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EFFECTS OF CYCLOSPORINE A ON CYCLIC AMP GENERATION AND GTP-BINDING PROTEINS IN ISOLATED ISLETS

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Abstract—The role of cyclosporine A (CsA) in cAMP generation and its relationship with guanine nucleotide-binding proteins (G-proteins) was investigated in isolated islets. cAMP accumulation in response to glucose, 3-isobutyl-1-methyl-xanthine (a phosphodiesterase inhibitor) and the calcium ionophore A23187 increased significantly ($P < 0.05$) in the presence of 0.5 μg/mL CsA. CsA (0.5 μg/mL) was unable to affect the 2.1-fold increase in cAMP formation induced by 30 μM forskolin (an adenylate cyclase complex activator). The pertussis toxin-induced cAMP generation in the presence of 20 mM glucose was suppressed by CsA by 34%. On the other hand, CsA enhanced cAMP levels in cholera toxin-treated islets. CsA caused a non-competitive inhibition of phosphodiesterase activity with half-maximal inhibition at 5 μg/mL CsA. CsA blocked the pertussis toxin ADP-ribosylation of a 41-kDa and a 21-kDa islet protein, but not the cholera toxin ADP-ribosylation of a 45-kDa and a 21-kDa islet protein. These data indicate that CsA increases cAMP content by a non-competitive inhibition of phosphodiesterase activity and by acting through G-proteins involved in the modulation of adenylate cyclase activity. An inhibitory effect of CsA on a 21-kDa pertussis toxin-sensitive G-protein was also observed.

Cyclosporine A (CsA†) is an immunosuppressive agent that has proved to be a highly effective drug for use in transplantation surgery [1]. CsA is also used in experimental therapy of recently diagnosed type I diabetes in humans, to suppress the autoimmune response [2]. In addition, several reports have observed that CsA decreases plasma insulin and brings about glucose intolerance [3]. Besides, it has been shown that CsA induces an inhibitory action of insulin release *in vitro* [4, 5]. However, the mechanisms of action of CsA in the β-cell remain to be established.

Some of the proposed signal molecules involved in stimulus–secretion coupling in isolated islets are cAMP, Ca^{2+}, inositol 1,4,5-trisphosphate (which releases calcium from intracellular stores) and 1,2-diacylglycerol [6–8]. The generation of each of these signals is dependent on guanine nucleotides. Guanine nucleotide-binding proteins (G-proteins) have a central role in both the activation and inhibition of cAMP formation [9]. In isolated islets, HIT cells and RINm5F cells G_s (guanine nucleotide-binding stimulatory protein) mediates increases in intracellular cAMP associated with hormone-induced stimulation of insulin secretion [10], and G_i (guanine nucleotide-binding inhibitory protein) mediates decreases in intracellular cAMP caused by inhibitors of insulin secretion [11]. Cholera and pertussis toxins are important investigational tools for studies of G-protein function and identification. In this context, cholera toxin can be used to amplify hormonal mechanisms that activate G_s and stimulate generation of cAMP. On the other hand, pertussis toxin can be used to block hormonal mechanisms that activate G_i and inhibit generation of cAMP.

Because cAMP and G-proteins play a dominant role in the regulation of pancreatic islet β-cell function, these studies were designed to identify the effects of CsA on the cAMP-dependent pathway and on the GTP-binding proteins system of the islet. We conclude that CsA is able to modify cAMP levels acting through guanine nucleotide-binding regulatory proteins and phosphodiesterase activity.

MATERIALS AND METHODS

Materials. Collagenase, D-glucose, pertussis toxin, cholera toxin, 3-isobutyl-1-methyl-xanthine (IBMX), 5′-nucleotidase, phosphodiesterase 3′:5′-cyclic nucleotide (from bovine heart, containing near saturation levels of both protein activator and Ca^{2+}), 5′-nucleotidase, cAMP, ATP, GTP, thymidine, nicotinamide, myokinase, NADP, forskolin, tissue culture fluids and other chemicals were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). A23187 was purchased from Calbiochem (La Jolla, CA, U.S.A.). Phosphocreatine and creatine phosphokinase were from Boehringer (Mannheim, Germany). Fiske–Subbarow reactive was purchased from Química Clínica Analítica (Tarragona, Spain). Electrophoresis reagents were from BioRad (Richmond, CA, U.S.A.). [adenylate-^{32}P]NAD was obtained from ICN Biomedicals

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† Abbreviations: CsA, cyclosporine A; G-protein, guanine nucleotide-binding protein; IBMX, 3-isobutyl-1-methyl-xanthine; KRB, Krebs–Ringer bicarbonate buffer; BSA, bovine serum albumin.

