAse (Sigma; $100 \mu \mathrm{~g} / \mathrm{ml}$ ) for 30 min in a dark chamber at room temperature. Sections were then washed with phosphate-buffered saline and costained by the immunoperoxidase method (Sternberger et al., 1970) for the detection of $\beta$ cells as described above. The $\beta$ cell death rate is expressed as the percentage of propidium iodide-labeled cells among the total $\beta$ cells counted.

## Immunofluorescence Labeling

Sections immunocytochemically stained for CKs by the $A B C$ method were subsequently costained by double (fluorescein and Texas red sulfonyl chloride) immunofluorescence with the antibodies raised against insulin, somatostatin, pancreatic peptide (PP), and glucagon as the primary serologic reagents to confirm the endocrine nature and the type of CK-positive cells in the extrainsular regions (Madsen et al., 1986). For this purpose, sections were washed three times each for 7 min in 10 -fold-concentrated phosphate-buffered saline. After these washes, the slides were first preincubated for 10 min with nonimmune serum of the same species as that of the secondary antibody diluted in Tris-buffered saline and then incubated for 1 h with the primary antibody in a humid chamber. The sections were next washed three times as before, dried, and incubated with the secondary, fluorescent antibody previously centrifuged for 45 min in a dark, humid chamber. The slides were finally washed three times each for 5 min and mounted with Tris-glycerol.

The first incubation of histologic preparations was with one of the following antibodies against the different pancreatic hormones: monoclonal antibody against human insulin (NovoClone HU-018) and against guinea pig somatostatin (NovoClone SOM018), both from Novo Nordisk A/S, Denmark, and rabbit polyclonal antibody against human PP and against porcine glucagon (Dako Corp., CA). Fluorescence labeling of each of these primary antibodies was accomplished through a second incubation with the two IgG-specific fluorophores fluorescein-conjugated affinity-purified goat antibodies against rabbit $\operatorname{IgG}$ (heavy plus light chains; Jackson ImmunoResearch Laboratories, Inc., West Baltimore Pike, U.S.A.) and Texas Red sulfonyl chloride (TRSC)-conjugated affin-ity-purified goat antibody against mouse IgG (heavy plus light chains; Texas Red; Jackson ImmunoResearch Laboratories, Inc.).

## Studies of Insulin Secretion in Vitro

Animals treated with glucose as described previously were used as pancreas donors for the insulin secretion studies. For each experiment, six 24 -h-fasted toads were pithed by severing of the spinal cord and pancreata were obtained as already described. Details of the entire incubation procedure have been previously reported (Francini and Gagliardino, 1995). In brief, the pooled pancreata were minced into small pieces and washed several times with cold Krebs buffer. Three to four pieces of the organ were then dropped into an open-ended plastic tube having a permeable bottom (consisting of a Whatman filterpaper disk covering the lower opening and fastened around it with a plastic washer). This tube was, in turn, placed vertically in another containing Krebs buffer with $1 \%(w / v)$ bovine serum albumin, 400 $\mathrm{U} / \mathrm{ml}$ aprotinin, and glucose ( 2 and 8 mM ). The Krebs buffer used throughout the procedure had the following composition (in mM ): $\mathrm{NaCl}, 111.2 ; \mathrm{KCl}, 1.87$; $\mathrm{CaCl}_{2}, 1.08 ; \mathrm{NaHCO}_{3}, 2.38$; glucose, 2. The buffer had been previously equilibrated with an $\mathrm{O}_{2}: \mathrm{CO}_{2}(95: 5 \%)$ gas mixture at a final pH of 7.4. The pieces of pancreas were incubated for 60 min at $25^{\circ} \mathrm{C}$; they were then removed, settled overnight at $37^{\circ} \mathrm{C}$, and weighed. The incubation medium was finally kept frozen at $-20^{\circ} \mathrm{C}$ until the measurement of insulin by radioimmunoassay (RIA), as described by Herbert et al. (1965).


FIG. 1. Serum glucose levels. Each set of bars depicts the serum glucose levels at days 3,5 , and 9 of treatment (open bars, control animals; gray bars, treated animals). Each value represents the mean $\pm \mathrm{SE}$ in $\mathrm{mg} / \mathrm{dl} ; n=12 .{ }^{*} P<0.05$.

