0026-895X/01/6004-873–879$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 4
910/930956
Mol Pharmacol 60:873–879, 2001
Printed in U.S.A.
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics

Diabetogenic Effect of Cyclosporin A Is Mediated by Interference with Mitochondrial Function of Pancreatic B-Cells

MARTINA DÜFER, PETER KRIPPEIT-DREWS, NICOLAS LEMBERT, LARS-ÅKE IDAHL, and GISELA DREWS

Department of Pharmacology, Institute of Pharmacy, University of Tübingen, Tübingen, Germany

Received February 23, 2001; accepted June 21, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Treatment of patients after organ transplantation with the immunosuppressive drug cyclosporin A (CsA) is often accompanied by impaired glucose tolerance, thus promoting the development of diabetes mellitus. In the present article we show that 2 to 5 μM CsA diminishes glucose-induced insulin secretion of isolated mouse pancreatic islets in vitro by inhibiting glucose-stimulated oscillations of the cytoplasmic free-Ca^{2+} concentration $[Ca^{2+}]_c$. This effect is not due to an inhibition of calcineurin, which mediates the immunosuppressive effect of CsA, because other calcineurin inhibitors, deltamethrin and tacrolimus, did not affect the oscillations in $[Ca^{2+}]_c$ of the B-cells. The CsA-induced decrease in $[Ca^{2+}]_c$ to basal values was not caused by a direct inhibition of L-type Ca^{2+} channels. CsA is known to be a potent inhibitor of the mitochondrial permeability transition pore (PTP), which we recently suggested to be involved in the regulation of oscillations. Consequently, CsA also inhibited the oscillations of the cell membrane potential, and it is shown that these effects could not be ascribed to cellular ATP depletion. However, the mitochondrial membrane potential $\Delta\Psi$ was affected by CsA by inhibiting the oscillations in $\Delta\Psi$. Interestingly, the observed reduction in $[Ca^{2+}]_c$ could be counteracted by the K^+_{ATP} channel blocker tolbutamide, indicating that the stimulus-secretion coupling was interrupted before the closure of K^+_{ATP} channels. It is concluded that CsA alters B-cell function by inhibiting the mitochondrial PTP. This terminates the oscillatory activity that is indispensable for adequate insulin secretion. Thus, CsA acts on different targets to induce the immunosuppressive and the diabetogenic effect.

Cyclosporin A (CsA) is a potent immunosuppressive drug widely used after organ transplantation of heart, kidney, liver, pancreas, and lung. Furthermore, efforts have been made to delay the autoimmune process leading to insulin-dependent diabetes mellitus by early CsA therapy (Canadian-European Randomized Control Trial Group, 1988; De Filippo et al., 1996). In contrast, there are many reports pointing to significant adverse effects of CsA on B-cell function. Thus, one general problem in immunosuppressive therapy with CsA is the elevated incidence of post-transplantation impaired glucose tolerance (Krentz et al., 1993) or overt diabetes mellitus (Yamamoto et al., 1991). Although no distinct relation between development of post-transplantation diabetes mellitus and CsA dosage can be described, it is obvious that the loss of glycemic control is more pronounced when CsA plasma levels are comparatively high (e.g., at the beginning of immunosuppressive therapy) and is, at least in part, reversible after dose reduction (Gunnarsson et al., 1984; Yamamoto et al., 1991) or withdrawal of CsA (Gunnarsson et al., 1984; Hahn et al., 1986). In this context, it is important to know that the lipophilic drug accumulates not only in the fat but also to a large extent in other tissues such as pancreas, liver, and kidney (Akagi et al., 1991). Thus, in the maintenance of therapeutical blood levels of about 100 to 400 ng/ml (Oellerich et al., 1995) the pancreatic CsA content can easily exceed 10-fold values (Akagi et al., 1991), thereby reaching micromolar concentrations. Moreover, it has been reported that several weeks after withdrawal, there are still measurable amounts of the drug in rat pancreatic tissue (Hahn et al., 1986).

It has been shown that glucose-induced insulin secretion is reduced in rat pancreatic B-cells and HIT cells after long-term incubation with CsA (Robertson, 1986; Draznin et al., 1988). This effect is ascribed partly to impaired insulin biosynthesis measured on the level of insulin DNA or mRNA synthesis as it has been shown for rat and mouse islets (Andersson et al., 1984; Hahn et al., 1986; Herold et al., 1993). Additionally, ultrastructural changes of the B-cells such as degranulation or vacuolization (Lucke et al., 1991) have been observed. However, the cellular mechanisms leading to deterioration in glucose metabolism (Gunnarsson et al., 1984) and to reduced insulin secretion after treatment of the B-cells with CsA (Robertson, 1986) remain to be clarified.

Apart from its immunosuppressive action CsA is also

This work was supported by grants of the Deutsche Forschungsgemeinschaft (Dr 225/4-1 and 4-2), the Deutsche Diabetesstiftung and the Svenska Sällskapet för Medicinsk Forskning.

ABBREVIATIONS: CsA, cyclosporin A; $[Ca^{2+}]_c$, cytoplasmic calcium concentration; Rh 123, rhodamine 123; KIC, α-ketoisocaproate; DAPP, diadenosinepentaphosphate; VC, voltage-clamp; CC, current-clamp.

known as a potent inhibitor of the mitochondrial permeability transition pore (PTP) (Halestrap and Davidson, 1990). Recently, it has been demonstrated that this voltage- and Ca^{2+}-activated ion channel localized at contact sites of the mitochondrial inner and outer membranes (Zoratti and Szabò, 1995; Duchen, 1999), responded to an increase in $[Ca^{2+}]_c$ with calcium-induced calcium release of the mitochondria (Ichas et al., 1997) and that it is involved in the regulation of Ca^{2+} homeostasis in intact cells (Fall and Bennett, 1999) or permeabilized cells (Evtodienko et al., 1994; Ichas and Mazat, 1998; Wood and Gillespie, 1998) in its low-conductance state. We recently proposed an important role for the PTP in a feedback mechanism that triggers the oscillatory activity of the B-cells (Krippeit-Drews et al., 2000), a prerequisite for normal cell function.

The aim of the present study was to investigate whether the diabetogenic side effects of CsA are caused by interactions with pancreatic B-cell function and whether they could be ascribed rather to an interference of the drug with the PTP than to its action on calcineurin, which is responsible for the desired immunosuppressive effect.

Materials and Methods

Preparation. The experiments were performed with islet cells of fed female NMRI mice (25–30 g) killed by cervical dislocation. Islets were isolated by collagenase digestion and dispersed in Ca^{2+}-free medium into single cells or small clusters. The cells were cultured up to 4 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. To determine mitochondrial ATP production mitochondria of islets from adult ob/ob mice were isolated as described previously (Lembert and Idahl, 1998).

