Effects of the immunosuppressants cyclosporin A and FK 506 on exocytosis in the rat exocrine pancreas *in vitro*

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1 We have examined the effects of the immunosuppressive drugs cyclosporin A (CsA) and FK 506 on exocytosis in two *in vitro* preparations of the exocrine pancreas—lobules and dispersed acini.

2 In lobules taken from starved rats and stimulated with the secretagogue caerulein, both CsA and FK 506, given shortly before stimulation, caused a dose-dependent inhibition of amylase secretion. In lobules from rats that had been pretreated *in vivo* with the protease inhibitor FOY-305 to stimulate secretion maximally, both CsA and FK 506 inhibited secretion of newly synthesized proteins, whereas only FK 506 inhibited caerulein-stimulated amylase release.

3 These different effects of the immunosuppressants on amylase release were reflected in their effects on degranulation, as revealed by electron microscopy. Control acinar cells in lobules from FOY-305-treated rats were almost completely degranulated, whereas treatment with FK 506, but not CsA, caused the accumulation of zymogen granules close to the apical plasma membrane.

4 In dispersed acini, stimulated with the cholinomimetic secretagogue bethanechol, both CsA and FK 506 reduced the secretory response, to about 45% of control; IC_{50} values were 50 nM and 3 nM, respectively. A similar partial inhibition of exocytosis was seen in acini permeabilized with the bacterial toxin streptolysin O and stimulated with $10 \mu\text{M}$ Ca^{2+} .

5 These results demonstrate that the immunosuppressants cause an inhibition of exocytosis in the exocrine pancreas that is both rapid in onset and potent. The loss of the inhibitory effect of CsA on amylase release in lobules taken from FOY-305-treated rats may reveal a change in the characteristics of exocytosis as a consequence of the high level of stimulation, and also indicates that CsA and FK 506 have subtly different effects on secretion. We suggest that these drugs might be useful tools in the dissection of the molecular mechanisms of exocytosis.

Keywords: Cyclosporin A; FK 506; exocytosis; amylase release; exocrine pancreas

Introduction

Cyclosporin A (CsA) and FK 506 are powerful immunosuppressive agents that are used clinically to prevent rejection of transplanted tissues and organs (Schreiber, 1991). Both CsA and FK 506 produce significant side-effects, including nephro- and hepatotoxicity and impairment of the function of both the endocrine and exocrine pancreas (Müller *et al.*, 1988; Carroll *et al.*, 1991; Doi *et al.*, 1992; Hirano *et al.*, 1992). Over the past few years, several long-term *in vivo* studies in animals (usually rats) have been carried out to examine the effects of CsA and FK 506 on pancreatic function. CsA has been found to cause lesions in pancreatic β -cells and a corresponding reduction in insulin secretion and glucose tolerance, resulting in the development of hyperglycaemia (Helmchen *et al.*, 1984; Fehmann *et al.*, 1987; Müller *et al.*, 1988; Suzuki & Oka, 1989). Structural damage to the acinar cells of the exocrine pancreas has also been observed, along with reductions in plasma amylase levels and in cholecystokinin (CCK)-stimulated output of amylase, lipase and trypsin (Stöckmann *et al.*, 1985; Fernandez-Cruz *et al.*, 1988; Müller *et al.*, 1988; Suzuki & Oka, 1989; Bani *et al.*, 1990; Lopez-Miranda *et al.*, 1991; Hirano *et al.*, 1992). There is some debate about the doses of CsA required to produce these effects, and in particular about whether they occur at therapeutic ($10 \text{ mg kg}^{-1} \text{ day}^{-1}$) or only at toxic ($50 \text{ mg kg}^{-1} \text{ day}^{-1}$) levels. The consensus seems to be that even therapeutic doses of CsA cause significant adverse effects on the pancreas. Similar effects on pancreatic structure and function have been found following treatment with FK 506 *in vivo*, although this drug is 10–100 times more potent than CsA (Ricordi *et al.*, 1991; Doi *et al.*, 1992;

Strasser *et al.*, 1992). For example, isolated pancreatic acini from rats receiving FK 506 over two weeks showed a reduction in CCK-stimulated amylase release (Doi *et al.*, 1992). Histological examination revealed that FK 506 treatment at $0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ caused the appearance of a small number of vacuoles in the cytoplasm of the acinar cells, while at ten times this dose numerous vacuoles and pyknotic nuclei were seen. A small number of studies on the effects of the immunosuppressants on the endocrine pancreas *in vitro* have also been carried out (Andersson *et al.*, 1984; Martin & Bedoya, 1990; Carroll *et al.*, 1991); inhibitory effects on β -cell function were observed, but no changes in cell structure were reported.

