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# CYCLOSPORIN-INDUCED INHIBITION OF INSULIN RELEASE

## POSSIBLE ROLE OF VOLTAGE-DEPENDENT CALCIUM TRANSPORT CHANNELS*

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**Abstract**—The exposure of normal pancreatic islets to cyclosporin-A (1 μg/ml) for 24 hr resulted in significant inhibition of glucose-induced (16.7 mM) insulin release from 197 ± 14 μU/10 islets/15 min (control) to 103 ± 14 μU/10 islets/15 min (Cy-A-treated islets; $P < 0.001$). Cy-A did not alter insulin release in the presence of non-stimulatory (1.7 mM) or submaximally effective glucose concentrations (9.2 mM). In parallel experiments, Cy-A reduced glucose-stimulated increases in cytosolic free calcium concentrations, $[Ca^{2+}]_i$ (217 ± 15 nM without and 137 ± 3 nM with Cy-A in the presence of 16.7 mM glucose, $P < 0.01$). To better define the site of Cy-A action, we studied its effect on insulin release and increases in $[Ca^{2+}]_i$ induced by either $K^+$ (50 mM), which promotes $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels, or by forskolin (20 μM), dibutyryl cyclic AMP (1 mM) or arachidonic acid (49 μM), all of which stimulate mobilization of intracellular $Ca^{2+}$ stores. Cy-A significantly inhibited $K^+$-induced changes (203 ± 13 nM without and 77 ± 6 nM with Cy-A, respectively, $P < 0.001$), but not those induced by forskolin, dibutyryl cyclic AMP or arachidonic acid. These observations suggest that Cy-A inhibits insulin release by interfering with $Ca^{2+}$ influx via voltage-dependent calcium channels.

Cyclosporin A (Cy-A), a potent immunosuppressive agent, is used widely following organ transplant surgery in general and, in particular, in diabetic patients receiving transplanted segments of pancreas or isolated pancreatic islets [1–3]. In addition, based upon theoretical considerations of the autoimmune nature of type I diabetes, Cy-A has been used in patients with newly diagnosed insulin-dependent diabetes as an experimental immunosuppressive therapy [4, 5].

Recently, several published reports have suggested that high concentrations of Cy-A exert adverse effects on pancreatic islet function both *in vivo* and *in vitro* [6–13]. Moreover, in patients with pancreatic transplants, Cy-A has been reported to cause deterioration of glucose tolerance [1, 2].

Several studies have revealed that Cy-A, in therapeutically relevant doses, directly inhibits insulin secretion from isolated human islets maintained in organ culture [12] and from a glucose-responsive clonal B-cell line (HIT cells) [13]. Since the total insulin content (after relatively short incubations of 1–16 hr) was not affected by Cy-A, it was suggested that within this time frame this drug directly influenced cellular pathways controlling insulin secretion.

A major event in the cascade of secretagogue-induced insulin secretion is an augmentation of cytosolic free calcium concentrations, $[Ca^{2+}]_i$. Cy-A could interfere with insulin secretion by adversely affecting the ability of secretagogues to increase $[Ca^{2+}]_i$. The aim of this study was to investigate the effect of Cy-A upon secretagogue-induced increases in $[Ca^{2+}]_i$ and insulin release from intact pancreatic islets from normal rats.

### MATERIALS AND METHODS

*Materials.* Cy-A was a gift from the Sandoz Co. Fura-2 and fura-2-AM were obtained from Molecular Probes, Inc., Junction City, OR. Culture medium (TCM-199), fetal calf serum, Dulbecco's phosphate-buffered saline (PBS) and antibiotics were purchased through the Grand Island Biological Co., Grand Island, NY. Collagenase (CLS-IV) was obtained from Worthington Biochemical, Freehold, NJ, and arachidonic acid (AA) from Nu-Chek Prep, Elysian, MN. All other biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO. Male Sprague–Dawley rats, weighing 250–350 g, employed in these studies were obtained from Simonson Laboratories, Gilroy, CA. The animals were allowed food and water *ad lib.* Anesthesia was induced with pentobarbital sodium (45 mg/kg body wt).

