THE ROLE OF EXTRACELLULAR CALCIUM IN THE SECRETORY RESPONSE OF THE EXOCRINE PANCREAS TO SECRETIN AND FORSKOLIN

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ABSTRACT

The influence of extracellular Ca^{2+} on secretin-induced secretory responses (enzyme output and juice flow) was examined in the isolated perfused rat pancreas. Secretin in the range of 100 pM to 10 nM produced dose-dependent secretory responses. The secretin-induced enzyme output was markedly inhibited when CaCl_2 was removed from the perfusing solution, whereas the juice flow by secretin at higher concentrations (1.0 and 10 nM) was not significantly inhibited. Not only the enzyme output but also the juice flow induced by 1 μM forskolin were inhibited in the Ca^{2+} -deficient environment. Continuous stimulation with 100 μM dibutyryl cAMP caused a gradual increase in the pancreatic secretory responses. The enzyme output was abolished but the juice flow was slightly inhibited in the Ca^{2+} -deficient environment. Continuous stimulation with 1 nM secretin induced a gradual increase in the amount of intracellular cyclic AMP, which was significantly decreased in the Ca^{2+} -deficient environment. The present results show that pancreatic enzyme output induced by secretin depends on the extracellular Ca^{2+} concentration. Two possible mechanisms involved in the Ca^{2+} -dependent enzyme output were discussed in relation to the two kinds of intracellular second messengers, Ca^{2+} and cyclic AMP.

The following views have been admitted to explain exocrine secretion of the pancreas, although the experimental basis for these has not been fully established (3). 1) The exocrine pancreas consists of two functional units: acinar cells, which secrete digestive enzymes and zymogens, and entero-acinar and terminal duct cells, which are responsible for electrolyte secretion; 2) secretin acts only on terminal duct cells and centro-acinar cells to stimulate an isotonic secretion rich in bicarbonate; and 3) cholecystokinin-pancreozymin (CCK) and acetylcholine act on acinar cells to cause the extrusion of the contents of zymogen granules by exocytosis. In the rabbit pancreas, Schulz, Yamagata and Weske (19) concluded that the entire ductular system is involved in electro-

lyte secretion induced by secretin, and the conclusion was supported principally by Swanson and Solomon (24) with the distinction that the latter considered the largest extralobular ducts to lack secretory capacity. These views have also been supported by experimental results from the cat pancreas (5, 3). In the rat pancreas, however, it has been shown that CCK and caerulein strongly stimulate not only the rate of enzyme output but also the rate of flow of pancreatic juice (9, 10, 12, 21). In isolated acinar cell preparation of the rat, De Pont *et al.* (7) have shown that CCK stimulates pancreatic enzyme output not only by increasing the cytosolic concentration of Ca^{2+} , $[\text{Ca}^{2+}]_c$, but also by activating adenylate cyclase to increase cytosolic cyclic AMP

concentration. They have also shown that secretin activates adenylate cyclase and induces pancreatic enzyme output. To elucidate the possible influence of extracellular Ca^{2+} , the isolated perfused preparation of rat pancreas may provide a promising clue: the perfusion technique has been used successfully in simultaneous measurements of the pancreatic enzyme release and juice flow in the environment containing various Ca^{2+} concentrations (13, 14). The results of the present study show that secretin, forskolin and dibutyl adenosine 3',5'-cyclic monophosphate (dbcAMP) produce a marked increase in both pancreatic protein output and fluid secretion, and that the protein output response is abolished but the fluid secretion response is only partially inhibited in the Ca^{2+} -deficient environment.

MATERIALS AND METHODS

Male Sprague-Dawley strain rats (about 200 g) were fasted but allowed water for 24 h before the start of each experiment. The isolated perfused pancreas was prepared as reported previously (14). Briefly, under ether anesthesia, the vascular system was cannulated and perfused through the superior mesenteric and coeliac arteries, with the portal vein as outlet. The rate of vascular perfusion was kept constant at 2 ml/min by a roller pump. The hepatic end of the duct was ligated and the pancreatic juice was collected from the duodenal end following cannulation with a stainless steel tube.

The composition of the standard Krebs-Henseleit solution used for perfusing and bathing the preparation was as follows (mM): NaCl, 131; KCl, 5.6; CaCl_2 , 2.5; MgCl_2 , 1; NaHCO_3 , 25; HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; Sigma, St. Louis), 1; glucose 5. In the Ca^{2+} -deficient solution, CaCl_2 was omitted. Dextran T-70 (Meito, Nagoya) was added to the perfusing solution at a final concentration of 5% (w/v). The solution was equilibrated with 5% CO_2 in O_2 and had a pH of 7.4. Pure natural porcine secretin (3,500 U/mg, by the courtesy of Professor V. Mutt, Karolinska Institute, Stockholm), forskolin (Calbiochem, La Jolla) and dbcAMP (Sigma, St. Louis) were added to the perfusing solution. dbcAMP was used with IBMX (isobutyl-methyl-xanthine; Calbiochem, La Jolla) to assure the effectiveness of dbcAMP.