CsA and FK 506 exert their major therapeutic effect by inhibiting T-cell activation (Schreiber, 1991; Schreiber & Crabtree, 1992). They bind to different sets of endogenous intracellular receptors, called immunophilins (Handschumacher *et al.*, 1984; Harding *et al.*, 1989; Siekierka *et al.*, 1989; Harrison & Stein, 1990), and the immunophilin-ligand complexes in turn bind to and inhibit the calcium/calmodulin-dependent serine/threonine protein phosphatase calcineurin (Liu *et al.*, 1991). Our interest in these agents was first aroused by a report that they were also able to inhibit IgE receptor-mediated degranulation in the mast cell line RBL-2H3 (Hultsch *et al.*, 1991), with IC_{50} values close to those for inhibition of T-cell receptor-mediated interleukin-2 transcription. This finding raises the possibility that they may be useful tools in the dissection of the molecular mechanisms of exocytosis.

The acinar cells of the exocrine pancreas synthesize and secrete a complement of 15–20 digestive enzymes and proenzymes (zymogens). The intracellular transport and discharge of these proteins involves a series of interrelated

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events, which have been described in the classic work of Palade (1975). After synthesis and translocation into the lumen of the rough ER, the proteins are transported vectorially through the Golgi complex. In the *trans* Golgi network, condensing vacuoles pinch off into the cytosol. These condensing vacuoles mature, with further condensation of their contents, to form zymogen granules. In the fasted state, the zymogen granules accumulate at the apical pole of the cell. Stimulation of pancreatic secretion is controlled by both neural and hormonal factors, such as acetylcholine and CCK. These secretagogues induce a rapid, oscillatory release of Ca^{2+} from intracellular stores (Tsunoda *et al.*, 1990), that results in the fusion of the zymogen granules with the apical domain of the plasma membrane. While the regulation of the synthesis and intracellular transport of the individual enzymes has been extensively studied (Vasiloudes *et al.*, 1991), little is known about the molecular details of exocytosis. It was of interest to us, therefore, to know whether the immunosuppressants CsA and FK 506 might shed light on this process in the pancreatic acinar cell. In this study, we have examined the effects of CsA and FK 506 on two *in vitro* preparations of the rat exocrine pancreas—lobules and isolated dispersed acini. Our aims were to establish precisely which cellular functions are affected by these agents (and in particular whether exocytosis is specifically inhibited), and also to determine the potency of the drugs and the rapidity of their effect. A preliminary account of this work has been presented to the British Pharmacological Society (Waschulewski *et al.*, 1992).

Methods

Preparation of pancreatic lobules

Pancreatic lobules were prepared from male Wistar rats (200–300 g; Zentralinstitut für Versuchstierkunde GmbH, Hannover, Germany) in which pancreatic exocytosis had either been inhibited by starvation for 18 h, or maximally stimulated by feeding with the protease inhibitor FOY-305. FOY-305 produces this effect by inhibiting the proteolytic activity of secreted trypsin and thereby removing the normal feedback inhibition of CCK release, so that CCK levels remain elevated for several hours (Rausch *et al.*, 1987). Animals received the FOY-305 at a dose of 50 mg kg^{-1} body weight in 1 ml tap water via an orogastric tube 6–8 h before they were killed. Rats were killed by exsanguination under light ether anaesthesia, and the pancreas was quickly removed and placed in Krebs-Ringer-HEPES (KRH) buffer, pH 7.4, containing physiological concentrations of amino acids. Pancreatic lobules were then prepared as described by Scheele & Palade (1975). For the preparation of lobules from FOY-305-treated rats, all buffers contained the CCK analogue caerulein (1 ng ml^{-1}) to maintain the exocytotic stimulus.