Patch-Clamp Recordings. Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical, Pangbourne, UK). Pipette resistance ranged between 3 and 5 MΩ when filled with pipette solution. Patch-clamp experiments were performed in the standard whole-cell configuration or in the perforated-patch mode by using an EPC 9 patch-clamp amplifier and software Pulse (HEKA, Lambrecht, Germany). Experiments were performed at 32°C. Membrane currents or potentials were recorded in the voltage-clamp (VC) or current-clamp (CC) mode, respectively. K^+_{ATP} currents were measured in the perforated-patch mode during 300-ms pulses to −80 and −60 mV at 15-s intervals from a holding potential of −70 mV. Perforation usually occurred within 10 min after seal formation (Rs $<$ 30 MΩ). Currents through L-type Ca^{2+} channels were elicited by 50-ms pulses from −70 to 0 mV in the perforated-patch mode and in the standard whole-cell configuration.

Measurement of $[Ca^{2+}]_c$. Cells were cultured on glass coverslips and incubated with fura-2/AM (5 μM) for 30 min at 37°C before the experiments. Fluorescence was measured on an Axiovert 100 microscope with software and equipment from TILL Photonics (Planegg, Germany). The excitation wavelength of 340 and 380 nm was adjusted by means of a diffractive grating and directed through the objective (PlanNeofluar 40×; Zeiss, Jena, Germany) by a glass fiber light guide and a dichroic mirror. The emitted fluorescence was filtered (LP 515 nm) and measured by a charge-coupled device camera. The ratio of the emitted light intensity at 340/380 excitation wavelength was used to calculate the corresponding concentration of $[Ca^{2+}]_c$ according to an in vitro calibration with fura-2 salt.

Determination of $\Delta\Psi$. Cells were loaded with rhodamine 123 (Rh 123, 10 μg/ml) for 10 min at 37°C. Fluorescence was excited at 480 nm. A depolarization of $\Delta\Psi$ was indicated by an increase in Rh 123 fluorescence (Duchen et al., 1993).

Quantification of Mitochondrial ATP Production. Mitochondria corresponding to the content of the cells of approximately one islet (obtained from a preparation of two ob/ob mice) were incubated in 1 ml of incubation medium (see below) at 37°C for 10 min. Oxidative ATP production was detected in incubations containing either pyruvate/malate (1/1 mM) or α-ketoisocaproate (KIC)/glutamate (0.1/10 mM) and ADP (50 μM), Ca^{2+} (200 nM), and the specific adenylate kinase inhibitor diadenosinepentaphosphate (DAPP, 1 μM). ATP production was stopped by addition of antimycin A (final concentration 0.5 μM). To normalize the ATP synthesis to the amount of intact mitochondria, ATP produced by mitochondrial adenylate kinase activity was measured in parallel incubations in the sole presence of ADP (50 μM). This reaction was stopped with DAPP (1 μM). ATP concentrations were determined in luciferin/luciferase assays as described previously (Lembert and Idahl, 1998). The normalized ATP production is defined as the ATP accumulation induced by oxidative phosphorylation divided by ATP accumulation induced by adenylate kinase.

Insulin Secretion. Batches of five islets were incubated with the indicated substrates at 37°C for 60 min. Insulin was determined by radioimmunoassay with rat insulin (Crystal Chem Inc., Chicago, IL) as the standard.

Solutions. K^+_{ATP} currents in the perforated-patch configuration were registered with a pipette solution containing 10 mM NaCl, 10 mM KCl, 70 mM K_2SO_4, 4 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM EGTA, 20 mM HEPES, 100 to 250 μM nystatin, pH 7.15. Bath solution was composed of 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5 mM glucose, 10 mM HEPES, pH 7.4. For measurement of Ca^{2+} currents in the perforated-patch mode the pipette solution contained 70 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 7 mM $MgCl_2$, 10 mM HEPES, 250 μg/ml amphotericin B, pH 7.15. Bath solution was composed of 115 mM NaCl, 1.2 mM $MgCl_2$, 10 mM $CaCl_2$, 20 mM tetraethylammonium-Cl, 10 mM HEPES, 0.1 mM tolbutamide, 15 mM glucose, pH 7.4. For determination of Ca^{2+} currents in the standard whole-cell configuration $CaCl_2$ was replaced by 10 mM $BaCl_2$ in the bath solution and the pipette solution contained 50 mM $CsCl_2$, 70 mM *N*-methyl-D-glucamine, 58 mM HCl, 4 mM $MgCl_2$, 3 mM Na_2ATP, 10 mM EGTA, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.15. To measure $[Ca^{2+}]_c$ and $\Delta\Psi$ the same bath solution as described for K^+_{ATP} currents was used. The incubation medium for determination of insulin secretion was composed of 122 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM HEPES, 0.5% bovine serum albumin, pH 7.4. The incubation medium for ATP determination consisted of 20 mM HEPES, 3 mM KH_2PO_4, 4 mM carnitine, 1 mM EGTA, 20 mM NaCl, 80 mM KCl, 0.3 mM Mg^{2+}, 0.5 mg/ml albumin, pH 7.10.

Chemicals. Fura-2/AM, fura-salt, and Rh 123 were obtained from Molecular Probes (Eugene, OR), and CsA, tolbutamide, KIC, pyruvate, malate, and glutamate from Sigma Chemical (Deisenhofen, Germany). Luciferase, ATP, and DAPP were purchased from Boehringer (Mannheim, Germany), D-Luciferin was from Biothema (Dalarö, Sweden), and tacrolimus was from Fujisawa (München, Germany). D600 was a kind gift from Knoll (Ludwigshafen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) in the purest form available.

Presentation of Results. Measurements are illustrated by recordings that are representative of the indicated number of experiments performed with different cells. Cells of at least three different preparations have been used for each series of experiments. Means ± S.E.M. are given in the text for the indicated number of experiments (n). When two samples were compared the statistical significance of differences between means was assessed by Student's t test for paired values. Multiple comparisons were made by analysis of variance followed by Student-Newman-Keuls test. $P \leq 0.05$ was considered significantly different.

Results

Influence of CsA on Insulin Secretion. CsA was tested for its effects on glucose-stimulated insulin release on freshly

prepared primary mouse B-cells in vitro over a 60-min incubation period. Figure 1 illustrates that insulin secretion was significantly diminished by 1 μM CsA. Maximal inhibition was observed with 2 μM CsA, and there was no further decrease at concentrations up to 10 μM CsA. On average, 2 μM CsA reduced glucose-stimulated insulin secretion from 51.0 ± 7.0 (n = 12) to 21.3 ± 2.0 pg/islet/min ($P < 0.001$, n = 11). To reveal the underlying mechanisms we first tested the effects of CsA on glucose-induced oscillations of $[Ca^{2+}]_c$.