(Costa Mesa, CA, U.S.A.). cAMP radioimmunoassay kit was from Amersham (U.K.). Autoradiography supplies were from Aldrich (Steinheim, Germany). CsA was from Sandoz (Basel, Switzerland), and was stored in a stock solution of 50 μg/mL in 1% dimethyl sulfoxide and 15% BSA at −20°.

Islet preparation. Islets from male Wistar rats weighing 180–200 g were isolated by collagenase procedure [12]. Briefly, in each experiment two pancreata were distended with Hanks solution, pH 7.4, 3 mM D-glucose at 4° and the tissue was minced and digested with collagenase for 10–15 min at 37° with manual gentle shaking. Collection of the islets was performed with finely drawn out Pasteur pipettes under stereomicroscope.

Protein determination. Protein content was assayed by the method of Bradford [13].

Studies on cAMP content in isolated islets. Batches of 10 islets were preincubated for 30 min at 37° in 1 mL of Krebs–Ringer bicarbonate buffer (KRB), 0.5% BSA, pH 7.4, gassed with $O_2 + CO_2$ (95:5) and containing 2.7 mM D-glucose. Then, the preincubation medium was removed and the islets were incubated for 30 min at 37° in 1 mL of KRB, 0.5% BSA, pH 7.4, gassed with $O_2 + CO_2$ (95:5), in the absence (vehicle alone) or in the presence of 0.5 μg/mL CsA, and in the presence of the desired test compounds.

To investigate the effect of pertussis toxin treatment on cAMP formation, batches of 200 islets aseptically isolated were cultured for 24 hr at 37° in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/mL penicillin–streptomycin, 250 μg/mL fungizone and 100 ng/mL pertussis toxin, in the absence (vehicle alone) or in the presence of 4 μg/mL CsA, and in a humidified atmosphere containing 5% CO_2. Control islets were cultured in the absence of pertussis toxin under identical conditions. For cholera toxin experiments, batches of 100 islets were pre-incubated for 4 hr at 37°, in RPMI-1640 medium supplemented with 10% fetal-calf serum, 2 mM L-glutamine, 50 μg/mL penicillin–streptomycin, 250 μg/mL fungizone and 200 ng/mL cholera toxin, in the absence (vehicle alone) or in the presence of 4 μg/mL CsA, and in a humidified atmosphere containing 5% CO_2. Control islets were cultured under the same conditions but in the absence of cholera toxin. Then, the culture medium was removed and the islets were incubated in batches of 10, for 30 min at 37° in 1 mL of KRB, 0.5% BSA, pH 7.4, gassed with $O_2 + CO_2$ (95:5), in the presence of 2 or 20 mM glucose.

Finally, at the end of the incubation periods, the tubes containing the islets were centrifuged at 1500 *g* for 5 min, the medium was removed and 0.5 mL of methanol was added. Then, they were sonicated, heated at 80° for 4 hr and stored at −20°. Intracellular cAMP was assayed by radioimmunoassay, using cAMP radioimmunoassay kit.

Phosphodiesterase assay. The two-step assay for enzymatic activity is similar to that described previously [14]. Phosphodiesterase (0.06 U/mL) was incubated in 40 mM Tris–HCl, 2.5 mM $MgCl_2$, 0.4 mM EGTA, pH 7.4 buffer containing different cAMP concentrations (in the kinetic studies, the substrate concentrations ranged from 4×10^{-5} to 4×10^{-3} M) in a total volume of 0.4 mL, and in the absence (vehicle alone) or in the presence of different CsA concentrations (1, 10, 100 μg/mL). After 60 min at 30°, the reaction was terminated by boiling for 5 min. Then, after cooling to 37°, 0.75 U/mL of 5′-nucleotidase was added with an additional incubation of 60 min at 37°. Finally, the inorganic phosphate (P_i) liberated was measured as described previously [15].