Exocytosis in lobules

Lobules were prepared either from starved rats or from FOY-305-treated rats. Batches of about 15–20 lobules were pre-incubated at 37°C for 20 min, with shaking, in 5 ml KRH buffer containing either CsA, FK 506 or vehicle alone (1% (v/v) final concentration). After pre-incubation, the lobules were washed with KRH buffer in a Buchner funnel and transferred to flasks containing 5 ml buffer together with appropriate drug concentrations and caerulein (1 ng ml^{-1}). The lobules were then incubated at 37°C for 90 min, with shaking, and a 1 ml sample was taken from the medium at 15, 30, 60 and 90 min and replaced with fresh medium. Oxygenation was carried out every 15 min. At the end of the incubation the lobules were homogenized in 5 ml of 0.05 M Tris-HCl, pH 8.8 containing 0.1% (v/v) Triton X-100.

Amylase in medium and homogenates was assayed by the

method of Bernfeld (1955). Secretion was expressed as a percentage of total amylase content. To assess the degree of cell damage, the release of the cytosolic marker enzyme lactate dehydrogenase (LDH) was also measured, by the method of Bergmeyer (1963). Again, release was expressed as a percentage of total LDH content.

Release of metabolically-labelled secretory proteins from lobules

Lobules prepared from FOY-305-treated rats were pre-incubated with immunosuppressant or vehicle as described above, and then pulse-labelled at 37°C for 8 min in methionine-free KRH buffer containing 100 μCi [^{35}S]-methionine in the continuous presence of caerulein (1 ng ml^{-1}). Lobules were transferred to Buchner funnels and washed with warm buffer containing a 100 fold excess of unlabelled methionine for 1–2 min. They were then incubated at 37°C, with shaking, in the same buffer. The incubation medium was sampled as described above for the measurement of amylase release. [^{35}S]-methionine-labelled proteins were quantitated by measuring trichloroacetic acid-insoluble radioactivity in medium and homogenates by the method of Mans & Novelli (1961). Secretion of labelled proteins was expressed as a percentage of the total contained in homogenized tissue samples.

Morphology of lobules

Lobules prepared from FOY-305-treated rats were pre-incubated with immunosuppressant or vehicle as described above. After incubation with caerulein (1 ng ml^{-1}) for 30, 60 or 90 min, lobules were fixed at 4°C for 2 h in a solution containing 2.5% (v/v) glutaraldehyde, 2.5% (w/v) paraformaldehyde, 0.05% (v/v) picric acid and 0.1% (w/v) sodium cacodylate, pH 7.4. Following post-fixation in reduced 1% (w/v) osmium tetroxide, tissues were stained in 0.5% (w/v) uranyl acetate in 50 mM malate buffer. Tissues were then dehydrated in a graded series of ethanol and embedded in Epon. Thin sections were cut with an Ultracut E microtome (Leica, Germany), stained in lead citrate solution and examined using a Zeiss EM 9S electron microscope.

Preparation of pancreatic acini

Pancreatic acini were prepared from male Sprague-Dawley rats (200–250 g), that had been starved overnight. Rats were killed by cervical dislocation and acini were prepared essentially as described by Rogers *et al.* (1988). A 5 ml volume of oxygenated, modified Krebs-Henseleit solution (isolation buffer, with the following composition, mM: NaCl 103, KCl 4.8, MgCl_2 1.2, CaCl_2 2.0, Na_2HPO_4 1.2, NaHCO_3 2.5, glucose 14, HEPES 25, glutamine 2, basal Eagle's medium amino acid supplement, bovine serum albumin (BSA) 2 mg ml^{-1} , soybean trypsin inhibitor 0.1 mg ml^{-1} , pH 7.2), containing collagenase (800 units ml^{-1}) was injected into the pancreas. The tissue was then incubated at 37°C for 10 min with shaking (60 cycles min^{-1}). The collagenase-containing buffer was removed and the tissue washed twice in fresh isolation buffer. It was then incubated for 10 min in isolation buffer and for two further 10 min periods in collagenase-containing buffer, separated by a 10 min wash in isolation buffer. The tissue was then dispersed by repetitive pipetting with a plastic Pasteur pipette and filtered through 200 μm mesh nylon gauze. The acinar cell suspension was layered onto 2 $\times$ 5 ml isolation buffer containing 4% (w/v) BSA and centrifuged at 500 r.p.m. in a bench centrifuge for 3 min. The pellets were washed twice in the same buffer and the cells were resuspended in 20 ml of isolation buffer. The cells were incubated in this buffer at 37°C for 30 min with shaking and oxygenation, and finally washed three times before use.