*Islet cell isolation technique.* Pancreatic islets (1000–3000 islets) were isolated using a standard Ficoll isolation technique as previously described [14, 15]. Following isolation, the islets were placed into 3 ml of TCM-199 tissue culture medium containing 10% fetal calf serum, 30 mg/dl glucose,

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100 units/ml of penicillin, and 100 μg/ml of streptomycin. The islets were incubated with 0 or 1 μg/ml Cy-A for 24 hr at 37° under 5% $CO_2$ in air [15]. On the day of the experiment, islets were disrupted into individual cells which were resuspended in 2 ml of Krebs/*N*-2-hydroxyethylpiperazine-*n'*-2-ethanesulfonic acid (HEPES) buffer, containing 118.4 mM NaCl, 4.69 mM KCl, 1.2 mM $MgCl_2$, 1.18 mM $KH_2PO_4$, 1.25 mM $NaHCO_3$, 20 mM HEPES, 5 mg/ml bovine serum albumin (BSA), and 30 mg/dl glucose. A cell count was then obtained in the presence of 0.04% trypan blue. The cell yield ranged between 250 and 400 cells per islet with cell viability over 85%.

*Measurement of intracellular calcium.* The intracellular loading of fura-2 was performed according to the method of Grynkiewicz *et al.* [16], as previously described [15]. The fluorescence of fura-2-loaded islet cells (F) was measured at an excitation wavelength of 340 nm and at an emission wavelength of 478 nm using a Turner model 430 spectrofluorometer fitted with a magnetic stirrer and a thermostatted cuvette holder. The fluorescence of the extracellular fura-2 was estimated by adding $MnCl_2$ (50 μM) which quenches extracellular fura-2. $MnCl_2$ was then chelated by the addition of 100 μM di-ethylenetriaminepentaacetic acid (DTPA). The estimate of extracellular fura-2 was made prior to stimulation of the cells with secretagogues. Fluorescence maximum ($F_{max}$) and minimum ($F_{min}$) were determined as previously reported [15, 17]. The cells were lysed with 0.04% Triton X-100, and the $F_{max}$ was measured in the presence of 1 mM $CaCl_2$. The $F_{min}$ was then measured in the presence of 2mM ethylene glycol bis(β-amino-ethylether)*N,N,N',N'*-tetraacetic acid (EGTA) and 50 mM Tris base (pH > 8.3). Neither Cy-A nor secretagogues used in this study affected the fluorescence of the fura-2–$Ca^{2+}$ complex.

*Insulin release.* Islet incubations for insulin release were performed using intact rat islets as previously described [18]. Most of the buffers, reagents, and culture conditions were identical to those used in the performance of the $[Ca^{2+}]_i$ measurements. However, the buffer used in studying insulin release was slightly different, containing 2.5 mM $CaCl_2$, 144 mM $Na^+$, 25 mM bicarbonate, and 0.5% BSA. In experiments with AA, BSA and $Ca^{2+}$ were omitted from the buffer and 0.5 mM EGTA was present.

*Statistical analysis.* The results of $Ca^{2+}$ studies are expressed as mean ± SEM of three to five experiments. Paired or unpaired Student's *t*-test was used to compare the mean values between experimental groups. Insulin data are presented as mean ± SEM for six to ten determinations, where each observation represent a batch of ten islets from a sample population of islets. The data were compared by nonpaired *t*-testing.

## RESULTS

We initially assessed the effect of Cy-A upon glucose-induced insulin release from normal rat pancreatic islets. The islets were cultured for 24 hr either with or without Cy-A. The total insulin content on the day of experiment was identical in control