ADP-ribosylation of isolated islets. The isolated islets (60 per group) were sonicated for 30 sec at maximal power in 50 mM potassium phosphate buffer (pH 7.4). Then, an amount of islet homogenate equivalent to 40 μg of protein was incubated for 60 min at 30° in 250 μL of a medium containing 50 mM potassium phosphate buffer (pH 7.4), 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 1 mM nicotinamide, 1 mM EGTA, 20 mM phosphocreatine, 20 U/mL creatine phosphokinase, 40 U/mL myokinase, 10 μCi [^{32}P]NAD, 20 μM NADP, and 30 μg/mL pertussis toxin or 90 μg/mL cholera toxin, in the absence (vehicle alone) and in the presence of 4 μg/mL CsA. Toxins were preactivated by treatment with 20 mM dithiothreitol for 10 min at 37°. The reaction was stopped by cooling at 4°, followed by centrifugation for 15 min at 40,000 *g*. The tubes and the surface of the pellet were rinsed with cold phosphate buffer. Then, 150 μL of 0.5 M Tris–HCl buffer (pH 6.8) containing 3% SDS, 0.7 M mercaptoethanol, 10% glycerol, 2 mM EDTA, 4 mM phenylmethylsulfonyl fluoride and 0.1% Bromophenol blue were added to the pellets and heated for 3 min at 100°. Finally, 130 μL of this mixture was subjected to PAGE by the method of Laemmli [16], using a 5% stacking gel and a 15% separating gel. The gels were stained with Coomassie brilliant blue G-250, dried and exposed to X-ray film for 10 days.

Statistical analyses. Results are presented as means ± SE. Statistical analysis was by the Student's *t*-test for unpaired data.

RESULTS

Effect of CsA on cAMP formation in isolated islets

In islets incubated in the presence of 10 mM D-glucose and 0.5 μg/mL CsA, a 2.5-fold increase in cAMP formation was observed when compared with controls (Table 1). Islets incubated with 10 mM D-glucose and 0.2 mM IBMX displayed a 2.6-fold increase in cAMP formation. This increase was enhanced 26% when islets were incubated in the presence of 0.5 μg/mL CsA. The 2.5-fold increase in cAMP formation induced by A23187 was also raised by 23% when 0.5 μg/mL CsA was present. Finally, CsA (0.5 μg/mL) was unable to affect the 2.1-fold increase in cAMP formation induced by 30 μM forskolin.

Effect of CsA on cAMP formation from pertussis toxin-treated intact islets

Table 2 shows that both control and toxin-treated islets responded to an elevation in glucose concentration from 2 to 20 mM with an increased rate of cAMP formation. Toxin-treated islets

Table 1. Effect of CsA on cAMP formation in isolated islets

	cAMP (fmol/islet/30 min)	
	Control	0.5 μg/mL CsA
Control	14.8 ± 2.8	37.7 ± 4.1*
0.2 mM IBMX	38.6 ± 3.5	52.5 ± 2.2*
10 μM A23187	38.2 ± 2.7	49.4 ± 3.7*
30 μM forskolin	31.6 ± 5.2	26.5 ± 4.3†

Mean values ± SEM are derived from eight experiments in all cases. As the control, in all cases the incubation medium contained 10 mM D-glucose.
* $P < 0.05$ vs control.
† Not significant vs control.

consistently showed a higher cAMP formation rate at 2 and 20 mM glucose than control islets. At 2 mM glucose, CsA (0.5 μg/mL) significantly increased cAMP levels in both control and toxin-treated islets. On the other hand, the stimulatory effect of CsA on cAMP formation at 20 mM glucose was only apparent in control islets. Pertussis toxin treatment seems to counteract the effect of both CsA and 20 mM glucose on cAMP levels.

