THE ROLE OF EXTRACELLULAR CALCIUM AND OTHER IONS IN THE SECRETORY RESPONSES OF THE EXOCRINE PANCREAS TO CHOLECYSTOKININ

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ABSTRACT

The influence of extracellular Ca^{2+} and other ions was examined in the isolated perfused rat pancreas. Continuous stimulation with cholecystokinin (CCK) at a physiological concentration (2×10^{-11} M) induced gradual increase in pancreatic protein output and juice flow. These CCK-induced secretory responses were completely abolished when Ca^{2+} was removed from the perfusing and bathing solution, and the responses reappeared when 2.5 mM CaCl_2 was reintroduced. Continuous stimulation with CCK at a higher concentration (3×10^{-10} M) induced secretory responses which consisted of two phases, an initial rising phase and a subsequent plateau. When Ca^{2+} was removed from the extracellular environment, the initial phase was decreased and the second phase was strongly inhibited. When all Cl^- in the perfusing solution was replaced by SO_4^{2-} , the secretory responses to CCK at physiological and higher concentrations were strongly inhibited. When Cl^- was replaced with Br^- , the secretory responses to CCK were slightly inhibited. Addition of 4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate to the perfusing solution induced inhibition of the CCK-induced secretory responses.

Secretion has been defined as a chain of processes in which the following three main steps may be observed: ingestion, synthesis, and extrusion (4). Although ingestion and synthesis are a relatively continuous process in the pancreatic acinar cell, extrusion of zymogen granules by exocytosis occurs episodically in response to food ingestion. Extrusion is physiologically controlled by the gut hormone cholecystokinin-pancreozymin (CCK) and the neurotransmitter acetylcholine (ACh). It is commonly believed that the extrusion effect of these secretagogues is exerted by increasing the cytoplasmic concentration of free, diffusible Ca^{2+} , $[\text{Ca}^{2+}]_i$. In fact, a recent study

showed that $[\text{Ca}^{2+}]_i$ is most effective in the comparatively narrow range between 0.4 and 1 μM (15).

Two views have been proposed on the nature of the process responsible for the secretagogue-induced rise in $[\text{Ca}^{2+}]_i$. The first view is that the rise may be produced by an increase in Ca^{2+} entry into the pancreatic acinar cell, and the second view is that the rise may be due to a release of Ca^{2+} from intracellular stores (1, 22). A compromise view in recent years is that the initial secretagogue action is to release intracellular Ca^{2+} followed by the sustaining phase of extrusion which may be due to a sustaining influx of extracellular Ca^{2+} (16, 22). Previous studies in our laboratory provided evidence that the amylase output induced by 5 mU/ml (3.7×10^{-10} M) CCK was inhibited by the addition of Mn^{2+} , an inhibitor of the Ca^{2+} influx (7), and that the amylase output induced by ACh at a low

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concentration (5×10^{-8} M) was strongly reduced in a Ca^{2+} -deficient environment, whereas the output induced by ACh at a high concentration (10^{-6} M) was not inhibited but rather enhanced by the Ca^{2+} removal (6). Although ACh is the chemical transmitter of the vagus nerve innervating the pancreas, the exact range of ACh concentration in physiological conditions is hard to determine. On the other hand, the physiological range of CCK level in the plasma can be determined, and the present experiments showed, for the first time, that the secretory responses induced by CCK at a physiological concentration (2×10^{-11} M) were completely inhibited in a Ca^{2+} -deficient environment.

MATERIALS AND METHODS

Isolation and Perfusion of the Pancreas

Sprague-Dawley strain rats (about 200 g) were fasted but allowed water for 24 h before each experiment. The isolated and perfused pancreas was prepared as reported previously (11). 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 flow 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 blood supplying the stomach, liver, and spleen was stopped by tying the arteries. The mesentery with its embedded whole pancreas and the attached duodenum was isolated, and then placed in a Lucite chamber containing 20 ml of a HEPES-buffered Ringer solution, maintained at 37°C.

Solution and Drugs

The composition of the standard HEPES-buffered Ringer solution used for perfusing and bathing the preparation was as follows (mM): NaCl, 145; KCl, 5.6; CaCl_2 , 2.5; MgCl_2 , 1; HEPES (*N*-2-Hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid: Sigma, St. Louis), 10; glucose, 5. In the Ca^{2+} -deficient solution, CaCl_2 was omitted. Dextran T-70 (Pharmacia, Uppsala) was added to the perfusing solution at a final concentration of 5% (w/v). The solution was equilibrated with O_2 and had a pH of 7.3. CCK (33 amino acid residues; 3,500 U/mg, by the courtesy of Professor Mutt, Karolinska Institute, Stockholm) was added to the perfusing solution. CCK was stored frozen at a

concentration of 30 μM in a stock physiological saline solution containing 0.5% bovine serum albumin (ICN Nutritional Biochemicals, Cleveland, U.S.A.) and 0.05% ammonium bicarbonate (Wako Pure Chemical Ind., Tokyo). This procedure assures the effectiveness of CCK (2).