Influence of CsA on $[Ca^{2+}]_c$ in Glucose-Stimulated B-Cells. The increase in the extracellular glucose concentration from 0.5 to 15 mM induced the well known triphasic response in $[Ca^{2+}]_c$ (Grapengiesser et al., 1988; Krippeit-Drews et al., 2000; Fig. 2): After an initial drop in $[Ca^{2+}]_c$ due to sarcoplasmic/endoplasmic reticulum ATPase activation, opening of L-type Ca^{2+} channels led to a drastic rise in $[Ca^{2+}]_c$ for a longer first period and eventually the characteristic glucose-induced oscillations in $[Ca^{2+}]_c$ occurred. Figure 2 shows that 1 μM CsA had no effect on glucose-induced oscillations in $[Ca^{2+}]_c$, but 2 to 5 μM CsA terminated the oscillatory activity and $[Ca^{2+}]_c$ was diminished to basal values (79 ± 2 nM with 2–5 μM CsA compared with 73 ± 8 nM with 0.5 mM glucose; N.S., n = 10).

The observation that CsA seems to be more effective in inhibiting insulin secretion than in suppressing oscillations in $[Ca^{2+}]_c$ may be due to the different preparations used. For hormone release measurements freshly prepared whole islets (five per batch) have been used and thus the result is an average answer from thousands of cells where paracrine and neural influences may alter the response to a drug. In contrast, the $[Ca^{2+}]_c$ measurements have been performed with small clusters of cells kept in cell culture for several days.

Inhibition of Calcineurin by CsA. CsA is known to evoke its immunosuppressive property by inhibition of calcineurin (Mijares et al., 1997), which is also present in pancreatic B-cells (Renström et al., 1996). To rule out that this mechanism accounts for the observed effects, the influence of deltamethrin, another potent inhibitor of calcineurin, that is maximally effective in the nanomolar concentration range (Enan and Matsumura, 1992; Renström et al., 1996) on $[Ca^{2+}]_c$ was examined. Figure 3A demonstrates that glucose-induced oscillations in $[Ca^{2+}]_c$ were not stopped after addition of 0.1 and 1 μM deltamethrin (n = 12) and even persisted in the presence of 10 μM deltamethrin in 9 of these 12 experiments. Moreover, we have tested the effect of tacrolimus, another efficacious immunosuppressive agent, on $[Ca^{2+}]_c$. Like CsA, tacrolimus acts via inhibition of calcineurin (Cardenas et al., 1995). Figure 3B illustrates that 5 μM tacrolimus did not alter glucose-induced oscillations in $[Ca^{2+}]_c$ (n = 5).

CsA Did Not Affect Whole-Cell Ca^{2+} Currents. To rule out whether CsA exerts its effects on $[Ca^{2+}]_c$ by an inhibition of L-type Ca^{2+} channels, an effect of CsA that has been described for cardiac myocytes (Mijares et al., 1997), we tested CsA for an effect on these channels in pancreatic B-cells. Experiments were performed in the standard whole-cell configuration where the cell interior was dialyzed with the pipette solution and in the perforated-patch mode where cell metabolism remains intact. Figure 4 shows that 5 μM CsA did not influence the current through L-type Ca^{2+} channels, which was 131 ± 13 pA under control conditions, 132 ± 12 pA in the presence of 5 μM CsA (N.S.), and 125 ± 11 pA after washout (n = 5) in the standard whole-cell configuration. When cell metabolism was intact, 5 μM CsA was also ineffective. In this series of experiments the Ca^{2+} current was 108 ± 9 pA under control conditions and 99 ± 9 pA with CsA (N.S., n = 5; data not shown).

Effects of CsA on Membrane Potential and K^+_{ATP} Current in Glucose-Stimulated B-Cells. Figure 5 shows in an experiment in the perforated-patch configuration with intact cell metabolism, the recording of the K^+_{ATP} current (Fig. 5A) under VC conditions and the corresponding membrane potential (Fig. 5B) in the CC mode. The stimulation of B-cells by an increase in glucose concentration from 0.5 to 15 mM resulted in a reduction in K^+_{ATP} current from 4 ± 1 pA (VC 1) to virtually zero (VC 2) (n = 4). Accordingly, the cell membrane depolarized from the resting membrane potential of −71 ± 3 mV to a plateau potential of −52 ± 6 mV ($P <$ 0.001) and displayed the characteristic spike activity that accompanies glucose-induced insulin secretion. Addition of 2 μM CsA terminated the electrical activity (Fig. 5B), but without any obvious increase in K^+_{ATP} current, which remains

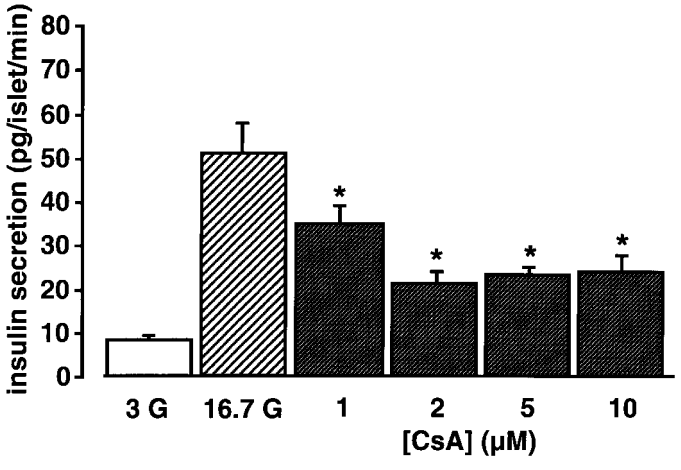

Fig. 1. Inhibition of glucose-stimulated insulin secretion by CsA. Batches of five islets were incubated for 60 min with 3 mM (3 G) or 16.7 mM glucose (16.7 G). CsA was added to the incubation medium (16.7 G) at the indicated concentrations. Values are means ± S.E.M. for 8 to 12 experiments from four separate preparations. The values with an * are statistically different from those observed with 16.7 mM glucose.