Effect of CsA on cAMP formation from cholera toxin-treated intact islets

Control and toxin-treated islets responded to an elevation in glucose concentration from 2 to 20 mM by increasing the rate of cAMP formation (Table 3). Toxin-treated islets showed a higher cAMP formation rate at 2 and 20 mM than control islets. CsA (0.5 μg/mL) exerted a significant increase in the cAMP formation in control islets in the presence of 2 and 20 mM glucose. By contrast, CsA elicited a 2.3- and 1.9-fold increase in cAMP formation when toxin-treated islets were exposed to 2 and 20 mM glucose, respectively.

Effect of CsA on phosphodiesterase activity

The phosphodiesterase 3′:5′-cyclic nucleotide exhibits Michaelis–Menten behavior (Fig. 1). This enzyme has an apparent K_m for cAMP hydrolysis of 4×10^{-4} M. CsA causes a non-competitive inhibition with respect to cAMP, with a K_i of 83 μM. The half-maximal inhibition calculated for CsA is 5 μg/mL.

Effect of CsA on cholera and pertussis toxin-ADP ribosylation

As shown in Fig. 2, incubation of homogenate of islets with [^{32}P]NAD in the absence of toxins resulted in no labelling of protein, with and without 4 μg/mL CsA (lanes 1 and 2 of Fig. 2A and B). Pertussis toxin catalyzed the [^{32}P]ADP-ribosylation of a 41,000-Da protein. A faintly labeled 39,000-Da band was also detected (Fig. 2A, lane 3). CsA at 4 μg/mL prevented the subsequent incorporation of radioctive ADP ribose in these protein fractions (Fig. 2A, lane 4). Cholera toxin ADP-ribosylated two islet proteins of 52,000- and 45,000-Da (Fig. 2B, lane 3). CsA at 4 μg/mL did not block this effect (Fig. 2B, lane 4). Finally, pertussis and cholera toxins ADP-ribosylated a 21,000-Da islet protein

Table 2. Effect of CsA on cAMP formation in pertussis toxin-treated intact islets

	cAMP (fmol/islets/30 min)	
	2 mM D-glucose	20 mM D-glucose
Control	8.2 ± 1.2	39.8 ± 3.6*
Control + 0.5 μg/mL CsA	19.6 ± 1.8	49.3 ± 1.41*
Pertussis toxin treated	24.5 ± 1.3	47.2 ± 1.8*
Pertussis toxin treated + 0.5 μg/mL CsA	47.2 ± 1.4	31.6 ± 2.4*

Mean values ± SEM are derived from seven experiments in all cases.
* $P < 0.01$ vs control.

Table 3. Effect of CsA on cAMP generation in cholera toxin-treated intact islets

	cAMP (fmol/islets/30 min)	
	2 mM D-glucose	20 mM D-glucose
Control	7.3 ± 1.7	38.6 ± 1.2*
Control + 0.5 μg/mL CsA	19.8 ± 1.2	49.4 ± 1.2*
Cholera toxin treated	48.4 ± 2.5	80.6 ± 3.1*
Cholera toxin treated + 0.5 μg/mL CsA	110.3 ± 3	154.1 ± 2.8*

Mean values ± SEM are derived from four experiments in all cases.
* $P < 0.002$ vs control.

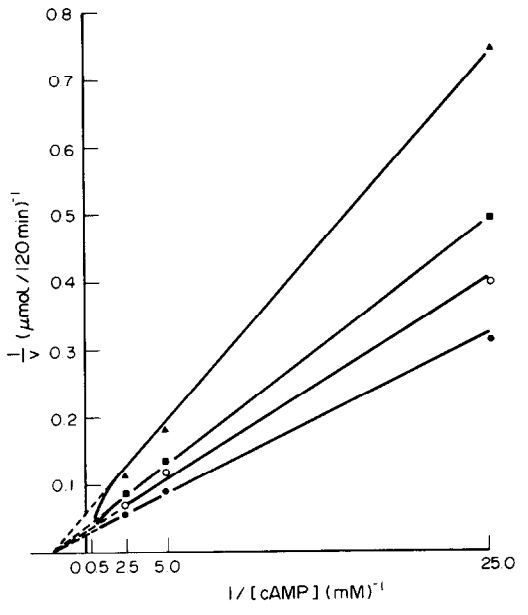

Fig. 1. Effect of CsA on phosphodiesterase 3′:5′-cyclic nucleotide activity. The substrate concentrations ranged from 4×10^{-5} to 4×10^{-3} M. The assay conditions are described in Materials and Methods. The representations were extrapolated to the abscissa to determine the apparent K_m. (●) Control. CsA concentrations: 1 μg/mL (○), 10 (□) and 100 μg/mL (▲).