This RIA employs a commercially available insulin antibody, ${ }^{125} \mathrm{I}$-labeled porcine insulin (Linde et al., 1980), and serial dilutions of highly purified rat insulin. Parallel antigen-antibody displacement curves with this same rat insulin standard were previously obtained (Francini and Gagliardino, 1995) and insulin was extracted from toad pancreata (Davoren, 1962). For routine purposes, rat insulin was therefore used as the antigen standard for these curves and the toad radioimmunoassayable insulin concentration in the incubation medium is expressed in terms of rat insulin equivalents per milligram wet weight of pancreas.

## Statistical Analysis

Statistical analysis of the data was performed by means of the ANOVA and the Student-NewmanKeuls tests.

## RESULTS

## Serum Glucose Levels

Treated animals showed a remarkable rise in blood glucose levels after the second glucose injection, attaining the peak level of hyperglycemia after four injections ( $122.6 \pm 16.7 \mathrm{mg} / \mathrm{dl}$ vs $23.5 \pm 1.26 \mathrm{mg} / \mathrm{dl}$ and $508.3 \pm 115.9 \mathrm{mg} / \mathrm{dl}$ vs $22.8 \pm 1.8 \mathrm{mg} / \mathrm{dl}$, at days 3 and 5 , respectively; $P<0.05$ ). Five days after the last glucose injection, the serum glucose values fell to levels comparable with those measured in control animals (Fig. 1).


FIG. 2. $\beta$ Cell area and number of islets $/ \mathrm{mm}^{2}$. (A) $\beta$ Cell area expressed as percentage of the total pancreatic free lobe area. (B) Number of islets $/ \mathrm{mm}^{2}$ of the total pancreatic free lobe area. In both cases, open bars represent control animals and gray bars represent treated animals. Each bar represents the mean of nine sections (three different levels from three separate animals) $\pm$ SE. ${ }^{*} P<0.05$.

## $\boldsymbol{\beta}$ C ell Population

The morphometrical analyses revealed significant differences with respect to the fractional area occupied by immunolabeled $\beta$ cells within the free lobe in control and treated animals. The injected toads showed a significantly lower percentage of immunolabeled $\beta$ cell Vvi than controls at all the time points sampled (Fig. 2A). This result agrees with that previously reported using immunofluoresence (Luna et al., 1999).

The number of islets $/ \mathrm{mm}^{2}$ was also significantly lower in treated than in control animals at every time point studied. However, the lowest islet number was registered after two glucose injections (i.e., day 3). It is interesting to note that from this point on, the number of islets $/ \mathrm{mm}^{2}$ increased significantly ( $117 \%$ ), without reaching, however, the values measured in control animals (Fig. 2B).

No significant differences occured between the average islet diameter of control and that of glucosetreated toads. However, due to the frequent irregular shape of the islets in both experimental groups, their diameter is difficult to assess with certainty; consequently, this lack of significance should be viewed with caution.

## $\boldsymbol{\beta}$ C ell Replication Rate

The percentage of BrdU-labeled cells among the total $\beta$ cells in glucose-injected animals was higher (two- to threefold) than in control animals at all the time periods sampled, even after removal of glucose administration, and blood glucose levels fell to normal values (Fig. 3A). There were no differences in percentage of BrdU-labeled ductal cells in control and treated animals.

## Cytokeratin Expression

Only a few cells labeled with the CK 19 antibody were seen in the pancreas of control animals, whereas the treated toads showed a strong positivity in cells located at the islet periphery and in a large number of endocrine cells scattered throughout the exocrine pancreas. Some of these latter positive cells were found near the ducts. The quantitative analysis showed that


FIG. 3. $\beta$ Cell replication rate, cytokeratin expression, and $\beta$ cell death (apoptosis) in glucose-injected (gray bars) and control (open bars) animals at all the time periods sampled (A) Percentage of BrdU-labeled cells among the total $\beta$ cells. (B) Pancreatic cells labeled with the CK 19 antibody. (C) Percentage of apoptotic $\beta$ cells. Each bar represents the mean of nine sections (three different levels from three separate animals) $\pm$ SE. ${ }^{*} P<0.05$.


FIG. 4. (A) $\beta$ Cell nuclei labeled with BrdU (arrows). (B) CK-positive cells located at the islet periphery (yellow arrows) and outside the islet (green arrow). (C) $\beta$ Cell nuclei located in the middle of the islet, labeled with propidium iodide. $\times 20$.
the CK-positive area in treated animals increased as a function of the days of treatment, showing the maximal value at day 9 of treatment, even when the glucose injections had been stopped 5 days before (Fig. 3B). Using the double-immunofluorescence technique on the same section, all these extrainsular CK-labeled cells were of an endocrine nature, as shown by their morphological features and their strong reactivity with either glucagon or PP antibodies.