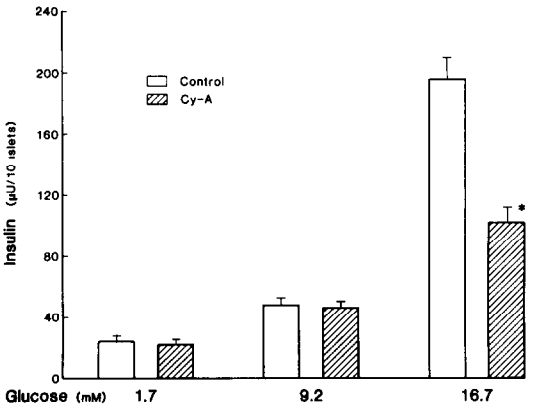


Fig. 1. Effect of Cy-A on glucose-induced insulin release. In these and subsequent experiments, the islets were incubated with or without 1 μg/ml Cy-A for 24 hr (as described in Materials and Methods) before being challenged with secretagogues in the continuous presence of Cy-A. In the depicted experiments, the islets were exposed to three concentrations of glucose for 15 min at 37°. * $P < 0.001$.

(45,992 ± 1,154 μU/20 islets) and Cy-A-treated islets (45,800 ± 2,427 μU/20 islets; P = NS; N = 6). The islets were then incubated with low (1.7 mM), intermediate (9.2 mM), and high (16.7 mM) glucose concentrations for 15 min. There was a dose-dependent increase in insulin secretion from the islets cultured in the absence of Cy-A (Fig. 1). As compared with basal insulin release at 1.7 mM glucose, insulin release doubled at 9.2 mM glucose and increased 8-fold in the presence of 16.7 mM glucose. Cy-A did not affect insulin release under non-stimulatory conditions or under stimulation with intermediate glucose concentrations. However, there was a significant ($P < 0.001$), albeit incomplete, inhibition of insulin release induced by a maximally effective glucose concentration.

We then assessed the effect of Cy-A on glucose-induced increases in $[Ca^{2+}]_i$ (Fig. 2). Similar to the experiments with insulin secretion, in the absence of Cy-A, both intermediate and high concentrations of glucose significantly augmented $[Ca^{2+}]_i$. However, the relative increments in $[Ca^{2+}]_i$ were of lesser magnitude than those seen with insulin release (Fig. 3).

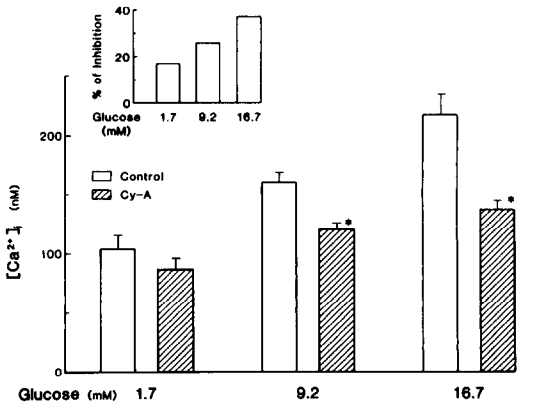


Fig. 2. Effect of Cy-A on glucose-induced increases in $[Ca^{2+}]_i$. The percent of inhibition of glucose effect by Cy-A is plotted on the inset. * $P < 0.01$.

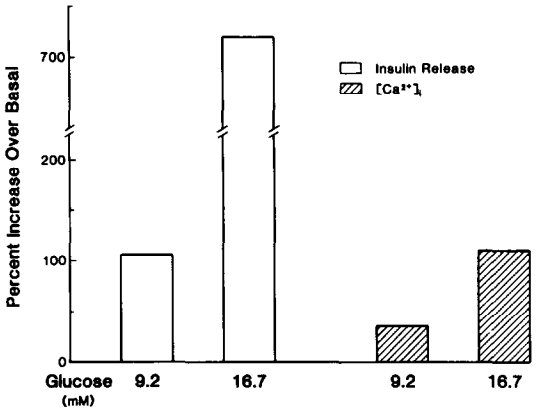


Fig. 3. Comparison of the magnitude of glucose-induced insulin release with that of glucose-induced increments in $[Ca^{2+}]_i$. The percent change is derived from the data shown in Figs. 1 (insulin release) and 2 $[Ca^{2+}]_i$.