Exocytosis in intact acini

Acinar cell suspensions, in isolation buffer, were distributed into microfuge tubes (final volume 1 ml) and incubated at 37°C for 10 min with either dimethylsulphoxide (DMSO, final concentration 0.1% (v/v), which by itself had no effect on exocytosis) or DMSO containing CsA or FK 506. An appropriate concentration of the cholinomimetic secretagogue bethanechol was then added and the acini were incubated at 37°C for a further 15 min, to allow exocytosis to proceed to completion (Edwardson *et al.*, 1990). At the end of the incubation, the tubes were centrifuged at 500 r.p.m. in a bench centrifuge to pellet the acini and a sample of the supernatant was taken for amylase determination.

Amylase was assayed by the method of Rinderknecht *et al.* (1967), using amylose azure as substrate, and porcine amylase as standard. All determinations were made in triplicate. Amylase release was expressed as a percentage of the total amylase content of the acini, determined by treating samples with 0.2% (v/v) Triton X-100. Results from several separate experiments were combined by use of a weighted means computer programme.

Figure 1 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of caerulein-stimulated amylase release from pancreatic lobules taken from starved rats. Lobules were pre-incubated for 20 min with vehicle (●), CsA at 10⁻⁶ M (▲) or 10⁻⁴ M (△), or FK 506 at 10⁻⁷ M (■) or 10⁻⁵ M (□), before stimulation with caerulein (1 ng ml⁻¹). Values are means $\pm$ s.e.mean from 3 (a) and 4 (b) separate experiments. Error bars are not shown where they lie within the dimensions of the symbols. **P* < 0.05 and ***P* < 0.01, when compared with control values.

Exocytosis in permeabilized acini

Acini were cooled on ice and washed twice in ice-cold buffer solution containing KCl (140 mM), MgCl₂ (1 mM), EGTA (1 mM) and HEPES (20 mM), pH 6.5. They were then incubated at 37°C for 10 min in the same buffer with either DMSO (0.1% v/v) or DMSO containing CsA or FK 506. The tubes were returned to ice and streptolysin O (0.2 u ml⁻¹) and either water or CaCl₂ (to give a final free Ca²⁺ concentration of 10 μ M (Fabiato & Fabiato, 1979)) were added. The tubes were then incubated at 37°C for 15 min. At the end of the incubation, the tubes were cooled on ice, centrifuged to pellet the acini and the amylase in the supernatants was assayed as above. Results are in the form of Ca²⁺-dependent amylase release, expressed as a percentage of total amylase content.

Drugs and reagents

CsA was kindly provided by Sandoz Pharmaceuticals, Basel, Switzerland, and was dissolved in ethanol:water:Tween-80, 50:48:2 by volume. FK 506 was kindly provided by Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan, and was dissolved in ethanol. FOY-305 (N,N-dimethylcarbamyldimethyl-*p*-(*p*-guanidinobenzoyloxy) phenylacetate-methansulphonate) was from Sanol-Schwartz, Monheim, Germany. Caerulein (Takus) was from Farmitalia, Freiburg, Germany. [³⁵S]-methionine was from Amersham Buchler, Braunschweig, Germany. Bovine serum albumin (BSA Type V), soybean trypsin inhibitor, collagenase (Type XI), bethanechol and porcine amylase (Type VI-B) were from Sigma, Poole, U.K. Streptolysin O was from Wellcome Diagnostics, Dartford, U.K.

Statistical evaluation of data

The statistical significance of differences between groups of data was assessed by Student's *t* test (paired for results with lobules and unpaired for results with acini). A *P* value of < 0.05 was considered to be significant. All values are expressed as mean $\pm$ s.e.mean.