Estimation of Digestive Enzyme

The collected juice sample was then diluted with 160 mM NaCl solution containing phosphate buffer (pH 7.1). The total protein in the pancreatic juice was assayed by the method of Lowry *et al.* (12), with bovine serum albumin as a standard.

Statistics

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 CCK at Different Concentrations

Continuous stimulation with CCK at a low concentration (2×10^{-11} M) induced gradual increases in pancreatic protein output and juice flow (Fig. 1). The total protein output in the 30 min of continuous stimulation in the standard solution was $3,147 \pm 199 \mu\text{g}$. The secretory responses were completely inhibited when Ca^{2+} was removed from the perfusing and bathing solution (Fig. 2). The total amount of CCK-induced protein output into the common duct collected in the Ca^{2+} -deficient environments was $198 \pm 70 \mu\text{g}$ per 30 min. Analysis of these two results showed that the diminution of CCK-induced protein output was significant ($P < 0.001$). The strong inhibition of secretory responses to continuous CCK stimulation in the Ca^{2+} -deficient environment was followed by detectable rises in pancreatic protein output and juice flow when 2.5 mM CaCl_2 was reintroduced to the perfusing and bathing solution in the absence of CCK (Fig. 2).

Continuous stimulation with CCK at a moderate concentration (5×10^{-11} M) induced secretory responses which consisted of two phases, an initial rising and a decline (Fig. 1). The total protein output in the 30 min of continuous stimulation in the standard solution was $5,707 \pm 252 \mu\text{g}$. When Ca^{2+} was removed from the per-

Fig. 1 Time course of changes in pancreatic juice flow and protein output during continuous perfusion of the isolated rat pancreas with 2×10^{-11} M (0.27 mU/ml), 5×10^{-11} M, and 3×10^{-10} M CCK in the standard solution. Each value represents the mean of four experiments. The open horizontal bar indicates the period of CCK perfusion.

Fig. 2 Time course of changes in pancreatic juice flow and protein output during continuous perfusion of the isolated rat pancreas with 2×10^{-11} M, 5×10^{-11} M, and 3×10^{-10} M CCK in a Ca²⁺-deficient solution (CaCl₂ was removed from the perfusing and the bathing solution). Responses induced by CCK in the standard solution containing 2.5 mM CaCl₂ (Fig. 1) are indicated by thin lines for reference. Each value represents the mean of four experiments. The open horizontal bar indicates the period of CCK perfusion.

fusing and bathing solution, the initial and second phases were both decreased to about one third of the respective controls obtained in the standard solution. The total amount of CCK-induced protein output into the common duct collected in the Ca -deficient environment was $1,650 \pm 317 \mu\text{g}$ per 30 min. Analysis of these two results showed that the diminution of CCK-induced protein output was significant ($P < 0.001$). The strong inhibition of secretory responses to continuous CCK stimulation was followed by marked increases in pancreatic protein output and juice flow when 2.5 mM CaCl_2 was reintroduced to the perfusing and bathing solution in the absence of CCK (Fig. 2).

Continuous stimulation with CCK at a higher concentration ($3 \times 10^{-10} \text{M}$) induced secretory responses which consisted of two phases, an initial rising phase and a subsequent plateau (Fig. 1). The total protein output in the 30 min of continuous stimulation in the standard solution was $5,923 \pm 618 \mu\text{g}$. When Ca^{2+} was removed from the perfusing and bathing solution, the initial phase was decreased to about two-thirds of the control obtained in the standard solution, and the second phase was strongly inhibited. The total amount of CCK-induced protein output into the common duct collected in the Ca^{2+} -deficient environment was $1,832 \pm 245$

μg per 30 min. Analysis of these two results showed that the diminution of CCK-induced protein output was significant ($P < 0.001$). The strong inhibition of secretory responses to continuous CCK stimulation was followed by marked rises in pancreatic protein output and juice flow when 2.5 mM CaCl_2 was reintroduced to the perfusing and bathing solution in the absence of CCK (Fig. 2).