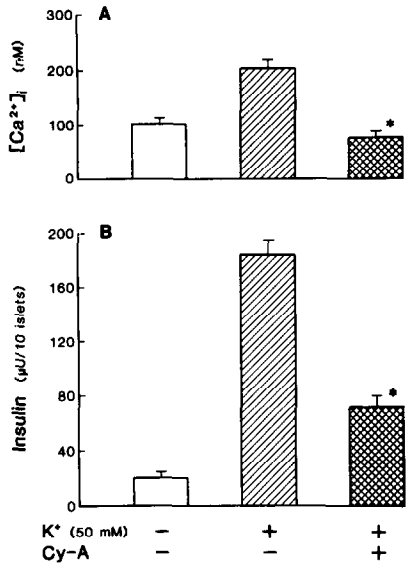


Fig. 4. Effect of Cy-A upon $D^+$-induced increases in $[Ca^{2+}]_i$ (A) and insulin release (B). * $P < 0.01$ vs the values observed with $K^+$ without Cy-A. In these and other experiments with non-glucose secretagogue, the buffer contained 1.7 mM glucose.

Culture of islets in the presence of Cy-A resulted in a minimal non-significant decrease in $[Ca^{2+}]_i$ under non-stimulatory conditions (1.7 mM glucose). However, glucose-induced increases in $[Ca^{2+}]_i$ were inhibited significantly by Cy-A. The percent inhibition of glucose-induced increases in $[Ca^{2+}]_i$ by Cy-A is shown on the inset of Fig. 2. This inhibitory effect of Cy-A was seen at both stimulatory glucose concentrations, but it was more prominent at the highest glucose concentration.

Comparing the responses in insulin release with those of $[Ca^{2+}]_i$ upon glucose stimulation (Fig. 3), one can see that the magnitude of stimulation of insulin release by glucose was much greater than that of cytosolic free $Ca^{2+}$. However, the percent inhibition by Cy-A was greater with regard to $[Ca^{2+}]_i$. There exists no simple quantitative correlation between these two variables. It appears that smaller increments in $[Ca^{2+}]_i$ resulted in much greater increments in insulin secretion.

To further characterize the mechanism of Cy-A action, we examined the effect of this immunosuppressive agent upon potassium-induced increases in $[Ca^{2+}]_i$ and insulin release. High concentrations of $K^+$ are known to depolarize the beta cell and promote $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels. In the absence of Cy-A, 50 mM $K^+$ increased $[Ca^{2+}]_i$ (Fig. 4A) and augmented insulin release (Fig. 4B). Cy-A completely blocked $K^+$-induced increases in $[Ca^{2+}]_i$ and significantly reduced insulin release. These observations suggested that Cy-A may interfere with $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels.

To examine whether Cy-A can also effect the mobilization of intracellular $Ca^{2+}$ stores, we assessed the influence of Cy-A on increases in $[Ca^{2+}]_i$ elicited by forskolin and dibutyryl cyclic AMP (d-cAMP). Forskolin and d-cAMP have been shown to increase $[Ca^{2+}]_i$ primarily by mobilizing intracellular $Ca^{2+}$ stores [15]. Both forskolin and d-cAMP elicited prompt increases in $[Ca^{2+}]_i$ (Fig. 5). These increases were not influenced by Cy-A, suggesting that this agent does not interfere with the mobilization of intracellular $Ca^{2+}$ stores.

Arachidonic acid (AA) is another agent that triggers insulin release and mobilizes intracellular $Ca^{2+}$ stores [19]. Its effect is particularly prominent in the absence of extracellular $Ca^{2+}$ [19]. Cy-A did not interfere with AA-induced increases in $[Ca^{2+}]_i$ or insulin release (Fig. 6). AA (49 $\mu$M) raised $[Ca^{2+}]_i$ 141 and 157% above the basal levels (without and with Cy-A respectively) and stimulated insulin release 72 and 92% above basal (without and with Cy-A respectively).