## $\boldsymbol{\beta}$ C ell D eath (A poptosis)

The percentage of apoptotic $\beta$ cells in the glucoseinjected animals was significantly higher (two- to sixfold) than that measured in control animals at every time period sampled, that is, even long after the glucose administration stopped and in the presence of normal blood glucose levels (Fig. 3C).
Figures 4A-4C show typical islet cells labeled with BrdU (replication rate), CKs (islet neogenesis), and propidium iodide (cell apoptosis). Similar material was used to perform a quantitative estimation of the corresponding parameters.

## Insulin Secretion in Vitro

Comparable amounts of insulin were secreted by the free lobe of control and treated toads in response to 2 mM glucose. Although slightly lower, the insulin released from pancreata of treated animals did not change significantly compared to the values obtained at the different time points tested during the treatment (Fig. 5A). Conversely, the output of insulin elicited by 8 mM glucose was significantly greater in controls than in treated animals (after 2 and 4 days of glucose treatment). However, a similar release of insulin in response to 8 mM glucose was recorded in both groups 5 days after
interruption of the glucose injection (Fig. 5B). At this time, the blood glucose levels recorded in treated animals were within the normal range.

## DISCUSSION

Glucose administration to normal adult toads-in the amount currently employed-produces an impor-


FIG. 5. Insulin secretion in the presence of $2 \mathrm{mM}(\mathrm{A})$ and 8 mM (B) glucose in control (open bars) and treated (gray bars) animals at days 3,5 , and 9 of treatment. Each value represents the mean $\pm \mathrm{SE}$ of the percentage increase in insulin output above basal values; $n=$ 20. Ten replicates from each variable were performed on 2 different days, and the insulin concentration in all samples was measured in a single radioimmunoassay. ${ }^{*} P<0.05$. Basal values at 2 mM glucose were $251 \pm 18 \mathrm{pg} / \mathrm{mg}$ dry pancreas $/ \mathrm{h}$.
tant alteration of glucose homeostasis, which results in marked and sustained hyperglycemia. The high serum glucose levels undoubtedly have a deleterious effect and can even kill the animals if glucose administration is prolonged for 7 days (von Lawzewitsch, 1962). In the period currently studied, the induced hyperglycemia was accompanied by a significant reduction in the proportion of immunolabeled $\beta$ cells and a significant decrease in insulin secretion in response to 8 mM glucose in vitro.

The decrease in the immunostained $\beta$ cell area was the consequence of a gross degranulation of these cells induced by the acute demand of insulin elicited by hyperglycemia, similar to that reported in the frog by Kumar and Khanna (1976). However, the higher rate of apoptosis currently observed in treated animals would suggest that cellular death may also be involved in the loss of immunostained $\beta$ cells. This increased apoptotic rate-which still remained high 5 days after the glucose injection was interrupted and even when blood glucose levels returned to normal values-might be ascribed to the $\beta$ cell glucose toxicity (Rossetti et al., 1990; Yki-Järvinen, 1992).

The increased $\beta$ cell replication rate found in the treated animals might represent an effort of the tissue to cope with the high demand of insulin. The high level of this replication rate found even in control animals compared with the low values frequently reported in rodents is noticeable (Bonner-Weir, 1994). This fact suggests that the rates of cellular growth would be particularly high in some vertebrate classes such as amphibians, here exemplified by the toad.

It has been proposed that CK immunostaining is a valuable tool for the study of cell differentiation in mammals (Bouwens et al., 1994). Treated toads showed an increased number of CK-positive cells located at the islet periphery, and scattered all throughout the exocrine pancreas, that increased as a function of the days of treatment. Maximal values were observed even after withdrawal of the glucose injections. Some of the positive cells appeared in close relation to the ducts. The endocrine origin of these CK-positive cells was demonstrated by their location (islets), shape, and reactivity with the glucagon or PP antibodies. The distribution pattern of the CK-positive cells found in the sugar-treated toads was similar to that reported by Bouwens et al. $(1994,1995,1996)$ in the neonatal rat pancreas and by our group in normal
hamsters fed with sucrose in the presence of an increased neogenetic rate (Del Zotto et al., 1999). Thus, these cells might indicate an active process of islet neogenesis that-as in the case of the $\beta$ cell replication rate-represents a compensatory mechanism to cope with the increased insulin demand. The expected decrease in islet diameter usually observed when the neogenesis process is triggered by a given stimulus in other species did not occur (Bouwens and Klöppel, 1996; Del Zotto et al., 1999). However, due to the peculiar irregular shape of toad islets (von Lawzewitsch, 1963, 1970; Francini et al., 1996), the precise measurement of this parameter is neither easy nor reliable. Thus, caution is needed when considering the lack of a significant difference in islet diameter in treated and control animals. It has to be noted that the capacity of the toad pancreas to increase its islet mass in the presence of a $\beta$ cell overload (partial pancreatectomy) had been early reported by Houssay and Biasotti (1933).

