Participation of Ca^{2+} and Calmodulin in Rat Pancreatic Enzyme Secretion Induced by Secretin, Forskolin, and Dibutyryl Cyclic AMP

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Abstract The role of Ca^{2+} and calmodulin in stimulation of the rat pancreatic acini induced by secretin, forskolin, and dibutyryl cyclic AMP (dbcAMP) was studied using W-7, a calmodulin antagonist, and a low Ca^{2+} medium. The time course of amylase secretion was studied in a perfusion system using dispersed rat pancreatic acini. The amylase release patterns of each secretagogue were as follows: a biphasic amylase release pattern under the stimulation of secretin, a one peak pattern during the stimulation of forskolin and a rapid response after cessation of the stimulation, and a gradual increased pattern during the stimulation of dbcAMP followed by a rapid response. The amylase release under the stimulation by forskolin and dbcAMP was slightly weaker as compared with that of secretin stimulation. The amylase secretion stimulated by secretin (5×10^{-7} M), forskolin ($50 \mu\text{M}$), and dbcAMP (2 mM) was inhibited by W-7 ($50 \mu\text{M}$). In a low Ca^{2+} medium ($4.7\text{--}5.1 \times 10^{-6}$ M), the secretory rate did not increase during the stimulation by secretin, forskolin, and dbcAMP, and a rapid amylase response remained after cessation of the stimulation of forskolin and dbcAMP. The pretreatment with EDTA (1 mM) suppressed both the gradual amylase release and the rapid response induced by dbcAMP in a low Ca^{2+} medium. These results suggested that each secretagogue, via cyclic AMP (cAMP), induced a different amylase secretory pattern dependent on an intracellular Ca^{2+} content, and was mediated by the Ca^{2+} -calmodulin complex.

Key words: Ca^{2+} -calmodulin, secretin, forskolin, dbcAMP, perfusion.

The presence of two pathways for the regulation of enzyme release from pancreatic acinar cell are known: One can be stimulated by octapeptide of cholecystokinin (CCK) or cholinergic agonists and this response is mediated by in-

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tracellular levels of Ca^{2+} (GARDNER, 1979; KANNO and HABARA, 1980; WILLIAMS and HOOTMAN, 1986). The other pathway can be stimulated by secretin or VIP and this response is mediated by cyclic AMP (cAMP). Therefore, both the increase of intracellular free Ca^{2+} concentration (GARDNER *et al.*, 1975; CASE, 1978; WILLIAMS, 1980) and cAMP content (KEMPEN *et al.*, 1977) are related to enzyme secretory processes. Calmodulin (CaM) reversibly binds calcium, and Ca^{2+} -CaM has been shown to regulate a wide variety of enzyme systems including phosphodiesterase, phosphorylase kinase, adenylate cyclase, protein kinase, and others (KAKIUCHI and YAMAZAKI, 1970; CHEUNG, 1980; MEANS and DEDMAN, 1980), and involved the enzyme secretory processes.

In this study we evaluated the role of the intracellular Ca^{2+} -CaM complex on the enzyme release process by assessing the effect of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (HIDAKA *et al.*, 1978, 1981), a specific antagonist of CaM and extracellular Ca^{2+} concentration on enzyme secretion via cAMP system, using a perfusion system (IMAMURA *et al.*, 1983) of dispersed rat pancreatic acini.

MATERIALS AND METHODS

Pancreatic acini from Wistar King albino rats (250–300 g body weight) were isolated by collagenase digestion and mechanical dispersion according to the method of AMSTERDAM and JAMIESON (1972, 1974) with some modifications and without the addition of a chelator such as EDTA. The pancreas was injected with the standard medium with digestive enzyme, 200 U/ml collagenase (Worthington, Type IV). The standard medium contained 20 mM HEPES, 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 (BSA), 0.01% soybean trypsin inhibitor, and Eagles minimum essential amino acids and vitamins (Nissui Seiyaku Co., Ltd., Tokyo) at pH 7.4 equilibrated with 100% O_2 . The pancreas was incubated for 15 min at 37°C in 5 ml of the standard medium with 100% O_2 . After the first digestion, the pancreas was washed with the standard medium and sequently incubated for 45 min with the standard medium with 200 U/ml collagenase. After the second digestion, the pancreas was dissociated by passage through 4 pipettes of decreasing orifice. Acini were prepared by filtration through one layer of gauze and centrifugation through the standard medium containing 4% BSA. The acini pellet was suspended in 10 ml of the standard medium. The perfusion system (IMAMURA *et al.*, 1983) consisted of a peristaltic mini pump, a perfusion chamber and a fraction collector. Pancreatic acini were injected into the perfusion chamber equipped with a filter (5.0 μm pore size, SNWP, Millipore Corp.). The secretagogues, dissolved in the perfusion buffer, were administered after an initial washout and equilibration in the preperfusion for 90 min. The W-7 was added to the perfusates from the beginning of the perfusion study. The medium was flowed at a rate of 0.6 ml/min through a silicon tubing of 1 mm diameter and was gassed continuously with 100% O_2 . The medium was changed to another by switching the stopcock. The basal stimulation levels were set