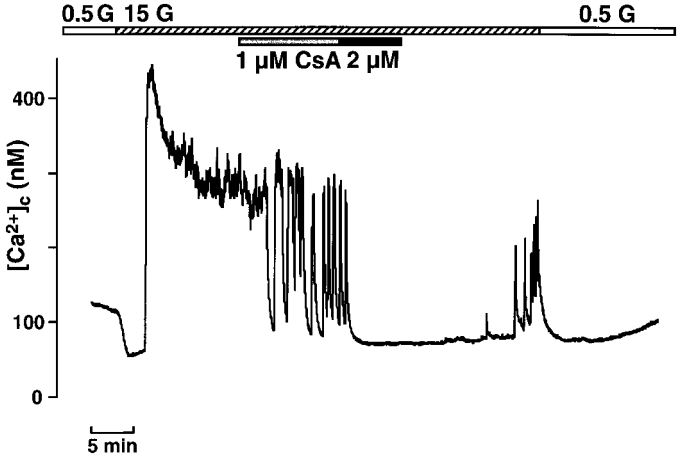

Fig. 2. Inhibitory effect of CsA on oscillations in $[Ca^{2+}]_c$. Cells were stimulated with 15 mM glucose and CsA (2–5 μM) was added after the appearance of the characteristic oscillatory pattern in $[Ca^{2+}]_c$. The recording is representative of 10 similar experiments.

virtually zero (Fig. 5A, VC 3) or hyperpolarization to the resting membrane potential (Fig. 5B). The effect of CsA was reversible and spike activity was restored after washout. On average, the cell membrane was hyperpolarized to −63 ± 3 mV from the plateau potential ($P < 0.001$, $n = 4$) in the presence of 2 to 5 μM CsA. In contrast, the inhibition of ATP synthesis by the cytochrome a_3 inhibitor NaN_3 (5 mM) or the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1 μM) drastically increased K^+_{ATP} current and hyperpolarized the cell membrane to the resting membrane potential (around −75 mV) (Düfer et al., 1999).

ATP Production in Isolated Mitochondria of Pancreatic B-Cells in Presence of CsA. To confirm that the effects of CsA were not owing to an ATP depletion of the B-cells, ATP synthesis was examined in isolated mitochondria (Fig. 6). Oxidative phosphorylation was induced either by 1 mM pyruvate/1 mM malate (open circles) or 0.1 mM KIC/10 mM glutamate (closed circles) in the presence of 50 μM ADP and 200 nM Ca^{2+}. Figure 6 illustrates that mitochondrial ATP synthesis was not decreased in a concentration range from 0.1 to 5 μM CsA. On average, malate/pyruvate-induced ATP production was 2.54 ± 0.17 under control conditions and 2.46 ± 0.27 with 5 μM CsA (N.S., $n = 4$). With KIC/glutamate as substrates ATP synthesis was 1.68 ± 0.11 under control conditions and 2.13 ± 0.29 in the presence of 5 μM CsA (N.S., $n = 6$). With 10 μM CsA a slight decrease in ATP production was observed for the malate/pyruvate-induced ATP production ($P < 0.05$).

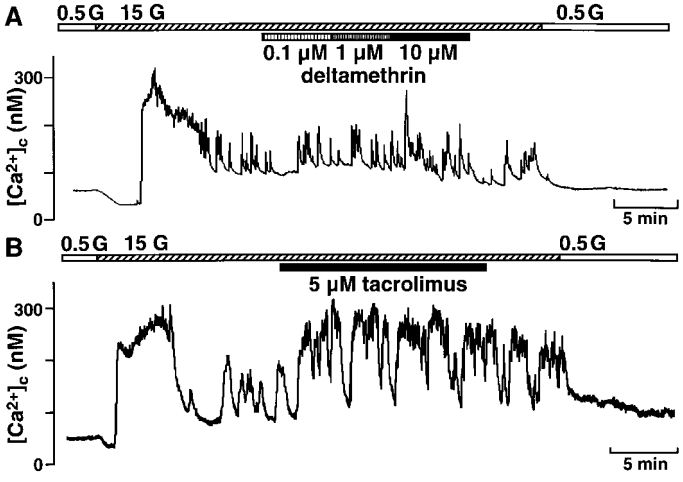

Fig. 3. Lack of effect of the calcineurin inhibitors deltamethrin and tacrolimus on glucose-induced oscillations in $[Ca^{2+}]_c$. Clusters of B-cells were stimulated with 15 mM glucose and test solutions were added as indicated by the horizontal bars. A, addition of 0.1 and 1 μM deltamethrin did not diminish oscillations in $[Ca^{2+}]_c$. In 9 of 12 cells even 10 μM deltamethrin was ineffective. B, addition of 5 μM tacrolimus did not influence the glucose-induced oscillations in $[Ca^{2+}]_c$. The recording is representative of five similar experiments.

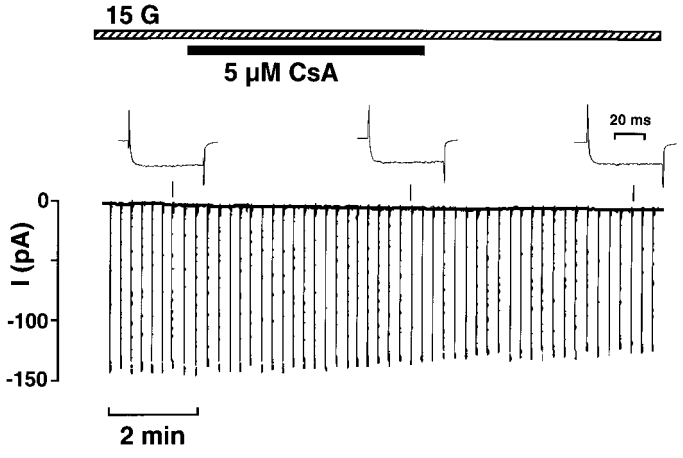

Fig. 4. Lack of effect of CsA on currents through L-type Ca^{2+} channels. Currents were elicited every 15 s by 50-ms voltage steps from −70 to 0 mV with Ba^{2+} as the charge carrier. CsA (5 μM) did not decrease the currents through L-type channels in the standard whole-cell configuration, where the cell interior is dialyzed. The insets show the depicted currents at an extended time scale. The recording is representative of five with similar results.

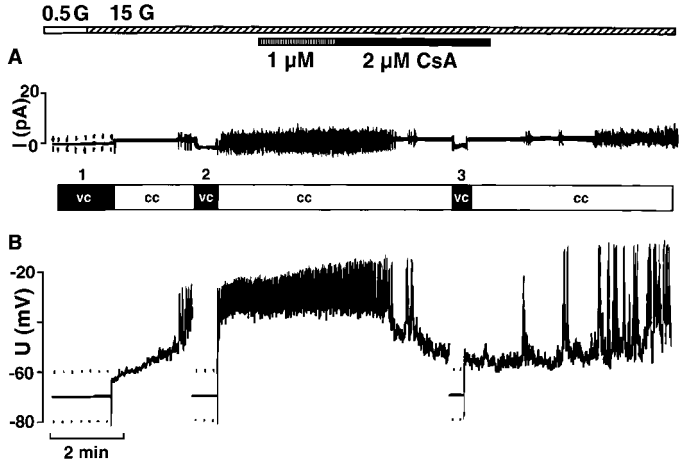

Fig. 5. Effect of CsA on B-cell membrane potential and K^+_{ATP} current. Experiments were performed in the perforated-patch configuration with intact cell metabolism. A, K^+_{ATP} currents measured in the voltage-clamp mode (VC 1, 2, and 3) during 300-ms pulses to −80 and −60 mV (lower and upper dashed lines in VC 1) from a holding potential of −70 mV (continuous line in VC 1). B, corresponding changes in cell membrane potential in the CC mode. With the nonstimulatory glucose concentration of 0.5 mM a small current through K^+_{ATP} channels was measured (A, VC 1). In the presence of 15 mM glucose the membrane potential was depolarized, spike activity occurred (B, CC), and the K^+_{ATP} current decreased to virtually zero (A, VC 2). Addition of 2 μM CsA reversibly inhibited electrical activity (B, CC) without an increase in K^+_{ATP} current (A, VC 3). The experiment is representative of four with similar results.