Results*Amylase release in lobules*

Lobules were taken either from starved rats, in which exocytosis would have been inhibited, or from rats that had been treated with the low molecular weight protease inhibitor FOY-305 to stimulate exocytosis maximally (Rausch *et al.*, 1987). In both cases, approximately 40% of the total amylase initially present in the lobules was released into the bathing medium over the 90 min incubation period used in these experiments (Figures 1 and 2). A 20 min pretreatment with either CsA or FK 506 inhibited amylase release from lobules taken from starved rats (Figure 1). The inhibition produced was dose-dependent, with FK 506 being about ten times more potent than CsA. For example, at the highest concentrations of the drugs used (10⁻⁴ M CsA and 10⁻⁵ M FK 506), amylase release over 90 min was reduced to 75 $\pm$ 7% and 69 $\pm$ 3% of control, respectively. A different picture emerged when lobules were taken from FOY-305-stimulated rats (Figure 2). In this case CsA (10⁻⁴ M) did not significantly inhibit amylase release, whereas FK 506 (10⁻⁵ M), although now less effective, still reduced release over 90 min, to 80 $\pm$ 9% of control.

The release of LDH from the lobules over the 90 min incubation period was always less than 5% of the total content, which indicates that the vast majority of the acinar cells maintained their integrity during the experiments. LDH release was not affected by treatment of the lobules with either CsA or FK 506. Hence, there was no evidence that the immunosuppressants caused any gross damage to the cells.

Figure 2 Effects of cyclosporin A (CsA, a) and FK 506 (b) on caerulein-stimulated amylase release from pancreatic lobules taken from FOY-305-treated rats. Experimental procedure and symbols are as for Figure 1. Values are means $\pm$ s.e.mean from 6 (a) and 4 (b) separate experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, when compared with control values.

Protein secretion in lobules

Secretion of proteins that had been pulse-labelled with [35 S]-methionine from lobules taken from FOY-305-treated rats was detected after a lag period of about 15 min, and 60% of the total labelled protein was released into the medium over 90 min (Figure 3). Secretion was inhibited by both CsA and FK 506; for example, secretion over 90 min was reduced to $75 \pm 3\%$ of control by CsA (10^{-4} M) and to $72 \pm 3\%$ of control by FK 506 (10^{-5} M).

Morphology of lobules

To determine whether the inhibitory effect of the immunosuppressants on exocytosis had any morphological correlate, lobules from FOY-305-treated rats were examined at the ultrastructural level. Figure 4a shows the appearance of control acinar cells after a 90 min incubation with vehicle alone. It is apparent that treatment of the rats with FOY-305 caused an extensive degranulation of the acinar cells; only a few electron-translucent secretory granules are found in the vicinity of the Golgi complex or near the acinar lumen. This degranulation was not affected by incubation of the lobules with CsA (10^{-4} M; Figure 4b). In contrast, acinar cells in

lobules that had been incubated with FK 506 (10^{-6} M) contained many zymogen granules, located primarily at the apical pole, indicating an inhibition of degranulation (Figure 4c). These granules, although of smaller size than those seen in control animals, revealed an electron-dense granule content, indicating maturation during storage (Figure 4d). Finally, neither FK 506 (Figure 5) nor CsA (not shown) had any effect on the morphology of the Golgi complex, or indeed on the structure of any other cellular organelle.

Figure 3 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of caerulein-stimulated release of pulse-labelled secretory proteins from pancreatic lobules taken from FOY-305-treated rats. Pre-incubation with drugs was for 20 min; symbols are as for Figure 1. Values are means $\pm$ s.e.mean from 6 (a) and 5 (b) separate experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, when compared with control values.

Figure 4 Effects of cyclosporin A (CsA) and FK 506 on caerulein-stimulated degranulation of acinar cells in lobules taken from FOY-305-treated rats. Lobules were pre-incubated for 20 min with vehicle (a), CsA at 10^{-4} M (b) or FK 506 at 10^{-6} M (c) or 10^{-5} M (d), and then incubated for 90 min in the presence of caerulein (1 ng ml^{-1}), before fixation and processing. RER, rough endoplasmic reticulum; L, acinar lumen; N, nucleus; ZG, zymogen granule. Bar $1 \mu\text{m}$ (a–c), 100 nm (d).