at the standard medium containing 2.5 mM Ca²⁺. The perfusion chamber was immersed in a water bath warmed to 37°C. Perfusion fraction samples were collected every min by a fraction collector and amylase activity was assayed by the method of ČESKA *et al.* (1969a, b), using the Phadebas reagent. In each experiment, a sample of the acini suspension was taken at the start of the perfusion, centrifuged and assayed to estimate the total amylase activity. Amylase release of each fraction was calculated as the percentage of the total amylase activity in the acini suspension. To evaluate the effect of extracellular calcium, the dispersed acini were washed 3 times with a standard medium without CaCl₂ (low Ca²⁺ medium). The Ca²⁺ concentration of acini suspension treated with a low Ca²⁺ medium, measured by using a calcium selective electrode (Corning Co., N. Y., U.S.A.) was $4.7\text{--}5.1 \times 10^{-6}$ M. Some of the dispersed acini were incubated for 10 min with a low Ca²⁺ medium containing 1 mM EDTA and then washed twice with low Ca²⁺ medium. Subsequently the above perfusion study was carried out using a low Ca²⁺ medium. The acini were exposed to the respective concentration of a secretagogue which was maximally effective in the static incubation of acini (data not shown). Drugs tested were W-7 and N-(6-aminoethyl)-1-naphthalenesulfonamide (W-5, Rikaken Co., Nagoya, Japan); carbamylcholine (Cch) and 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical Co., St. Louis, Mo); secretin (Protein Research Foundation, Minoh, Japan); forskolin (Calbiochem., La-Jolla, CA); dibutyryl cyclic AMP (dbcAMP, Boehringer Mannheim GmbH., West Germany); and collagenase (Type IV, Worthington, Freehold, N.J.).

Statistical significance was tested using Student's *t*-test.

RESULTS

A) Effect of W-5 and W-7 on Cch-stimulated amylase release

Stimulation under 10^{-5} M Cch after preperfusion during 90 min caused a biphasic amylase release pattern with $0.46 \pm 0.03\%$ (mean $\pm$ S.E.) at peak in the initial burst (first) phase and $0.35 \pm 0.02\%$ at 20 min (from the beginning of the stimulation) in the sustained (second) phase (Fig. 1). In the presence of W-7 at concentrations of 30, 50, or 100 μ M, the amylase release stimulated by 10^{-5} M Cch was inhibited in the dose-dependent manner, in both the first and second phase. To exclude the non-specific effect of W-7, the perfusion study was carried out using W-5, of which the structure was similar to W-7 and the inhibitory effect was about 13% compared to that of W-7 (HIDAKA *et al.*, 1981). The amylase secretion stimulated by Cch after the removal of W-7 restored normal secretion (data was not shown). Therefore, in the present study, W-7 was used at the concentration of 50 μ M to exclude the toxic effect of W-7. It seemed that the first phase was strongly inhibited compared with the second phase in the concentration of 50 μ M W-7. As shown in Fig. 2, W-7 proved to be a potent inhibitor of amylase release stimulated by Cch. Fifty μ M W-7 suppressed 70.5% of amylase release in the first phase and 48.5% in the second phase.

Fig. 1. Effect of 10^{-5} M carbamylcholine on amylase release from perfused rat acini in the presence of various concentrations of W-7. W-7 was added at the beginning of the perfusion. Values are mean $\pm$ S.E. (control: $n=12$, $30 \mu\text{M}$: $n=4$, $50 \mu\text{M}$: $n=3$, $100 \mu\text{M}$: $n=2$).

B) Effect of IBMX on secretin-induced amylase release

Amylase release induced by 5×10^{-7} M secretin showed a biphasic secretion pattern with an increase in amylase release of $0.37 \pm 0.02\%$ at peak in the initial burst phase and $0.18 \pm 0.01\%$ at 20 min (from the beginning of the stimulation) in the sustained phase. The combination of 5×10^{-7} M secretin and 1 mM IBMX caused a 2–3 fold increase in the amylase release (Fig. 3).

Fig. 2. Effect of W-7 (●) and W-5 (○) at various concentrations on inhibition of amylase release. The percent of inhibition induced by 10^{-5} Cch in the standard medium without W-7 and W-5 was expressed as 0% at the peak value of the first phase and at 20 min value from the beginning of the stimulation in the second phase.