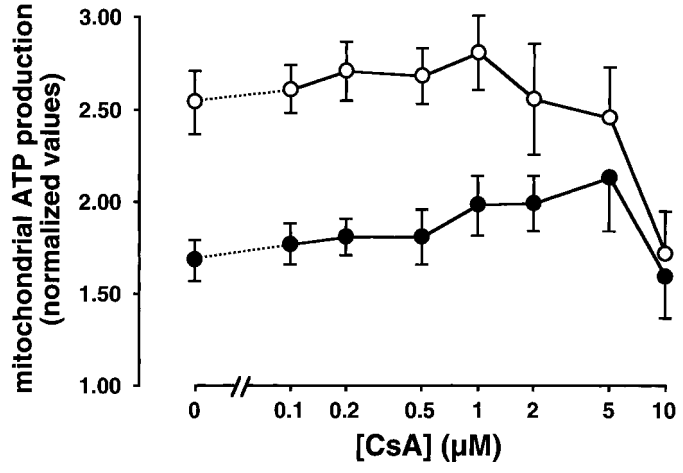

Fig. 6. ATP production in isolated B-cell mitochondria. Isolated mitochondria were incubated for 10 min with 1 mM pyruvate/1 mM malate (open circles) or 0.1 mM KIC/10 mM glutamate (closed circles) in the presence of 50 μM ADP and 200 nM Ca^{2+} with or without various concentrations of CsA. ATP synthesis was not decreased by CsA in concentrations up to 5 μM. Values are means ± S.E.M. of four (pyruvate/malate) and six (KIC/glutamate) experiments, respectively.

Influence of CsA on ΔΨ in Glucose-Stimulated B-Cells. Although no direct effect on ATP synthesis was observed, CsA was tested for an effect on mitochondrial function by measuring the mitochondrial membrane potential ΔΨ (Fig. 7). The experiment is the same as shown in Fig. 2. $[Ca^{2+}]_c$ and ΔΨ were recorded simultaneously in a cluster of B-cells preincubated with fura-2/AM and Rh 123. To ensure that the effects are not influenced by interactions between the two fluorescent dyes, series of control experiments were performed where the cells were loaded with one dye only. As for $[Ca^{2+}]_c$ the increase in the extracellular glucose concentration from 0.5 to 15 mM induced a triphasic response in ΔΨ (Krippeit-Drews et al., 2000): the glucose-induced activation of mitochondrial respiratory chains hyperpolarized ΔΨ (Duchen et al., 1993) as indicated by a decrease in Rh 123 fluorescence. The following elevation in $[Ca^{2+}]_c$, in turn, led to a partial depolarization of ΔΨ and thereafter oscillations in $[Ca^{2+}]_c$ were followed by oscillations in ΔΨ as previously described in detail (Krippeit-Drews et al., 2000).

Although 1 μM CsA had no effect on glucose-induced oscillations in ΔΨ, the addition of 2 to 5 μM CsA terminated the oscillatory activity (Fig. 7, $n = 10$). Rh 123 fluorescence showed a gradual slow increase in the presence of CsA. However, as described above, this observation is not coupled to obvious changes in ATP production. The inset shows for comparison the depolarizing effect of 5 μM CsA and the mitochondrial inhibitor NaN_3 (5 mM) on ΔΨ ($n = 3$).

Influence of Tolbutamide on CsA-Induced Drop in Glucose-Elevated $[Ca^{2+}]_c$. To examine whether the CsA-induced inhibition of $[Ca^{2+}]_c$ is due to interference with a step before the closure of K^+_{ATP} channels the sulfonylurea tolbutamide was added to a test medium containing 15 mM glucose and 5 μM CsA. Figure 8 shows that in glucose-stimulated cells, where $[Ca^{2+}]_c$ was first decreased by 5 μM CsA from 299 ± 45 to 90 ± 15 nM ($P < 0.001$), 100 μM tolbutamide was still effective, leading to an elevation in $[Ca^{2+}]_c$ to a peak value of 383 ± 50 nM ($P < 0.001$, $n = 5$).

Influence of Tolbutamide on $[Ca^{2+}]_c$ and ΔΨ in Thapsigargin-Pretreated B-Cells. To rule out that the observed tolbutamide-induced increase in $[Ca^{2+}]_c$ may be owing to Ca^{2+} release from mitochondria as proposed by Smith et al. (1999), we have tested the effects of tolbutamide on $[Ca^{2+}]_c$ and ΔΨ in Ca^{2+}-free medium in cells pretreated with 1 μM thapsigargin. In some of the eight cells tested 100 μM tolbutamide induced a minute depolarization of ΔΨ (Fig. 9A). The parallel measurements of $[Ca^{2+}]_c$ demonstrate that there was no increase in $[Ca^{2+}]_c$ by 100 μM tolbutamide under these conditions (Fig. 9B, $n = 8$). Similar results were obtained when Ca^{2+} influx was inhibited by 100 μM D600 instead of removal of extracellular Ca^{2+} with otherwise identical protocol (data not shown; $n = 6$). Taken together, these results clearly speak against tolbutamide-induced Ca^{2+} release from mitochondria. After readdition of Ca^{2+} to the medium tolbutamide increased $[Ca^{2+}]_c$ and depolarized ΔΨ as expected due to Ca^{2+}influx ($n = 5$).

Discussion

Deterioration in glucose tolerance is frequently observed in human transplant recipients treated with CsA (Gunnarsson et al., 1984). This study examines whether and how CsA impairs B-cell function in a therapeutically relevant concentration range. CsA-induced deterioration of glucose tolerance can be caused either by an increased insulin resistance of the tissue (Gunnarsson et al., 1984; Krentz et al., 1993) or by an impaired insulin secretion (Yamamoto et al., 1991). The latter view is supported by many studies showing a marked decrease in glucose-induced insulin secretion in human (Nielsen et al., 1986) and rat islets (Carroll et al., 1991; Martin and Bedoya, 1991; Herold et al., 1993) pretreated or acutely treated with CsA in a concentration range of the drug that can be achieved during immunosuppressive therapy. Additionally, it has been described for βTC3 cells that the reduced insulin secretion coincided with a decrease in insulin mRNA (Herold et al., 1993), thus explaining the reduced