## DISCUSSION

Previous investigations have demonstrated inhibition of glucose-induced insulin release by Cy-A [6, 8, 9, 12, 13]. In the present study, we focused on the effects of acute (24 hr) administration of Cy-A. In this setting, the major impact of Cy-A seemed to be on insulin release. In contrast, Cy-A administered orally for 7 days induces degranulation of the beta cells accompanied by hypoinsulinemia and

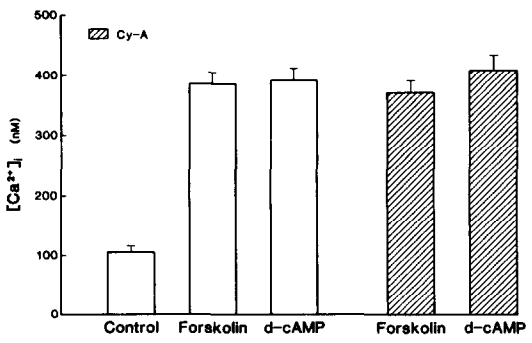


Fig. 5. Effect of Cy-A upon forskolin- and dibutyryl cyclic AMP-induced increases in $[Ca^{2+}]_i$.

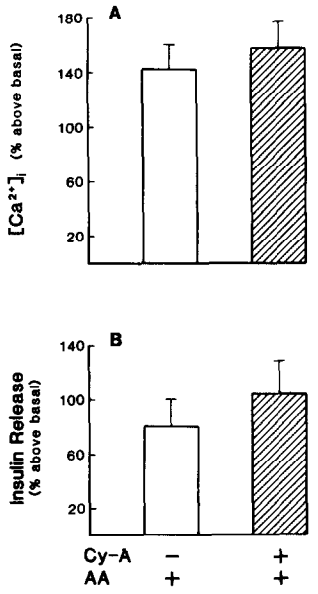


Fig. 6. Effect of Cy-A upon AA (49 μM)-induced increases in $[Ca^{2+}]_i$ (A) and insulin release (B). The results are expressed as the percent above basal values and represent the mean ± SEM of four experiments. The basal levels of $[Ca^{2+}]_i$ were 103 ± 8 and 89 ± 9 nM without and with CyA respectively. The basal levels of insulin release were 81 ± 17 and 156 ± 13 μU/ml without and with CyA respectively.

hyperglycemia in Wistar rats [20]. This latter effect was attributed to an inhibitory effect of Cy-A on both DNA and mRNA synthesis [20]. Interestingly, the Cy-A effect on mRNA synthesis is rapidly reversible and is not related to a direct interaction of the drug with the transcription site *per se* [20].

The present observations demonstrate that Cy-A inhibited insulin release under conditions wherein the influx of extracellular $Ca^{2+}$ was stimulated. Thus, Cy-A abrogated glucose- and $K^+$-induced insulin release and increases in $[Ca^{2+}]_i$. The inhibition of the glucose-induced increases in $[Ca^{2+}]_i$ by Cy-A was, however, incomplete. Glucose probably promotes $Ca^{2+}$ influx by mechanisms other than just the activation of voltage-dependent $Ca^{2+}$ channels. Furthermore, a part of the glucose effect on $[Ca^{2+}]_i$ is related to its ability to mobilize intracellular $Ca^{2+}$ stores. This latter effect may be mediated by the combined actions of inositol trisphosphate [21], AA [19, 22], and cyclic AMP [15, 23].

Cy-A exerted no effect upon the mobilization of intracellular $Ca^{2+}$ stores. Cy-A did not inhibit mobilization of $[Ca^{2+}]_i$ by forskolin or d-cAMP. Similarly, Cy-A did not suppress rises in $[Ca^{2+}]_i$ or insulin release elicited by AA, an agent which does not promote $Ca^{2+}$ influx. These results are in agreement with the observations in lymphocytes [24, 25] where Cy-A did not interfere with the mobilization of intracellular $Ca^{2+}$.

Taken as a whole, the data suggest that the mechanism by which Cy-A inhibits $Ca^{2+}$ influx in pancreatic islet cells is likely to involve the voltage-dependent $Ca^{2+}$ channels, although other possible sites of action cannot be excluded. Fehmann *et al.* [26] have reported recently that the calcium channel blocker diltiazem diminishes the suppression of insulin release induced by Cy-A in pancreatic islet cells.