Pancreatic protein output and juice flow were estimated as reported previously (13). Content of cyclic AMP was estimated as reported by Brown *et al.* (2) with the aid of an assay kit for cyclic AMP (Amersham, England). Results are expressed as the mean $\pm$ SE of several experiments, and are analyzed by Student's *t*-test.

RESULTS

Influence of Ca^{2+} -Removal on Secretory Responses Induced by Secretin

The isolated pancreas was perfused first with standard solution for 15 min to reach steady levels, after which the resting levels of secretory responses (pancreatic juice flow and protein output) were estimated. The pancreas was then stimulated continuously by perfusing it with secretin. Continuous stimulation with secretin at 0.1 or 0.2 nM induced a gradual increase in juice flow and a transient rise in protein output (Fig. 1). The secretin-induced juice flow increased gradually to reach a plateau level of several times greater than the corresponding resting level 20 min after starting the continuous stimulation, maintained the plateau level during the continuous stimulation and then gradually decreased toward the resting level after termination of the stimulation. The secretin-induced protein output, however, increased transiently to about three times greater than the resting level 10 min after starting the continuous stimulation followed by a gradual decrease to the resting level during the stimulation.

The protein output and the juice flow in response to the continuous stimulation with 1 or 10 nM secretin gradually reached the plateau levels, which were maintained as long as the stimulation was continued (Fig. 1).

When Ca^{2+} was removed from the perfusing and bathing solution, the protein output induced by continuous stimulation with 0.1, 0.2, 1 or 10 nM secretin was strongly inhibited (Fig. 2). The juice flow induced by the stimulation with 0.1, 0.2, 1 or 10 nM secretin, however, was only partly inhibited in the Ca^{2+} -deficient environment. The levels of the plateau phase of juice flow induced by stimulation with 0.1, 0.2, 1, and 10 nM secretin in the Ca^{2+} -deficient environment were about 54, 54, 81, and 76% of the corresponding levels recorded in the Ca^{2+} -sufficient environment.

To examine further the influence of extracellular Ca^{2+} , secretin-induced protein output

Fig. 1 Time course of changes in pancreatic protein output and juice flow during continuous stimulation of the isolated rat pancreas with 0.1, 0.2, 1 and 10 nM secretin in a standard solution containing 2.5 mM Ca^{2+} . Each value represents the mean of four experiments. The open horizontal bar indicates the period of secretin stimulation.

Fig. 2 Time course of changes in pancreatic protein output and juice flow during continuous stimulation of the isolated rat pancreas with 0.1, 0.2, 1, and 10 nM secretin in a Ca^{2+} -deficient environment (CaCl_2 was removed from the perfusing and the bathing solution). Each value represents the mean of four experiments. The open horizontal bar indicates the period of secretin stimulation.

and juice flow in the Ca^{2+} -containing or Ca^{2+} -deficient environment were plotted in Fig. 3. Larger protein output was obtained when a higher secretin concentration was used in the environment containing 2.5 mM Ca^{2+} , whereas the output remained very small even when a higher secretin concentration was used in the Ca^{2+} -deficient environment. On the contrary, a dose-dependent increase in juice flow was obtained whether the extracellular solution contained Ca^{2+} or not. The response to a lower secretin concentration in the Ca^{2+} -deficient environment was significantly smaller than that in the Ca^{2+} -containing environment, but the difference between the responses in the Ca^{2+} -deficient and Ca^{2+} -containing environments to higher secretin

concentration was not significant.

Ca^{2+} -dependent Secretory Responses to Forskolin

It is commonly believed that the secretin-induced secretory responses are exerted by increasing the intracellular concentration of cyclic AMP (3), which is also known to mediate the responses to forskolin (20). In the following experiments, we used 1 μM forskolin, which was shown to induce pancreatic secretory responses comparable to those to 1 nM secretin.

Continuous stimulation with 1 μM forskolin induced sustaining increases in juice flow and protein output in the environment

Fig. 3 Pancreatic protein output and juice flow in response to doses of secretin in a Ca^{2+} -containing and a Ca^{2+} -deficient environment. The protein output and juice flow are the total amount in a sample collected during 40 min of stimulation with secretin. Each value shown by the filled circles (●) indicates the respective experiments carried out in a Ca^{2+} -containing environment as in Fig. 1, and that shown by open circles (○) indicates the mean value of corresponding experiments carried out in a Ca^{2+} -deficient environment as in Fig. 2. Asterisks (*) underneath the open circles represent significant ($P < 0.05$) difference between the values immediately above.

containing 2.5 mM Ca^{2+} (Fig. 4). In the Ca^{2+} -deficient environment, the stimulation induced only a small and transient increase in protein output, and a diminished but sustaining increase in juice flow (Fig. 4). The maximum plateau level of forskolin-induced juice flow in the Ca^{2+} -deficient environment was about 1/4 of that in the Ca^{2+} -containing environment.