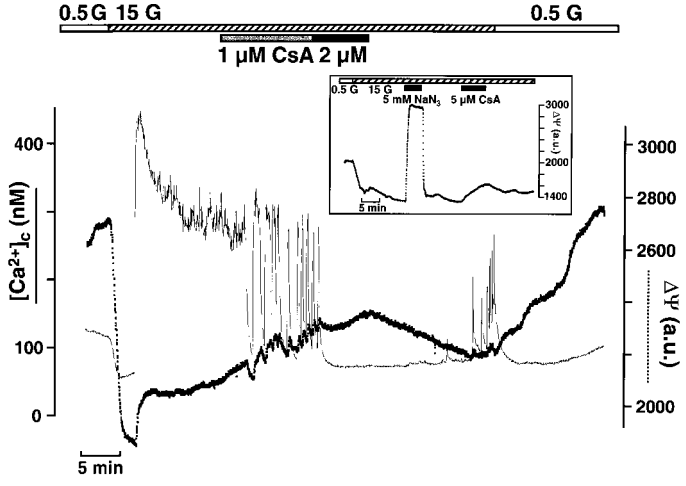

Fig. 7. Inhibitory effect of CsA on oscillations in ΔΨ (fat dotted line). Same experiment as in Fig. 2. The graph showing $[Ca^{2+}]_c$ (thin solid line) is added for comparison. For simultaneous recording of $[Ca^{2+}]_c$ and ΔΨ cells were loaded with fura-2/AM and Rh 123. A decrease in Rh 123 fluorescence corresponds to a hyperpolarization of ΔΨ. Cells were stimulated with 15 mM glucose, leading to the characteristic oscillatory pattern in $[Ca^{2+}]_c$ and ΔΨ. CsA (2–5 μM) abolished these oscillations. Additionally, CsA induced a slow increase in Rh 123 fluorescence. The recording is representative of 10 similar experiments. The inset shows for comparison the depolarizing effect of 5 μM CsA and the mitochondrial inhibitor NaN_3 (5 mM) on ΔΨ ($n = 3$).

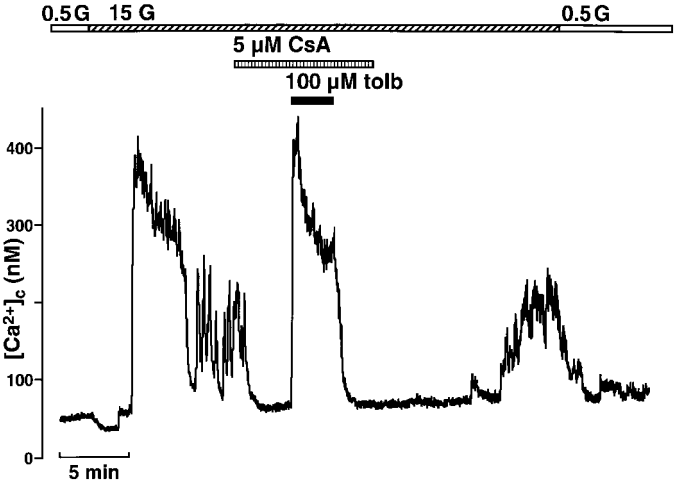

Fig. 8. Effect of the sulfonylurea tolbutamide on $[Ca^{2+}]_c$ in CsA-treated B-cells. Test solutions were added as indicated by the horizontal bars. Cells were stimulated with 15 mM glucose and oscillations in $[Ca^{2+}]_c$ were stopped by 5 μM CsA. The addition of 100 μM tolbutamide in the presence of CsA reversibly elevated $[Ca^{2+}]_c$. The experiment represents five with similar results.

insulin content observed in mouse islets (Andersson et al., 1984).

Our data clearly show the interference of CsA with B-cell secretory function because we observed a significant decrease in glucose-stimulated insulin secretion of about 55% after a 60-min incubation period with 1 to 10 μM CsA. These values are in accordance with the few data available in the literature for short-term incubations, where insulin release was reduced by 30 to 60% in the presence of 0.42 to 83 μM CsA (Robertson, 1986; Carroll et al., 1991; Martin and Bedoya, 1991). CsA inhibited oscillations of $[Ca^{2+}]_c$, $\Delta\Psi$, and membrane potential that are known to regulate pulsatile insulin secretion in intact islets (Santos et al., 1991; Gilon et al., 1993; Krippeit-Drews et al., 2000) and are impaired in diabetes mellitus (Meissner and Schmidt, 1976; Polonsky et al., 1998). CsA abolished glucose-induced B-cell activity in those experiments that were performed with single cells or small clusters of cells. At a first glance this contrasts with the incomplete inhibition of insulin secretion. However, the secretion experiments were made with whole islets where paracrine effects of other cells (e.g., A cells) may support B-cell function or CsA may not reach and influence all B-cells during the incubation period.

The data presented in this study show that the effects of CsA are neither due to a direct interference of the drug with the current through voltage-dependent Ca^{2+} channels nor to an indirect action via calcineurin. The specific calcineurin inhibitors deltamethrin and tacrolimus were without any effect on glucose-induced oscillations of $[Ca^{2+}]_c$. This latter finding is important because the protein phosphatase calcineurin is the target of CsA, which is responsible for the immunosuppressive action. CsA blocks the phosphatase activity of calcineurin via binding to the cellular immunophilin cyclophilin (Zoratti and Szabò, 1995). Thus, desired and side effects of CsA seem to be mediated by different pathways.

The observation that the CsA-induced decrease in $[Ca^{2+}]_c$ was reversed by the sulfonylurea tolbutamide strengthens the view that the CsA-provoked decrease in Ca^{2+} influx is owing to changes in the stimulus-secretion coupling upstream to cell membrane depolarization. The mitochondria might represent such a target and Fig. 7 shows that indeed CsA affects $\Delta\Psi$.

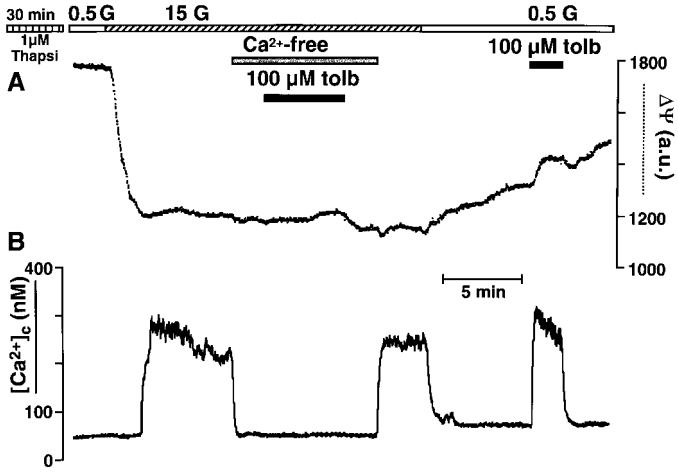

Fig. 9. Effects of tolbutamide on $\Delta\Psi$ and $[Ca^{2+}]_c$ in thapsigargin-pretreated cells. Tolbutamide was added in the presence of 15 mM glucose in a nominally Ca^{2+}-free incubation medium or in the presence of 0.5 mM glucose in Ca^{2+}-containing medium as indicated by the horizontal bars. A, $\Delta\Psi$ was only marginally depolarized by 100 μM tolbutamide in Ca^{2+}-free medium. In contrast, in the presence of Ca^{2+} the depolarizing effect of tolbutamide was clearly visible. B, parallel measurement of $[Ca^{2+}]_c$. In contrast to Ca^{2+}-containing medium 100 μM tolbutamide did not alter $[Ca^{2+}]_c$ in Ca^{2+}-free medium. The experiment is representative of eight with similar results ($n = 5$ for the effects of tolbutamide in the presence of Ca^{2+}).

