Dynamic Analysis of Secretagogue-induced Amylase Secretion from Rat Pancreatic Acini Studied by Perifusion System

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Abstract A perifusion system was applied for the study on stimulus-enzyme secretion coupling in dispersed pancreatic acini. The system is highly simple, preserves the acini up to more than 3 hr, and makes feasible clear-cut examination on the time course of enzyme secretion caused by secretagogues.

Caerulein (10^{-9} M) and carbamylcholine (10^{-5} M) caused a biphasic amylase secretory pattern consisting of an initial burst secretion and a sustained one. Caerulein induced a persistent amylase release even after cessation of the stimulation, while carbamylcholine-stimulated amylase release returned to basal levels. Atropine inhibited completely carbamylcholine-stimulated amylase release and the successive stimulation by caerulein evoked the amylase secretion with a decreased initial burst secretion. In calcium free medium, caerulein and carbamylcholine induced only a slight secretion, particularly in the sustained secretion phase and a gradual increase occurred with the addition of calcium.

Key Words: pancreas, perifusion, amylase, caerulein, carbamylcholine.

Recently, dispersed pancreatic acini have been used to investigate the effect of secretagogues on pancreatic enzyme secretion (PEIKIN *et al.*, 1978; WILLIAMS *et al.*, 1978). In studies of static incubation, however, it was difficult to examine the time course of enzyme secretion in detail. In the present study, we applied the perifusion which had originally been used for pancreatic segments by BURR *et al.* (1970), and developed for isolated islets by LACY *et al.* (1972), to dispersed rat pancreatic acini to elucidate the kinetics of enzyme secretion enhanced by secretagogues.

Using the perifusion system, we examined minutely the time course of enzyme secretion from dispersed acini caused by cholecystokinin (CCK) analogue caerulein

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and cholinergic agonist carbamylcholine. Many investigators previously demonstrated the requirement of extracellular calcium for CCK or cholinergic agents-induced enzyme secretion (ARGENT *et al.*, 1973; BENZ *et al.*, 1972; CASE and CLAUSEN, 1973; HEISLER *et al.*, 1972; HOKIN, 1966; KANNO, 1972; KANNO and NISHIMURA, 1976; ROBBERECHT and CHRISTOPHE, 1971). Some investigators proposed the hypothesis that the triggering of stimulation is independent of extracellular calcium but is dependent on calcium from intracellular stores (GARDNER *et al.*, 1979; PETERSEN and UEDA, 1976; SCHEELE and HAYMOVITS, 1979; SCHULZ *et al.*, 1981). Thus, we examined the effect of removing extracellular calcium on enzyme secretion caused by caerulein and carbamylcholine in the perfusion study.

MATERIALS AND METHODS

Perifusion of pancreatic acini. Rat pancreatic acini were prepared by a modification of the method of AMSTERDAM and JAMIESON (1972, 1974). One male Wistar King albino rat (250 to 350 g) was decapitated after an overnight fast. The pancreas was removed and trimmed of fat, lymphnodes, and mesentery. Five ml of the standard medium (composition described below) with digestive enzyme, 200 U/ml collagenase (Type IV, Millipore Corp., Freehold, N.J.) was injected into the gland using a 27 gauge needle. The standard medium contained 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, Wako Pure Chemical Industries, Ltd., Osaka), 120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 , 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 5 mM glucose, 0.2% bovine serum albumin (fraction V, Armour Pharmaceutical Co., Phoenix, Ariz.), 0.01% soybean trypsin inhibitor (Type I-S, Sigma Chemical Co., Saint Louis, Mo.), and Eagles minimal essential amino acids and vitamins (Nissui Seiyaku Co., Ltd., Tokyo), adjusted to pH 7.4 and equilibrated with 100% O_2 . After the first digestion at 37°C for 15 min, the medium was discarded and the tissue was washed with the standard medium. The pancreas was incubated at 37°C with 5 ml of digestion medium for another 40–45 min and washed 3 times with the standard medium. Pancreatic acini were liberated by sequential passage through 4 pipettes of different tip diameters (3–1 mm) in the standard medium containing 0.1% soybean trypsin inhibitor and filtered through one layer of gauze. The suspension of dispersed acini was laid over the standard medium containing 4% albumin and centrifuged for 3 min at 20 *g*. The supernatant was discarded and the acini were resuspended in the standard medium, filtered again through one layer of gauze and washed twice with the medium. Finally the acini were suspended in 10 ml of the standard medium.