Nagineni *et al.* [27] have also presented evidence in favor of close interaction between Cy-A and $Ca^{2+}$ channels. They have observed that $Ca^{2+}$ channel blockers diminish uptake of Cy-A by the renal proximal tubular cells. Although Cy-A did not reduce $Ca^{2+}$ influx in these cells, the authors postulated a close spatial relationship between Cy-A binding sites and $Ca^{2+}$ channels. Furthermore, Matyus *et al.* [28] have shown that Cy-A reduces depolarization of mouse lymphocytes induced by ionomycin or A23187. Their finding suggested incorporation of Cy-A into the cellular membrane, resulting in changes in ion fluxes. The present study lends further support to the possibility of direct interaction between Cy-A and $Ca^{2+}$ channels. Attention needs to be focused upon whether Cy-A is altering either the function or the number of voltage-dependent $Ca^{2+}$ channels.

An alternate possibility is that Cy-A inhibits voltage-dependent $Ca^{2+}$ influx indirectly via opening $K^+$ channels and thereby hyperpolarizing the cell. Recent studies have suggested that glucose opens $Ca^{2+}$ channels, at least in part, by closing the ATP-dependent $K^+$ channels [29, 30]. The possibility that Cy-A inhibits glucose-induced increases in both $[Ca^{2+}]_i$ and insulin release by opening such $K^+$ channels needs to be investigated further.

It is evident that Cy-A did not inhibit completely glucose-induced insulin release. Cy-A did not decrease insulin release at 9.2 mM glucose, whereas it did inhibit increases in $[Ca^{2+}]_i$ at this level. This incomplete inhibition of insulin release supports the view that glucose is a complex secretagogue which also stimulates events responsible for insulin secretion other than increasing $[Ca^{2+}]_i$. As an example, the production of diacylglycerol and the consequent activation of protein kinase C may stimulate insulin release independently of changes in the levels of $[Ca^{2+}]_i$ and may potentiate the effects of increases in $[Ca^{2+}]_i$ [31]. The involvement of other modulatory steps, besides an increase in $[Ca^{2+}]_i$, in the process of insulin secretion may also explain the discrepancies between the magnitude of incremental insulin release and incremental change in $[Ca^{2+}]_i$. This lack of quantitative correlation between these two variables is not unexpected and has been shown previously [32]. For example, we have demonstrated that increases in $[Ca^{2+}]_i$ elicited by forskolin and d-cAMP trigger only minimal insulin release [32]. The present data indicate that a decrease in $[Ca^{2+}]_i$ with Cy-A did not inhibit insulin release induced by intermediate (9.2 mM) glucose concentrations. Moreover, considerable decreases in $[Ca^{2+}]_i$ as seen in the experiments with Cy-A and 16.7 mM glucose, given together, did not lead to commensurate decreases in insulin release. These data confirm several previous observations that the levels of $[Ca^{2+}]_i$ may not quantitatively correlate with the magnitude of insulin release [32]. Smaller increments in $[Ca^{2+}]_i$ are accompanied by much greater increments in insulin release, due to the synergistic effects of other transduction signals with the $[Ca^{2+}]_i$ signal. Part of the problem may relate to the different time intervals of sampling. In this study, insulin measurements

reflect integrated secretion over 15 min, whereas $[Ca^{2+}]_i$ measurements reflect a single point in time.

The effect of Cy-A cannot be ascribed to non-specific toxicity of this drug on $Ca^{2+}$-dependent exocytosis, since it did not decrease AA-induced or middle level (9.2 mM) glucose-induced insulin release. Furthermore, the failure to inhibit increases in $[Ca^{2+}]_i$ induced by AA, forskolin and d-cAMP suggest that Cy-A does not merely stimulate $Ca^{2+}$ extrusion mechanisms, quench $Ca^{2+}$, or interfere with fura-2 fluorescence.

Cyclosporin A has proven to be an extremely useful and potent immunosuppressive agent. In therapeutic doses, cyclosporin suppressed glucose-induced insulin release and mRNA synthesis. A possible mechanism to explain, at least, this suppression of insulin release may be related to the effect of cyclosporin on $Ca^{2+}$ transport through voltage-dependent channels.

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