Exocytosis in intact acini

Bethanechol caused a stimulation of amylase release from intact acini of approximately 4% over a 15 min period, with half-maximal stimulation occurring at a concentration of

10^{-5} M. The percentage of the total amylase released from the acini is similar to the extent of release from lobules over the same period (see Figure 1). The difference between the two preparations is that release from acini is complete after 15 min (Edwardson *et al.*, 1990), whereas release from

Figure 5 Effect of FK 506 on ultrastructure of acinar cells in lobules taken from FOY-305-treated rats. Lobules were pre-incubated for 20 min with vehicle (a) or FK 506 at 10^{-5} M (b), and then incubated with caerulein (1 ng ml^{-1}) for 30 min. RER, rough endoplasmic reticulum; G, Golgi complex. Bar $1 \mu\text{m}$.

Figure 6 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of bethanechol-stimulated amylase release from dispersed intact pancreatic acini taken from starved rats. Acini were pre-incubated for 10 min with dimethylsulphoxide vehicle (●), or with CsA at 10^{-6} M (▲) or FK 506 at 10^{-7} M (■), before stimulation with various concentrations of bethanechol for 15 min. Values are means $\pm$ s.e. mean from 8 (a) and 10 (b) separate experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, when compared with control values.

lobules continues beyond 90 min. A 10 min pretreatment of the acini with either CsA (10^{-6} M) or FK 506 (10^{-7} M) caused a reduction in this bethanechol-dependent amylase release (Figure 6). CsA did not reduce basal amylase release, whereas FK 506 did cause a small reduction. The concentration-dependencies of the effects of the immunosuppressants on exocytosis are shown in Figure 7. Neither drug completely abolished exocytosis, even at high concentrations. Exocytosis was reduced to $43 \pm 8\%$ (CsA) and $45 \pm 5\%$ (FK 506) of control, with IC_{50} values of 50 nM (CsA) and 3 nM (FK 506).

Exocytosis was reduced to $43 \pm 8\%$ (CsA) and $45 \pm 5\%$ (FK 506) of control, with IC_{50} values of 50 nM (CsA) and 3 nM (FK 506).

Figure 7 Concentration-dependence of the inhibitory effects of cyclosporin A (CsA, a) and FK 506 (b) on bethanechol-stimulated amylase release from intact pancreatic acini taken from starved rats. Experimental procedure was as for Figure 6, except that the bethanechol concentration was kept constant at 10^{-4} M and the concentration of immunosuppressant was varied. Values are means $\pm$ s.e.mean from 10 (a) and 17 (b) separate experiments.

Exocytosis in permeabilized acini

In acini permeabilized with the bacterial toxin, streptolysin O, exocytosis can be triggered by raising the free Ca^{2+} concentration (Edwardson *et al.*, 1990). In this study, the mean Ca^{2+} -dependent exocytosis was 3.4% of total cellular amylase over a 15 min period. Pretreatment of the acini with the immunosuppressive drugs, at concentrations that were maximally effective in intact acini, 1 μM CsA and 100 nM FK 506, caused a significant reduction in the extent of exocytosis observed, to $44 \pm 6\%$ of control with CsA and $66 \pm 6\%$ of control with FK 506 (Figure 8).

Discussion

In this study, we have examined the effects of CsA and FK 506 on secretion from lobules taken either from starved

Figure 8 Inhibition by cyclosporin A (CsA, 10^{-6} M) and FK 506 (10^{-7} M) of Ca^{2+} -stimulated release of amylase from streptolysin O-permeabilized pancreatic acini taken from starved rats. Amylase release was allowed to proceed to completion (15 min). Values are means $\pm$ s.e.mean from 19 (control), 14 (CsA) and 18 (FK 506) separate experiments. *** $P < 0.001$, when compared with control values.