(lanes 3 of Fig. 2A and B). CSA 4 μg/mL blocked the pertussis toxin ADP-ribosylation of the 21,000-Da islet protein (Fig. 2A, lane 4), but not the cholera toxin ADP-ribosylation of this protein (Fig. 2B, lane 4).

DISCUSSION

Nucleotide-binding regulatory proteins, G_s and G_i, modulate receptor-mediated stimulation and inhibition of cAMP synthesis in several cells systems, including pancreatic islets [9, 17]. On the other hand, cAMP degradation is controlled by a Ca^{2+}-calmodulin dependent phosphodiesterase [18]. This study describes the effects of CsA on the islet cAMP system and provides insights into the mechanisms by which CsA modifies cAMP levels in pancreatic islets, namely through inhibition of phosphodiesterase activity and interaction with G-proteins.

Significant rises in islet cAMP content have been detected following glucose stimulation [19]. Since glucose does not increase cAMP in the absence of external Ca^{2+} [20], fuel-induced cAMP accumulation results from a rise in cytosolic Ca^{2+} which, in combination with calmodulin, activates adenylate cyclase [18]. Other studies have reported that calmodulin enhanced cAMP phosphodiesterase activity [21]. Our data show that CsA enhances cAMP formation in the presence of glucose and this effect is potentiated by the phosphodiesterase inhibitor IBMX and by the Ca^{2+} ionophore A23187 (Table 1). CsA interacts with calmodulin and blocks