Procedure for perifusion. The perifusion system was a modification of the method used for pancreatic segments by BURR *et al.* (1970) and pancreatic islets by LACY *et al.* (1972). As shown in Fig. 1, acini in a plastic chamber was perfused with a medium through tubing by a pump and perfusates were collected

Fig. 1. Diagram of the perfusion system (see text for details).

using a fraction collector. Before the experiment, the system was filled and flowed with the standard medium for 30 min. After isolation of pancreatic acini, a peristaltic pump (SJ-1215, Mitsumi Scientific Industry, Tokyo) was stopped and a connector was removed from the chamber (plastic filter holder, Swinnex, Millipore Corp., Bedford Massachusetts) with a filter ($5\ \mu\text{m}$ pore size, SMWP, Millipore Corp.). One ml of acini suspension was immediately injected with a plastic syringe into the chamber through the top and air was cleared; acini then settled on the filter. The syringe was removed and the connector was rapidly put into the chamber. The chamber was immersed and fixed in a water bath controlled at 37°C and the pump was started. The medium flowed at a rate of $0.6\ \text{ml/min}$ through a silicon tubing of $1\ \text{mm}$ diameter. The medium was changed one to another by switching the stopcock. Glass flasks were used for reservoirs and gassed with 100% O_2 throughout the experiment. Perfused medium was collected every min by the fraction collector (SF-100G, Tokyo Kagaku Sangyo Co., Ltd., Tokyo) and stored for amylase assay.

Amylase activity was measured by the method of CESKA *et al.* (1969a, b) using Phadebas Amylase Test (Pharmacia Diagnostics, Piscataway, N.J.). To determine total amylase, $1\ \text{ml}$ of acini suspension, the same volume as that injected into the chamber, was sonicated and centrifuged at $1,500\ g$ for 10 min. Amylase release of each fraction was expressed as the percentage of the total amylase. Number of acinar cells that were perfused could not be counted, therefore we measured their protein content. To determine protein content, $1\ \text{ml}$ of acini suspension was washed 3 times with $2\ \text{ml}$ of saline and resuspended in $1\ \text{ml}$ of saline. The acini suspension was sonicated and centrifuged at $1,500\ g$ for 10 min. The protein concentration of the supernatant was measured by the method of LOWRY *et al.* (1951). The protein content of acini which were injected to the chamber was about $1\ \text{mg}$.

Carbamylcholine (carbachol) and atropine were obtained from Sigma Chemical Co., and caerulein was from Kyowa Hakko Kogyo Co., Tokyo.

RESULTS

In the perfusion study, the rate of amylase release was relatively high at the beginning as a result of the pre-existing amylase extruded from the degraded acinar cells through isolation. As the rate became steady after the 90 min wash period, the acini were stimulated at 90 min from the start of the perfusion in each experiment. In this system, there was a dead space of 3 ml, which corresponded to 5 min, taking into account the flow rate.

Caerulein-stimulated amylase release

Acini were stimulated by caerulein at the concentration of 10^{-10} M, such being maximally effective in the static incubation (data not shown). The basal rate of amylase release was about 0.1% of the total amylase for 1 min. The response was slow and reached a plateau in several minutes after the stimulation

Fig. 2. Effect of caerulein on amylase release in perfusion of dispersed acini. Black bars indicate the period perfused with standard medium containing caerulein at a concentration of 10^{-10} M (A, $\circ$) and 10^{-9} M (B, $\bullet$). In experiments B, acini were also perfused with standard medium (controls, $\triangle$). Amylase release for 1 min was expressed as the percentage of total amylase. Values are means from four experiments in A, seven in B, and four in controls. Vertical bars represent S.E.

because of the dead space. When caerulein was withdrawn, the rate of amylase release decreased to near the basal level. Subsequent stimulation with 10^{-10} M caerulein produced again a slow release of amylase and after reaching a plateau there was a decline after cessation of the stimulation (Fig. 2A). When acini were stimulated by 10^{-9} M caerulein, a biphasic secretory pattern was observed. It consisted of an initial burst secretion and a sustained secretion. The latter persisted even after cessation of the stimulation and subsequent stimulation with 10^{-9} M caerulein induced no further increase of amylase release.

Fig. 3. Effect of carbamylcholine (carbachol) on amylase release in perfusion of dispersed acini. White bars indicate the period perfused with standard medium containing carbamylcholine at concentration of 10^{-5} M (A, $\circ$) and 10^{-4} M (B, $\bullet$). Values are means from four experiments. Vertical bars represent S.E.

To exclude the possibility that the sustained amylase release was the result of degradation of the acinar cells, acini were simultaneously perfused with the standard medium containing no caerulein. There was no significant increase of amylase release up to 210 min (Fig. 2B).