Insulin secretion in response to a high glucose concentration ( 8 mM ) was significantly lower in glucoseinjected than in control animals during the first 4 days of treatment (sustained hyperglycemia). However, the response in the former group reassumed values comparable to those found in control animals 5 days after the interruption of glucose administration (recovery period), when blood glucose levels reverted to normal in treated animals. Since at this time the percentage $\beta$ cell area is still lower ( $35 \%$ ) in treated than in control animals, the similar insulin output observed in both groups might be either the consequence of $\beta$ cell hyperfunction induced by the previous hyperglycemia in the treated toads or the fact that in pancreata obtained from normoglycemic animals, total $\beta$ cell mass was not necessary to cope with the in vitro challenge of 8 mM glucose.

Our results support early reports on the importance of the pancreas function to maintain glucose homeostasis in amphibians as it occurs in mammals (Penhos and Ramey, 1973). There are, however, differences among species in the way that the pancreas accomplishes such function. Intravenous administration of insulin to amphibians induces hypoglycemia, but compared with mammals the action of insulin is delayed and prolonged, depending on the dose employed (from 10 h to 2 days postinjection) (reviewed by Penhos and Ramey, 1973). Further, it has been
shown that intravenous administration of glucose to male B. arenarum produced marked hyperglycemia that returned to normal values after 8-24 h (Houssay and Rietti, 1956). Thus, the amphibian pancreas would be less efficient than the mammalian pancreas in handling a glucose load. This characteristic could be ascribed to a peculiar response of $\beta$ cells to a glucose stimulus. In this regard, we have already reported that (a) though the glucose dose:insulin response curve follows a similar sigmoidal pattern in mammals and toads, maximal insulin release was obtained in the latter at a significantly lower concentration of hexose (8 vs 17 mM glucose, respectively) and (b) whereas at a low glucose concentration the toad pancreas releases more insulin than the rat pancreas, the opposite effect was observed in the presence of a high glucose concentration, probably conditioned by a higher hexokinase/glucokinase ratio in the toad $\beta$ cells (Francini et al., 2000). This higher ratio could explain the lower blood glucose levels measured in normal toads and the lower capacity of the amphibian pancreas to cope with an increased demand of insulin elicited by a sustained glucose load. These low blood glucose levels would protect the animal from the deleterious effect of the high extracellular glucose concentration that negatively affects body growth and the enzyme activity in different organs (Francini et al., 2000) and can even kill the animals in a short period of time (1 week) (von Lawzewitsch, 1962). The different dynamics of insulin action in the periphery-slow hypoglycemic effect of insulin at least when exogenously administered (see above)—could also play a role in this lower adaptative capacity of the toad to glucose stress.

Together, these results showed for the first time that (a) hyperglycemia triggered marked morphological changes in the toad pancreas (increase in $\beta$ cell replication rate, islet neogenesis, and $\beta$ cell apoptotic rate); (b) these changes, which remained at high levels even after removal of glucose administration and when blood glucose reverted to normal values, were similar to those elicited by high serum glucose in the pancreas of several rodents and were sufficient to overcome the deleterious effect of a hyperglycemia maintained for a relatively short period (4 days); (c) CK immunolabeling constitutes an adequate tool for the study of cell differentiation in this group; and (d) these changes were accompanied by a marked reduction in the insulin response to high glucose that reverts to normal
after removal of the administration of glucose. Thus, these results and others previously reported (Francini and Gagliardino, 1995, 1998; Francini et al., 1996, 2000; Luna et al., 1999) support the usefulness of the toad as an alternative easily handled model to study the growth and secretory function of the endocrine pancreas.

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