C) *Effect of extracellular Ca²⁺ and W-7 on secretin-induced amylase release*

In $50 \mu\text{M}$ W-7 containing medium, the secretin-induced amylase release was inhibited. However, the inhibition of the second phase was smaller than that of the first phase (Fig. 4A). The removal of extracellular Ca²⁺ caused a little alteration of the basal amylase secretion, secretin-induced amylase release was suppressed, in both the first and second phase (Fig. 4B).

D) *Effect of extracellular Ca²⁺ and W-7 on forskolin-induced amylase release*

In standard medium, $50 \mu\text{M}$ forskolin induced one peak of amylase release during the stimulation, following a rapid amylase response after cessation of the stimulation. All the secretory responses were significantly inhibited with the addition of $50 \mu\text{M}$ W-7 (Fig. 5A). In a low Ca²⁺ medium, the amylase release during the stimulation by forskolin did not increase significantly, but the response after cessation of the stimulation showed the same pattern as that in Ca²⁺ present (Fig. 5B).

E) *Effect of extracellular Ca²⁺ and W-7 on dbcAMP-induced amylase release*

DbcAMP-induced amylase release showed the gradual increased pattern different from secretin-induced amylase release. After cessation of the stimulation, a

Fig. 3. Potentiation of secretory response to 5×10^{-7} M secretin by 1 mM IBMX from perfused pancreatic rat acini. Values are mean $\pm$ S.E. ($n=4$, $*p<0.05$).

rapid amylase response was observed. The addition of $50 \mu\text{M}$ W-7 significantly inhibited the amylase release during and after the stimulation of dbcAMP (Fig. 6A). In a low Ca^{2+} medium, dbcAMP did not increase the secretory rate during the stimulation, but a rapid amylase response was observed after cessation of the stimulation. In a perfusion study of acinar preparations pretreated with 1 mM EDTA, a rapid amylase response after cessation of stimulation in a low Ca^{2+} medium was not observed (Fig. 6B).

DISCUSSION

The perfusion system used in this study facilitates an examination of the kinetics of a minute amount of enzyme releases caused by secretagogues, *in vitro*. We obtained an interesting response pattern that was difficult to achieve in the static

Fig. 4. (A) Effect of 5×10^{-7} M secretin on amylase release from perfused pancreatic rat acini in the presence of $50 \mu\text{M}$ W-7, added at the beginning of the perfusion. Values are mean $\pm$ S.E. ($n=4$, $*p<0.05$). (B) Effect of removing extracellular calcium on 5×10^{-7} M secretin induced amylase release from perfused pancreatic rat acini. Acini were perfused with a low calcium ($\circ$) or standard medium containing 2.5 mM calcium ($\bullet$) ($n=4$).

Fig. 5. (A) Effect of 50 μM forskolin on amylase release from perfused pancreatic rat acini in the presence of 50 μM W-7, added at the beginning of the perfusion. Values are mean $\pm$ S.E. ($n=4$, $*p<0.05$). (B) Effect of removing extracellular calcium on 50 μM forskolin-induced amylase release from perfused with a low calcium ($\circ$) or standard medium containing 2.5 mM calcium ($\bullet$).

Fig. 6. (A) Effect of 2 mM dbcAMP on amylase release from perfused pancreatic rat acini in the presence of 50 μ M W-7, added at the beginning of the perfusion. Values are mean $\pm$ S.E. ($n=4$, $* p < 0.01$). (B) Effect of removing extracellular calcium on dbcAMP-induced amylase release from perfused pancreatic rat acini. Acini were perfused with a low calcium medium ($\circ$), standard medium containing 2.5 mM calcium ($\bullet$), or EDTA treated calcium-free medium ($\blacktriangle$).

study (IMAMURA *et al.*, 1983). Various secretagogues, which cause Ca^{2+} release from intracellular stores (GARDNER *et al.*, 1979; WILLIAMS, 1980) and subsequent uptake of Ca^{2+} from the extracellular fluid, can evoke pancreatic enzyme secretion by a rise of intracellular Ca^{2+} concentration. But the distal step regulating enzyme secretion is not defined. Some investigators (BURNHAM and WILLIAMS, 1982; FREEDMAN and JAMIESON, 1982) reported that CCK and cholinergic agonists altered the phosphorylation of specific endogenous pancreatic proteins. Furthermore, Ca^{2+} binds with regulatory protein such as CaM and regulates a multitude of intracellular enzyme systems which influence secretory processes (KAKIUCHI and YAMAZAKI, 1970; CHEUNG, 1980; MEANS and DEDMAN, 1980).