Fig. 4. (A) Effect of prior exposure to carbamylcholine (carbachol) on subsequent response of amylase release to caerulein in perfusion of dispersed acini. Acini were previously exposed to 10^{-5} M carbamylcholine and subsequently perfused with standard medium containing 10^{-9} M caerulein. Values are means from four experiments. Vertical bars represent S.E. (B) Effect of atropine on amylase release caused by carbamylcholine and subsequent response to caerulein in perfusion of dispersed acini. Acini were perfused with standard medium containing 10^{-5} M carbamylcholine for 15 min, then 10^{-5} M atropine was added to the medium. After 30 min, the medium was changed to standard medium containing 10^{-9} M caerulein. Hatched bar indicates the period perfused with 10^{-5} M atropine. Values are means from four experiments. Vertical bars represent S.E.

Carbamylcholine-stimulated amylase release

Carbamylcholine caused a biphasic pattern of amylase release at the concentration of 10^{-5} M, such also being maximally effective in the static incubation (data not shown). The rate of amylase release rapidly increased to the maximum in several minutes and then decreased to a steady level. Withdrawal of stimulation resulted in a return of the amylase release to the basal rate. Subsequent stimulation with 10^{-5} M carbamylcholine induced secretion but the magnitude of the second response was less in the initial burst secretion than that of the first one (Fig. 3A). When acini were stimulated with 10^{-4} M carbamylcholine, a pattern similar to that seen with a dose of 10^{-5} M soon became apparent and the residual stimulatory effect was not observed (Fig. 3B).

Effect of atropine on amylase release

The effect of a prior exposure to carbamylcholine on subsequent response to caerulein was examined. Prior stimulation with 10^{-5} M carbamylcholine produced a biphasic pattern of amylase release and sustained constant for 45 min. Successive stimulation with 10^{-9} M caerulein maintained the rate of amylase release (Fig. 4A). When 10^{-5} M atropine was added to the medium, the

Fig. 5. Effect of removing extracellular calcium on caerulein induced amylase release in perfusion of dispersed acini. Acini were perfused with calcium free medium (●) or standard medium containing 2.5 mM calcium (○). Black bar indicates the period perfused with 10^{-9} M caerulein. Dotted bar indicates the period perfused with 2.5 mM calcium in the experiment without calcium. Values are means from six experiments. Vertical bars represent S.E.

carbamylcholine-induced amylase secretion was inhibited, and acini responded again to successive stimulation with 10^{-9} M caerulein. However, the magnitude of the second response was less in the initial burst secretion (Fig. 4B).

Effect of extracellular calcium on amylase release

The removal of extracellular calcium did not alter the basal rate of amylase secretion. In a calcium free medium, however, 10^{-9} M caerulein induced only a small amount of amylase secretion, and the peak was about 1/5 that seen in the standard medium containing 2.5 mM calcium. In particular, the sustained amylase release was reduced in the calcium free medium. When the calcium concentration returned to 2.5 mM, the secretory rate increased gradually, particularly in the sustained phase (Fig. 5). Secretory response of

Fig. 6. Effect of removing extracellular calcium on carbamylcholine (carbachol)-induced amylase release in perfusion of dispersed acini. Acini were perfused with calcium free (●) or standard medium containing 2.5 mM calcium (○). White bar indicates the period perfused with 10^{-5} M carbamylcholine. Dotted bar indicates the period perfused with 2.5 mM calcium in the experiment without calcium. Values are means from six experiments. Vertical bars represent S.E.

acini to 10^{-5} M carbamylcholine in a calcium free medium was similar to that seen with caerulein, and was also increased gradually with the addition of calcium (Fig. 6).

DISCUSSION

In the present study, we modified the method of BURR *et al.* (1970) and LACY *et al.* (1972). GUDERLEY and HEISLER (1980) used a cell column suspension system in which dispersed rat pancreatic acinar cells were mixed with Biogel P-4 beads. FRANDSEN (1980) also described a perfusion system using a cell column in which isolated rat pancreatic acini were suspended with Biogel P-2. The Biogel served the dual purpose of retaining and separating the acini, thus preventing clogging during perfusion. The perfusion system we used did not require such cell columns and made feasible clear-cut examination of kinetics of minute enzyme release caused by secretagogues, *in vitro*. In control studies of perfusion, there was no significant increase of amylase release that would imply a degradation of acinar cells up to 210 min. Cell viability was also confirmed as the acini responded well to the secretagogues and the enzyme secretion returned to the basal rate after cessation of stimulation (except for the case of 10^{-9} M caerulein).