It is well documented that CsA interferes with mitochondrial function by inhibiting the PTP of the mitochondria (Zoratti and Szabò, 1995) and there is increasing evidence that this voltage- and Ca^{2+}-regulated ion channel of the inner and outer mitochondrial membranes plays an important role in Ca^{2+} homeostasis. It has been documented that there are at least two different conductance states of the PTP (Ichas and Mazat, 1998). In its high-conductance state that is irreversibly induced by a Ca^{2+} overload of the cells the PTP is involved in the cascade of apoptotic cell death (Zamzami et al., 1996), whereas a physiological role in the regulation of cellular Ca^{2+} homeostasis is proposed for the low-conductance state (Ichas and Mazat, 1998). We have recently proposed that the PTP is important in regulating the oscillatory activity of pancreatic B-cells (Krippeit-Drews et al., 2000), which is a prerequisite for adequate insulin secretion. The observation that an inhibitor of the PTP such as CsA interrupts oscillations of $\Delta\Psi$, the membrane potential, and $[Ca^{2+}]_c$ fits well with this hypothesis. However, further experiments are needed to clarify the exact mechanism how the inhibition of the PTP finally suppresses oscillations and Ca^{2+} influx.

Interference of CsA with the PTP may influence mitochondrial Ca^{2+} handling and/or ATP production. It has been proposed that disturbance of the mitochondrial Ca^{2+} homeostasis directly influences inactivation of different Ca^{2+} influx pathways in the plasma membrane because intact mitochondria serve as a Ca^{2+} sink, which rapidly removes Ca^{2+} from the cytoplasm (Hoth et al., 2000). In insulin-secreting cells changes in $[Ca^{2+}]_m$ participate in the regulation of cellular Ca^{2+} homeostasis: It has been shown that the glucose-induced rise in $[Ca^{2+}]_c$ is followed by a drastic increase in $[Ca^{2+}]_m$ (Kennedy and Wollheim, 1998) and that inhibition of mitochondrial Ca^{2+} uptake prevents insulin secretion in permeabilized INS-1 cells (Maechler et al., 1997). Possibly a direct interaction between mitochondrial Ca^{2+} handling and Ca^{2+} influx across the plasma membrane exists, but a direct prove for such an interaction in B-cells is still lacking.

We have shown that the direct inhibition of K^+_{ATP} channels by tolbutamide could overcome the CsA effect on $[Ca^{2+}]_c$, pointing to an interference of the drug with the K^+_{ATP} current. The experiments presented in Fig. 9 allow us to rule out that tolbutamide increases $[Ca^{2+}]_c$ by Ca^{2+} release from mitochondria. However, the effect of CsA on the K^+_{ATP} current has to be a minute one, just sufficient to repolarize the membrane potential below the threshold for the opening of Ca^{2+} channels. A dramatic drop in ATP synthesis by CsA can be excluded for the following reasons. First, there is neither an increase in K^+_{ATP} current causing a repolarization to the resting membrane potential nor an immediate, steep depolarization of $\Delta\Psi$ that would indicate a drop in ATP synthesis as observed with NaN_3 and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Düfer et al., 1999; Krippeit-Drews et al., 2000; Fig. 7, inset). Second, ATP depletion of the cells would result in a sustained elevation of $[Ca^{2+}]_c$ because

ATP-dependent sequestration of Ca^{2+} would be abolished. This is in contrast to the observed reduction of $[Ca^{2+}]_c$ to basal values in the presence of CsA. Third, ATP production of isolated mitochondria is not affected at concentrations up to 5 μM CsA. One possible explanation for the action of CsA is that it inhibits a mitochondrial factor other than ATP, which normally decreases K^+_{ATP} channel activity. On the other hand, we cannot rule out that CsA marginally diminishes ATP synthesis, which, however, might be too small to be resolved as a measurable change in the K^+_{ATP} current amplitude or in the overall ATP production of mitochondria. The small and slow increase in Rh 123 fluorescence observed with CsA may be taken as a hint for this assumption. At a high glucose concentration (e.g., 15 mM) almost all K^+_{ATP} channels are closed and therefore membrane resistance is extremely high. Under such conditions rare openings of a few K^+_{ATP} channels induced by CsA may evoke a small repolarization in the membrane potential, which can be crucial to determine whether Ca^{2+} channels are open or closed.

In conclusion, the data presented in this study indicate that CsA decreases insulin secretion by interference with the mitochondrial permeability transition pore and not by an action on calcineurin.

Acknowledgments

We thank I. Breuning (Tübingen, Germany) and B. Borgström (Umeå, Sweden) for skillful technical assistance.

References

Akagi H, Reynolds A, and Hjelm M (1991) Cyclosporin A and its metabolites, distribution in blood and tissues. *J Int Med Res* **19:**1–18.

Andersson A, Borg H, Hallberg A, Hellerström C, Sandler S, and Schnell A (1984) Long-term effects of cyclosporin A on cultured mouse pancreatic islets. *Diabetologia* **27:**66–69.

Canadian-European Randomized Control Trial Group (1988) Cyclosporin-induced remission of IDDM after early intervention. Association of 1 yr of cyclosporin treatment with enhanced insulin secretion. *Diabetes* **37:**1574–1582.

Cardenas ME, Muir RS, Breuder T, and Heitman J (1995) Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. *EMBO (Eur Mol Biol Organ) J* **14:**2772–2783.

Carroll PB, Boschero AC, Li MY, Tzakis AG, Starzl TE, and Atwater I (1991) Effect of the immunosuppressant FK506 on glucose-induced insulin secretion from adult rat islets of Langerhans. *Transplantation* **51:**275–278.

De Filippo G, Carel J-C, Boitard C, and Bougnères P-F (1996) Long-term results of early cyclosporin therapy in juvenile IDDM. *Diabetes* **45:**101–104.

Draznin B, Metz SA, Sussman KE, and Leitner JW (1988) Cyclosporin-induced inhibition of insulin release. Possible role of voltage-dependent calcium transport channels. *Biochem Pharmacol* **37:**3941–3945.