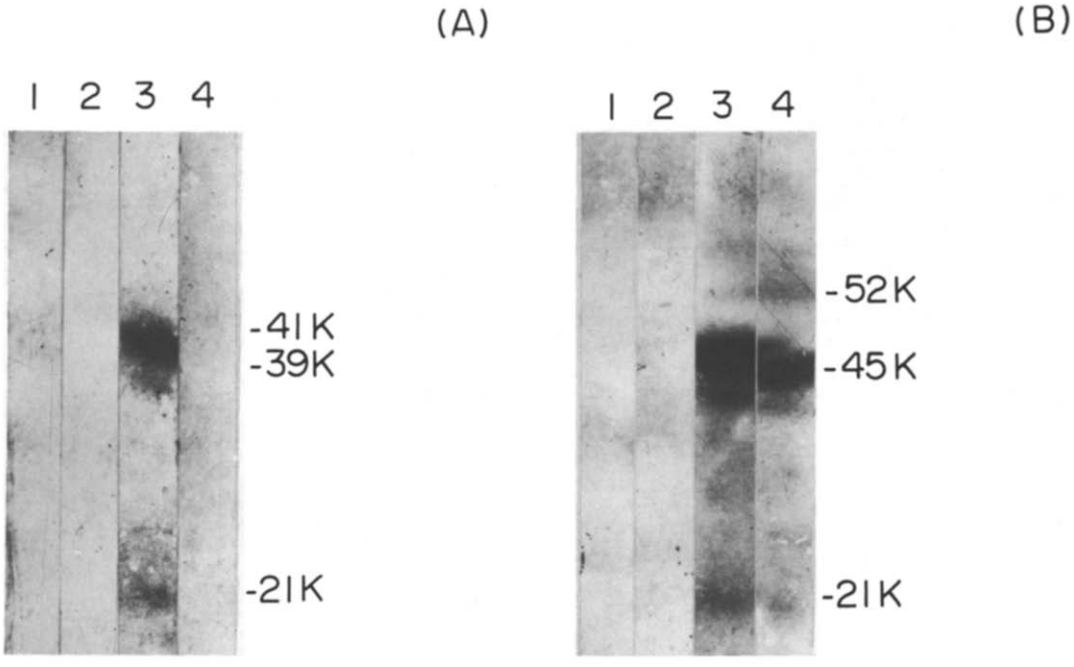

Fig. 2. Effect of CsA on cholera toxin- and pertussis toxin-induced [^{32}P]ADP-ribosylation of islet homogenate proteins. Homogenate of islets was incubated with [^{32}P]NAD, in the presence of 4 μg/mL CsA (lanes 2 and 4 of A, B) and in the absence of CsA (lanes 1 and 3 of A, B), with no toxin (lanes 1 and 2 of A, B), with 30 μg/mL pertussis toxin (lanes 3 and 4 of A), and with 90 μg/mL cholera toxin (lanes 3 and 4 of B) as described in Materials and Methods. Radiolabeling of membrane proteins was analysed by SDS–PAGE followed by autoradiography. Migration of molecular mass markers is indicated (right).

its regulatory function [22]. Thus, activation of both adenylate cyclase and phosphodiesterase by calmodulin can be blocked by CsA. Our results indicate that phosphodiesterase plays a prominent role in islets and this is blocked by CsA. When bovine heart Ca^{2+}-calmodulin-containing phosphodiesterase was assayed in the presence of increasing concentrations of cAMP, a non-competitive inhibition by CsA was observed (Fig. 1). The half-maximal inhibition calculated for CsA is 5 μg/mL. This value is 50-fold higher than the value reported for phosphodiesterase containing non-saturating levels of both Ca^{2+} and calmodulin [22]. Our experiments were performed at saturating levels of both Ca^{2+} and calmodulin. This may account for the higher levels of CsA needed for half-maximal inhibition. This value is also 10-fold higher than the concentration used in experiments with intact islets. Since CsA accumulates in the pancreas [23], it is feasible that CsA inhibits phosphodiesterase activity in intact islets.

Forskolin raises cAMP in islets by activating the adenylate cyclase complex [24]. In this context, the failure of CsA to affect the forskolin-induced cAMP formation (Table 1) is consistent with he idea that CsA does not exert a direct effect on the adenylate cyclase complex.

Pertussis toxin treatment of pancreatic islets leads to an increase in the islet cAMP content at both low and high glucose (Table 2). While CsA exerts an additive effect on cAMP content in pertussis toxin-treated islets at low glucose, it inhibits cAMP accumulation at high glucose. This finding clearly suggests that CsA also acts on the islet cAMP system through a pertussis toxin-sensitive mechanism. Cholera toxin treatment of pancreatic islets leads to increases in islet cAMP content at both low and high glucose (Table 3). CsA potentiates this effect at both low and high glucose. While CsA inhibition of phosphodiesterase can account for this effect at low glucose, an additional stimulatory effect of CsA on G_s is proposed at high glucose. Additionally, CsA did not block the ADP-ribosylation of the 45-kDa α subunit of G_s catalysed by cholera toxin (Fig. 2B, lane 4). It has been proposed that GTP and low molecular mass G-protein, (G_{Ei}) mediate the inhibition of insulin secretion at a late stage following activation of inhibitory α_2-adrenergic receptors [25]. Moreover, when GTP is provided to permeabilized RINm5F cells, clonidine inhibits both Ca^{2+}- and diglyceride-induced secretion, an effect that is abolished by pretreatment with pertussis toxin [26]. These observations and our finding that CsA blocks the pertussis toxin ADP-ribosylation of a 21-kDa protein (Fig. 2A, lane 4) in islets lead us to propose a feasible action of CsA on a pertussis toxin-sensitive 21-kDa G-protein coupled with exocytosis.

We conclude from these studies that: (1) CsA increases cAMP production by a non-competitive inhibition of phosphodiesterase activity; (2) CsA inhibits a pertussis toxin-sensitive G_i protein and stimulates a cholera toxin-sensitive G_s protein; and (3) CsA may exert an inhibitory action on a pertussis toxin-sensitive 21-kDa G-protein in islets.

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