In the perfusion of acini, carbamylcholine caused a biphasic secretory pattern, that consisted of an initial burst secretion and a sustained secretion at maximal and supramaximal concentrations which had been determined in static incubation. Though the supramaximal concentration of caerulein also caused a biphasic secretory pattern, a monophasic pattern was observed with the maximal concentration. There is the possibility that the concentration of 10^{-10} M of caerulein is actually submaximal. The biphasic secretory pattern of the pancreatic enzyme was also reported by other workers (FRANDSEN, 1980; GARDNER *et al.*, 1979; GUDERLEY and HEISLER, 1980; KANNO and NISHIMURA, 1976; MATTHEWS *et al.*, 1973; PETERSEN and UEDA, 1976; SCHEELE and HAYMOVITS, 1979; SCHREURS *et al.*, 1976; SCHULZ *et al.*, 1981).

In the present series, caerulein had different effects on amylase release, as compared with carbamylcholine. A ten times higher supramaximal concentration of caerulein induced a persistent amylase secretion even after cessation of stimulation. It seems unlikely that this effect was due to a relatively high concentration of caerulein, because residual stimulation was not observed with 10^{-4} M carbamylcholine, a dose which was equally ten times higher than its maximal concentration. A similar result was found with the octapeptide of CCK (CCK-OP) in a static incubation of dispersed pancreatic acini from guinea pig (COLLINS *et al.*, 1981). In their studies, CCK-OP induced a persistent amylase release at relatively high concentrations, whereas carbamylcholine produced no residual stimulation.

Desensitization is a phenomenon involving prior exposure to an agonist which reduces the subsequent response to the same agonist. This phenomenon was found in dispersed pancreatic acini from guinea pig by ABDELMOUMENE and GARDNER (1980). We also found that the second secretory response of acini to repeated stimulation with caerulein or carbamylcholine was less than the first. Interestingly, the initial burst secretion was reduced. A similar explanation may apply to the reduced response seen with caerulein after exposure to carbamylcholine, as these agents stimulate enzyme secretion through a common mechanism involving the same intracellular calcium pool. The caerulein-stimulated initial burst secretion was still reduced even when prior carbamylcholine-induced amylase release was inhibited by atropine, an antagonist of carbamylcholine.

The effect of omission of extracellular calcium on pancreatic enzyme secretion stimulated by CCK or cholinergic agents has been studied by many investigators. In a calcium free medium containing EGTA or EDTA, CCK or cholinergic agonist-stimulated enzyme secretion was abolished in the isolated pancreas (ARGENT *et al.*, 1973; KANNO, 1972), pancreatic slices (HOKIN, 1966) and fragments (BENZ *et al.*, 1972; CASE and CLAUSEN, 1973), or decreased in pancreatic fragments (HEISLER *et al.*, 1972; ROBBERECHT and CHRISTOPHE, 1971). Some investigators found that the initial secretion evoked by cholinergic agents of CCK was not affected yet the subsequent secretion was decreased or absent in the calcium free solution containing EGTA, in cases of the isolated pancreas (SCHREURS *et al.*, 1976), pancreatic segments (PETERSEN and UEDA, 1976), lobules (SCHEELE and HAYMOVITS, 1979), dispersed acinar cells (SCHULZ *et al.*, 1981), and acini (GARDNER *et al.*, 1979). In the present study, the chelating agent was not added to the calcium free medium, but caerulein and carbamylcholine induced only a slight release of amylase and the sustained amylase release in particular was reduced in the calcium free medium. It is known that CCK and cholinergic agents increase calcium efflux in the absence of extracellular calcium (CASE and CLAUSEN, 1973; CHRISTOPHE *et al.*, 1976; SCHREURS *et al.*, 1976; WILLIAMS and CHANDLER, 1975) and this calcium is thought to be released from intracellular stores (GARDNER, 1979; WILLIAMS, 1980). Thus, the stimulated enzyme secretion is probably triggered by the efflux of intracellular calcium followed by calcium re-uptake and the sustained secretion requires an influx of extracellular calcium into the cells occurred probably by an increased plasma membrane permeability to calcium (KONDO and SCHULZ, 1976; SCHULZ and STOLZE, 1980). Namely, the initial burst secretion observed in our perfusion study corresponds to calcium efflux due to calcium release from an intracellular calcium trigger pool and the sustained enzyme secretion corresponds to an increased calcium influx into the cells. In this study, the basal rate of amylase release was not influenced by removal of the extracellular calcium in agreement with findings of other workers (BENZ *et al.*, 1972; GARDNER *et al.*, 1979; SCHREURS *et al.*, 1976; SCHULZ *et al.*, 1981; WILLIAMS *et al.*, 1976). Some investigators did find a reduction in basal enzyme secretion in the absence of extracellular calcium (ARGENT

et al., 1973; CASE and CLAUSEN, 1973; HEISLER *et al.*, 1972; ROBBERECHT and CHRISTOPHE, 1971; WILLIAMS and CHANDLER, 1975).

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