Duchen MR (1999) Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J Physiol (Lond)* **516:**1–17.

Duchen MR, Smith PA, and Ashcroft FM (1993) Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. *Biochem J* **294:**35–42.

Düfer M, Drews G, and Krippeit-Drews P (1999) Oscillations in intracellular calcium provoke oscillations in the mitochondrial membrane potential of B-cells (Abstract). *Diabetologia* **42:**A140.

Enan E and Matsumura F (1992) Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. *Biochem Pharmacol* **43:**1777–1784.

Evtodienko YuV, Teplova V, Khawaja J, and Saris NE (1994) The Ca^{2+}-induced mitochondrial permeability transition pore is involved in Ca^{2+}-induced oscillations. A study on permeabilised Ehrlich ascites tumour cells. *Cell Calcium* **15:**143–152.

Fall CP and Bennett JP (1999) Visualization of cyclosporin A and Ca^{2+}-sensitive cyclical mitochondrial depolarizations in cell culture. *Biochim Biophys Acta* **1410:**77–84.

Gilon P, Shepherd RM, and Henquin J-C (1993) Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. *J Biol Chem* **268:**22265–22268.

Grapengiesser E, Gylfe E, and Hellman B (1988) Dual effect of glucose on cytoplasmic Ca^{2+} in single pancreatic β-cells. *Biochem Biophys Res Commun* **150:**419–425.

Gunnarsson R, Klintmalm G, Lundgren G, Tydén G, Wilczek H, Östman J and Groth CG (1984) Deterioration in glucose metabolism in pancreatic transplant recipients after conversion from azathioprine to cyclosporine. *Transplant Proc* **16:**709–712.

Hahn H-J, Dunger A, Laube F, Besch W, Radloff E, Kauert C, and Kotzke G (1986) Reversibility of the acute toxic effect of cyclosporin A on pancreatic B cells of Wistar rats. *Diabetologia* **29:**489–494.

Halestrap AP and Davidson AM (1990) Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem J* **268:**153–160.

Herold KC, Nagamatsu S, Buse JB, Kulsakdinun P, and Steiner DF (1993) Inhibition of glucose-stimulated insulin release from βTC3 cells and rodent islets by an analog of FK506. *Transplantation* **55:**186–192.

Hoth M, Button DC, and Lewis RS (2000) Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. *Proc Natl Acad Sci USA* **97:**10607–10612.

Ichas F, Jouaville LS, and Mazat J-P (1997) Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* **89:**1145–1153.

Ichas F and Mazat J-P (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta* **1366:**33–50.

Kennedy ED and Wollheim CB (1998) Role of mitochondrial calcium in metabolism-secretion coupling in nutrient-stimulated insulin release. *Diabetes Metab* **24:**15–24.

Krentz AJ, Dousset B, Mayer D, McMaster P, Buckels J, Cramb R, Smith JM, and Nattrass M (1993) Metabolic effects of cyclosporin A and FK 506 in liver transplant recipients. *Diabetes* **42:**1753–1759.

Krippeit-Drews P, Düfer M, and Drews G (2000) Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells. *Biochem Biophys Res Commun* **267:**179–183.

Lembert N and Idahl LÅ (1998) α-Ketoisocaproate is not a true substrate for ATP production by pancreatic β-cell mitochondria. *Diabetes* **47:**339–344.

Lucke S, Radloff E, Laube R, and Hahn HJ (1991) Morphology of the endocrine pancreas in cyclosporine-treated glucose-intolerant Wistar rats. *Anat Anz* **172:**351–358.

Maechler P, Kennedy ED, Pozzan T, and Wollheim CB (1997) Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic β-cells. *EMBO (Eur Mol Biol Organ) J* **16:**3833–3841.

Martin F and Bedoya FJ (1991) Mechanisms of action of cyclosporin A on islet α-and β-cells. Effects on cAMP-and calcium-dependent pathways. *Life Sci* **49:**1915–1921.

Meissner HP and Schmidt H (1976) The electrical activity of pancreatic β-cells of diabetic mice. *FEBS Lett* **67:**371–374.

Mijares A, Malécot CO, Peineau N, and Argibay JA (1997) *In vivo* and *in vitro* inhibition of the L-type calcium current in isolated guinea-pig cardiomyocytes by the immunosuppressive agent cyclosporin A. *J Mol Cell Cardiol* **29:**2067–2076.

Nielsen JH, Mandrup-Poulsen T, and Nerup J (1986) Direct effects of cyclosporin A on human pancreatic β-cells. *Diabetes* **35:**1049–1052.

Oellerich M, Armstrong VW, Kahan B, Shaw L, Holt DW, Yatscoff R, Lindholm A, Halloran P, Gallicano K, Wonigeit K, et al. (1995) Lake Louise Consensus Conference on cyclosporin monitoring in organ transplantation: report of the consensus panel. *Ther Drug Monit* **17:**642–654.

Polonsky KS, Sturis J, and Van Cauter E (1998) Temporal profiles and clinical significance of pulsatile insulin secretion. *Horm Res* **49:**178–184.

Renström E, Ding WG, Bokvist K, and Rorsman P (1996) Neurotransmitter-induced inhibition of exocytosis in insulin-secreting β cells by activation of calcineurin. *Neuron* **17:**513–522.

Robertson RP (1986) Cyclosporin-induced inhibition of insulin secretion in isolated rat islets and HIT cells. *Diabetes* **35:**1016–1019.

Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B, and Valdeolmillos M (1991) Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflueg Arch Eur J Physiol* **418:**417–422.

Smith PA, Proks P, and Moorhouse A (1999) Direct effects of tolbutamide on mitochondrial function, intracellular Ca^{2+} and exocytosis in pancreatic β-cells. *Pflueg Arch Eur J Physiol* **437:**777–588.

Wood PG and Gillespie JI (1998) Evidence for mitochondrial Ca^{2+}-induced Ca^{2+} release in permeabilised endothelial cells. *Biochem Biophys Res Commun* **246:**543–548.

Yamamoto H, Akazawa S, Yamaguchi Y, Yokota A, Yamasaki H, Nakanishi T, Tahara D, Matsuya F, Saito Y, and Nagataki S (1991) Effects of cyclosporin A and low dosages of steroid on posttransplantation diabetes in kidney transplant recipients. *Diabetes Care* **14:**867–870.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gómez-Monterrey I, Castedo M, and Kroemer G (1996) Mitochondrial control of nuclear apoptosis. *J Exp Med* **183:**1533–1544.

Zoratti M and Szabò I (1995) The mitochondrial permeability transition. *Biochim Biophys Acta* **1241:**139–176.

Address correspondence to: Dr. Peter Krippeit-Drews, Department of Pharmacology, Institute of Pharmacy, Auf der Morgenstelle 8, University of Tübingen, D-72076 Tübingen, Germany. E-mail: peter.krippeit-drews@uni-tuebingen.de