CaM antagonists such as neuroleptic drugs (LEVIN and WEISS, 1977; WONG and CHEUNG, 1979; HEISLER *et al.*, 1980) may not be suitable for use in cell biology because they are highly toxic to intact cells and are more soluble in the hydrophilic than in the aqueous phase (SEEMAN *et al.*, 1974). In the present study we used W-7 (HIDAKA *et al.*, 1978) which entered cells more readily and bound to CaM with greater affinity than neuroleptic drugs. And also in this study, the normal secretory pattern was restored by the removal of W-7 (data not shown). Therefore it was indicated that there was little irreversible damage to the acini in the concentration of $50\text{ }\mu\text{M}$ W-7. The results that every secretory phase under the stimulation by secretin, forskolin, and dbcAMP was inhibited strongly by a CaM antagonist (W-7), suggested the involvement of the Ca^{2+} -CaM complex in the cAMP regulated pathways. These results coincide with the finding that the Ca^{2+} -CaM complex phosphorylates the CaM-dependent protein in a low Ca^{2+} concentration in islet cells (MACDONALD and KOWLURU, 1982).

Secretin stimulates enzyme secretion by the mechanism of the cAMP-regulated pathways in the exocrine pancreas in rat and guinea-pig (GARDNER and JACKSON, 1977; PEARSON *et al.*, 1981), but the effects of Ca^{2+} -CaM complex on the cAMP-regulated pathways have not yet been clarified (BURNHAM and WILLIAMS, 1984). WILLIAMS *et al.* (1978) reported that the cAMP-mediated agents potentiate the action of secretagogues which act through Ca^{2+} . We examined the effects of various secretagogues on the cAMP-regulated pathways; secretin acting via the receptor (JENSEN *et al.*, 1983; OKUMURA *et al.*, 1983), forskolin (SEAMON *et al.*, 1981a, b), IBMX inhibiting phosphodiesterase, and dbcAMP. Amylase release showed a biphasic pattern under stimulation with secretin (Fig. 3), as seen in the case of stimulation with Cch (Fig. 1). However, amylase release under stimulation with secretin was slightly weak as compared with Cch. The amylase release under the stimulation with forskolin (HEISLER, 1983; DEHAYE *et al.*, 1985) and dbcAMP (HEISLER and FORTIN, 1983) was poor in the static study, but a slightly significant response was found in the this study. IBMX showed a potentiated effect on the secretin-induced amylase release (KORMAN *et al.*, 1980), and so it was confirmed that the increase of intracellular cAMP content evoked the amylase release. And the forskolin- and dbcAMP-induced amylase response were slower as compared with that of secretin, and after cessation of each secretagogue a rapid amylase response

was observed.

To study the Ca²⁺ dependence on cAMP mediated amylase release, we examined the amylase release under stimulation by secretagogues regulated by cAMP in a low Ca²⁺ medium. In a low Ca²⁺ medium, the amylase releases during the stimulation by secretin, forskolin, and dbcAMP were inhibited, and the pretreatment with EDTA suppressed the rapid response after cessation of dbcAMP-stimulation. And it has been reported that, in pancreatic islet cell, forskolin increased the intracellular cAMP content in a low Ca²⁺ medium (Niki *et al.*, 1983). Furthermore, it has been reported that secretin and VIP neither evoked changes in ⁴⁵Ca outflux nor caused membrane depolarization (GARDNER and JACKSON, 1977; PEARSON *et al.*, 1981). On the other hand, regarding the regulation of intracellular Ca²⁺ mobilization by cAMP, it has been known that the increase of intracellular cAMP content caused the decrease of cytosol Ca²⁺ concentration (OWEN *et al.*, 1981) and Ca²⁺-uptake into isolated membrane vesicles was stimulated in the presence of cAMP in human platelets (KASER-GLANZMANN *et al.*, 1979). From this point of view, our data might suggest as follows: the stimulation of the endogenous and exogenous cAMP induced Ca²⁺ uptake into the intracellular Ca²⁺ pool following the decrease of cytosol Ca²⁺ concentration, so that forskolin or dbcAMP did not evoke amylase release during the stimulation, but after cessation of these stimulations the cytosol Ca²⁺ concentration increased and caused the rapid amylase response in a low Ca²⁺ medium. And when acini was pretreated with EDTA, both Ca²⁺ concentration of the cytosol and intracellular Ca²⁺ pool were lower than that of low Ca²⁺ medium. Consequently, it was thought that the rapid amylase response was suppressed after cessation of dbcAMP-stimulation.

In this study we can clarify that the amylase releases by the cAMP-mediated secretagogues are strongly influenced by Ca²⁺-CaM complex, and postulate that the first phase of the secretin-induced amylase release is interacted strongly by Ca²⁺-CaM complex, the second phase of secretin-induced amylase release and forskolin- and dbcAMP-induced amylase release are interacted by both of the cAMP passage and intracellular Ca²⁺ pool, and the rapid amylase response after cessation of forskolin- and dbcAMP-stimulation is influenced by cytosol Ca²⁺ concentration derived from intracellular Ca²⁺ pool.

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