Ca^{2+} -dependent Secretory Responses to dbcAMP

Since cyclic AMP is a possible intracellular messenger of secretin- and forskolin-induced secretory responses, the influence of extracellular Ca^{2+} on the secretory responses to dbcAMP, which is known to penetrate into a cell (6), was examined. dbcAMP ($100 \mu\text{M}$) and $10 \mu\text{M}$ IBMX were added to the perfusing solution. IBMX was used to prevent possible deterioration of dbcAMP. In a preliminary test, IBMX was found to be ineffective on the secretory responses.

Continuous stimulation with $100 \mu\text{M}$ dbcAMP induced a very slow increase in protein output and juice flow (Fig. 5). In the Ca^{2+} -deficient environment, the dbcAMP-induced protein output was significantly inhibited ($P < 0.01$), whereas the difference between dbcAMP-induced juice flow in the Ca^{2+} -deficient and the Ca^{2+} -containing environments was not significant.

Change in Cyclic AMP Content Induced by Secretin and Forskolin

As shown in Figs. 3 and 4, both secretin- and forskolin-induced protein output were strongly inhibited in the Ca^{2+} -deficient environment. The following experiments were carried out to examine the influence of extracellular Ca^{2+} on the secretagogue-induced change in cyclic AMP content of the pancreas.

Continuous stimulation with 1 nM secretin gradually increased cyclic AMP content of the pancreas in the Ca^{2+} -containing environment. In the Ca^{2+} -deficient environment, the stimulation failed to increase cyclic AMP content (Fig. 6).

Continuous stimulation with $1 \mu\text{M}$ forskolin significantly increased cyclic AMP content of the pancreas 40 min after starting the stimulation in the Ca^{2+} -containing environment (Fig. 6). In the Ca^{2+} -deficient environment, the stimulation failed to increase cyclic AMP

Fig. 4 Time course of changes in pancreatic protein output and juice flow during continuous stimulation of the isolated rat pancreas with $1 \mu\text{M}$ forskolin in a Ca^{2+} -containing and Ca^{2+} -deficient environment. Each value represents the mean of four experiments. The horizontal open bar indicates the period of forskolin stimulation, and the shaded horizontal bar indicates the period of Ca^{2+} -removal from the perfusing and bathing solution.

content.

DISCUSSION

Secretin-induced Secretory Responses

It is commonly believed that secretin acts on the ductal tissue (terminal duct cells and centro-acinar cells) of the pancreas to induce juice flow and NaHCO_3 secretion (3, 21), and that binding of secretin with secretin receptor activates adenylate cyclase, which in turn increases intracellular cyclic AMP.

The range of plasma secretin level before and after eating has not yet been estimated in the rat, but when estimated by radioimmunoassay, the plasma secretin level was found to be about 20 pM when diluted HCl was infused into the duodenal lumen in the anesthetized dog (16). The secretin concentrations used in the present experiments ranged from 100 pM to 10 nM, which may be in the range of the physiological and pharmacological concentration.

The present experiments demonstrated that in the isolated perfused rat pancreas, continuous stimulation with secretin (over the concentration range 0.1–10 nM) induced a dose-dependent increase in juice flow as well as in protein output, and that the secretin-induced protein output was almost nullified when Ca^{2+}

was removed from the perfusing and bathing solution. The protein output may be due to secretin-induced stimulus-secretion coupling in the pancreatic acinar cells, since zymogen granules can be detected morphologically in the acinar cells but not in the cells of ductal tissue. Secretin-induced pancreatic enzyme output was already demonstrated in the pancreas of rats (1). Secretin-specific receptors were demonstrated not only in the cells of the ductal tissue (4), but also in acinar cell (1). A commonly accepted view is that the binding of secretin with the receptors may activate adenylate cyclase (18) and the synthesis of cyclic AMP, and in turn, may induce extrusion of pancreatic enzyme proteins (7, 8, 15). This view may be compatible with the following results in the present experiments: 1) continuous stimulation with 1 nM secretin, and with $1 \mu\text{M}$ forskolin, gradually increased cyclic AMP content of the whole pancreas (Fig. 6); 2) the stimulation induced sustaining secretory responses in isolated perfused rat pancreas (Figs. 1 and 4); and 3) dbcAMP *per se* caused secretory responses (Fig. 5).