rats or from rats treated with the low molecular weight protease inhibitor FOY-305. Caerulein-stimulated secretion of newly-synthesized proteins from lobules taken from starved rats has previously been shown to occur at a low rate (20% of pulse-labelled protein released over 90 min (Vasiloudes *et al.*, 1991)). Hence, the amylase release occurring in these experiments (40% of total cell content over 90 min) must largely represent release of pre-formed amylase that had been stored in zymogen granules. The inhibition by CsA and FK 506 of this release, therefore, indicates a direct effect of these drugs on the final stage in the exocytotic pathway, that is, the fusion of the granules with the plasma membrane. Treatment of rats with FOY-305 results in the release of endogenous CCK, which in turn causes persistent stimulation of secretion in the exocrine pancreas (Rausch *et al.*, 1987). It has also been shown (Vasiloudes *et al.*, 1991) that pre-stimulation *in vivo* with caerulein for 24 h results in a five fold increase in the rate of secretion of pulse-labelled proteins in response to caerulein *in vitro*, but that the proteins still pass through zymogen granules en route to the plasma membrane. As expected from these previous results, it was found in the present study that about 60% of pulse-labelled protein was released over 90 min from lobules taken from FOY-305-treated rats. In these lobules, the storage of amylase in zymogen granules will be greatly reduced and the amylase released will largely be newly-synthesized. Inhibition of amylase release, therefore, could result from an effect at any point on the exocytotic pathway. It was found that CsA did not significantly inhibit amylase release from lobules taken from FOY-305-treated rats, but that FK 506, although now less effective than in lobules taken from starved rats, still had an inhibitory effect. This picture was corroborated by morphological experiments conducted in parallel, which showed that FK 506, but not CsA, inhibited the degranulation of acinar cells produced by caerulein. These results indicate that in lobules taken from FOY-305-treated rats, only FK 506, and not CsA, has an effect on the fusion of zymogen granules with the plasma membrane. Hence, the two drugs have subtly different actions in the acinar cell.

Since both drugs inhibit the release of pulse-labelled proteins, and the inhibition caused by FK 506 is greater than its effect on amylase release, it would appear that both drugs have an additional effect at a point earlier in the exocytotic pathway, although there was no indication of any ultrastructural changes in cytoplasmic organelles, such as the rough ER or the Golgi complex, occurring in their presence. At present, it is not clear why the inhibitory effect of CsA on amylase release should be lost in lobules taken from FOY-305-treated rats, or how in these lobules CsA can inhibit the release of newly synthesized proteins without affecting amylase release. These results do suggest, however, that the properties of the exocytotic event may be changed as a consequence of persistent stimulation.

It is difficult to relate the concentrations that are effective here to the doses required to affect pancreatic function *in vivo*, because of pharmacokinetic complications. It is clear, however, that effects on acinar cell function occur at concentrations that do not cause any detectable adverse effects on cell integrity or morphology. Although the concentrations of the immunosuppressants required to produce maximal inhibition of secretion in lobules have not been determined here, our experiments show that dispersed acinar cell suspensions are considerably more sensitive than lobules to the effects of the immunosuppressants. For example, the inhibitory effect of CsA on exocytosis in acini is maximal at 10^{-6} M, a concentration which in lobules from starved rats produces only about 50% of the inhibition of amylase release caused by CsA at 10^{-4} M. The reasons for this difference in potency are unclear, although access of the drug to the cells may be a problem in the lobules. Despite these complications, the potency of FK 506 in both lobules and acini is of the order of ten times that of CsA, as reported in other systems.

Recent interest has focused on the potential utility of these

immunosuppressive drugs in probing intracellular communication pathways (McKeon, 1991). For example, the inhibition by CsA and FK 506 of exocytosis in RBL-2H3 cells occurs at concentrations similar to those required to inhibit T-cell receptor-mediated interleukin-2 transcription (Hultsch *et al.*, 1991). Both drugs appear to act at a step distal to the early events at the plasma membrane, such as IgE receptor aggregation, phosphatidyl inositol bis-phosphate hydrolysis and elevation of intracellular Ca^{2+} concentration, but proximal to late events, such as exocytotic membrane fusion. The results of the present study, and in particular the close correspondence between the IC_{50} values for the drugs in the two systems, indicate a similar type of effect in pancreatic acinar cells. The inhibition of exocytosis by CsA and FK 506 is seen both in lobules stimulated by the CCK analogue caerulein and in acini stimulated by the cholinomimetic secretagogue bethanechol. The immunosuppressants also inhibit exocytosis in permeabilized acini triggered directly by Ca^{2+} . These results provide clear evidence for a site of action downstream of receptor occupation and second messenger production. We concur, therefore, with the idea that these drugs may prove to be important probes of the mechanisms of intracellular membrane transport in general and of regulated exocytosis in particular.

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