Ca^{2+} -dependent Secretory Responses to Secretin

The results that the removal of extracellular

Fig. 5 Time courses of changes in pancreatic protein output and juice flow during continuous stimulation with $100 \mu\text{M}$ dbcAMP in a Ca^{2+} -containing and a Ca^{2+} -deficient environment. Each value represents the mean of four experiments. The open horizontal bar indicates the period of dbcAMP stimulation, and the shaded horizontal bar indicates the period of Ca^{2+} removal from the perfusing and the bathing solution.

Fig. 6 Change in cyclic AMP content of the isolated pancreas perfused with 1 nM secretin or $1 \mu\text{M}$ forskolin in a Ca^{2+} -containing or Ca^{2+} -deficient environment. Each value represents the mean of four preparations.

Ca^{2+} almost completely abolished the secretory responses to secretin, forskolin, and dbcAMP resembles the results of previous experiments in which the removal of extracellular Ca^{2+} almost completely abolished the secretory responses to continuous stimulation with CCK at a physiological concentration ($2\text{--}20 \text{ pM}$; 11, 13, 22). The similarity between the present and previous results led us to conclude that the influx of extracellular Ca^{2+} is

the dominant source of the increase in cytoplasmic Ca^{2+} concentration when the pancreatic acinar cell is stimulated with secretin at a pharmacological concentration and with CCK at a physiological concentration.

The question now arises as to which of these two intracellular messengers, Ca^{2+} or cyclic AMP, plays a major role in the stimulus-secretion coupling activated by secretin? For the answer, at least two views may be proposed.

The first view is that the final event in the coupling may be $[Ca^{2+}]_i$ -induced exocytosis of the zymogen granules, and the final event may be potentiated by the increase in cyclic AMP. The second view is that cyclic AMP may trigger the process of exocytosis, and extracellular Ca^{2+} may play a role as an activator of adenylate cyclase in the plasma membrane of acinar cells. A support for the second view is the fact that lowering extracellular Ca^{2+} is known to inhibit the activity of adenylate cyclase in many types of cells (17). The second view may harmonize with the present result that the removal of Ca^{2+} nullified secretin- and forskolin-induced increase in cyclic AMP content (Fig. 6).

However, the second view does not agree with the present result that dbcAMP-induced protein output was also markedly diminished in the Ca^{2+} -deficient environment. This result can be explained by the first view. If secretin can induce increases in both cyclic AMP and Ca^{2+} influx in the acinar cell, then the synarchic interaction between these two intracellular messengers may greatly potentiate $[Ca^{2+}]_i$ -dependent exocytosis of zymogen granules.

In the course of preparing this paper, the following two papers appeared and suggested that the first view may be more valid. Trimble *et al.* (25) demonstrated that, besides cyclic AMP, other second messengers such as inositol trisphosphate, diacylglycerol, and intracellular Ca^{2+} are increased by secretin in isolated rat acini. They proposed that these four messengers must be taken into account when considering the mechanism(s) by which secretin promotes amylase output from pancreatic acinar cells. Shinozaki *et al.* (23) showed that in the dispersed rat pancreatic acini, the amylase released by secretin, forskolin, and dbcAMP was inhibited by W-7, a calmodulin antagonist, and by the removal of Ca^{2+} from the medium. They postulated that the first phase of the secretin-induced amylase release is interacted strongly by Ca^{2+} -calmodulin complex, and the second phase of secretin-induced amylase release and forskolin- and dbcAMP-induced amylase release is interacted by both of the cyclic AMP passage and intracellular Ca^{2+} pool. The results obtained by Shinozaki *et al.* (23) however, are different from ours in the following aspects. 1) They used dispersed acini, and stimulated by secretagogues at much higher concentrations (500 nM secretin, 50 μ M forskolin, and 2 mM dbcAMP),

which are 5,000, 50, and 10 times higher than concentrations of the respective secretagogues we used. Previous studies in our laboratory provided evidence that the influence of extracellular Ca^{2+} and the cellular mechanism of stimulus-secretion coupling in the acinar cell are not identical among the secretory responses to physiological, pharmacological, and toxic concentrations of CCK (11, 22). Such dose dependency of extracellular Ca^{2+} and of the cellular mechanism may also be recognized in the responses to secretin. In fact, amylase released by 500 nM secretin in the dispersed acini showed a biphasic secretory pattern, which resembled the pattern induced by CCK stimulation at the pharmacological and toxic concentrations in the perfused pancreas (11, 13, 22). 2) The secretory response of dispersed acini was much smaller than that of the perfused pancreas. Amylase released by 500 nM secretin in dispersed acini was about 2 times larger than the resting level, whereas protein released by 0.1 nM secretin in the perfused pancreas was 6 times larger than the resting level. 3) The present study demonstrated the effects of secretin on both protein output and juice flow, and the unique influence of extracellular Ca^{2+} on secretin-induced protein output. The Ca^{2+} -resistant component of secretin-induced juice flow may possibly be due to the secretory response of the ductal tissue (4).

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