Influence of Cl^- Replacement on Secretory Responses Induced by CCK at Different Concentrations

When all Cl^- in the perfusing solution was replaced by SO_4^{2-} , not only the secretory responses to continuous CCK stimulation but also the responses to CaCl_2 reintroduction were strongly inhibited (Fig. 3). The inhibitory influence of SO_4^{2-} may be attributable to decrease in the $[\text{Ca}^{2+}]_o$, although we used 10 mM CaSO_4 to compensate for the low Ca^{2+} ion concentration that exists in the presence of sulphate. When all Cl^- was replaced by Br^- , however, the secretory responses to $2 \times 10^{-11} \text{M}$ or $5 \times 10^{-11} \text{M}$ CCK were slightly and insignificantly inhibited, but the responses to $3 \times 10^{-10} \text{M}$ CCK were significantly inhibited ($P < 0.02$; Fig. 4).

Fig. 3 Time course of changes in pancreatic juice flow and protein output during continuous perfusion of the isolated rat pancreas with $2 \times 10^{-11} \text{M}$, $5 \times 10^{-11} \text{M}$, and $3 \times 10^{-10} \text{M}$ CCK in a Cl^- -free solution (Cl^- was replaced with SO_4^{2-}). Responses induced by CCK in the standard solution (Fig. 1) are indicated by thin lines for reference. Each value represents the mean of two experiments. The open horizontal bar indicates the period of CCK perfusion.

Fig. 4 Time course of changes in pancreatic juice flow and protein output during continuous perfusion of the isolated rat pancreas with 2×10^{-11} M, 5×10^{-11} M, and 3×10^{-10} M CCK in a Cl^- -free solution (Cl^- was replaced with Br^-). Responses induced by CCK in the standard solution (Fig. 1) are indicated by thin lines for reference. Each value represents the mean of four experiments. The open horizontal bar indicates the period of CCK perfusion.

Fig. 5 Time course of changes in pancreatic juice flow and protein output during continuous perfusion of the isolated rat pancreas with 2×10^{-11} M, 5×10^{-11} M, and 3×10^{-10} M CCK in a solution containing organic acids (4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate). Responses induced by CCK in the standard solution (Fig. 1) are indicated by thin lines for reference. Each value represents the mean of four experiments. The open horizontal bar indicates the period of CCK perfusion.

Influence of Organic Acids on Secretory Responses Induced by CCK at Different Concentrations

The solution superfusing or bathing the pancreas fragments, dispersed acini or acinar cells frequently contains organic acids. For example, solution containing 4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate has been used to superfuse the pancreatic fragments (13, 14, 16). The influence of the addition of these organic acids to the perfusing and bathing solution on the secretory responses to CCK was examined in the isolated perfused rat pancreas (Fig. 5). When 4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate were added to the standard solution, the protein output induced by continuous stimulation with 2×10^{-11} M CCK was slightly inhibited. The difference between the total amount of CCK-induced protein output in the presence of the organic acids and that in the absence of the organic acids was not significant ($P > 0.1$). The pancreatic juice flow induced by 2×10^{-11} M CCK in the presence of the organic acids was significantly smaller than in the absence of organic acids ($P < 0.02$). The protein output and the juice flow induced by 5×10^{-11} M or 3×10^{-10} M CCK in the presence of the organic acids were significantly smaller than the corresponding values obtained in the absence of organic acids ($P < 0.01$).

DISCUSSION

Physiological, Pharmacological, and Pathological Effects of CCK

Grossman (3) has proposed the following criteria for the physiological effect of gastroenteric hormones: 1) the effect should be seen with doses of exogenous hormone equivalent to the ED_{50} (dose inducing half-maximum responses) for primary action; 2) application by continuous intravenous infusion, not by bolus; and 3) the action should be seen with endogenous release of the hormone. We have no information about the range of plasma CCK level before and after eating in the rat, but it has been estimated by bioassay that the vascular perfusate of the isolated perfused rat duodenum contained about 7 mU/ml (5.1×10^{-10} M) CCK after intraduodenal infusion of a synthetic trypsin inhibitor, FOY 305 (100 mg/kg body weight), which is known to be a most effective stimulus for endogenous CCK release (10). CCK in the portal vein is known to be partly inactivated in the plasma and during the pass through the liver,

and further diluted about three times with venous blood passed through organs other than the liver. It seems reasonable that the rise in CCK level in response to food ingestion should be far below the level of 5×10^{-10} M in the arterial blood perfusing the pancreas *in situ*, and the secretory response to 2×10^{-11} M CCK is regarded as a physiological effect. CCK in the range from 5×10^{-10} M to 2×10^{-9} M may induce pharmacological effects. CCK in the range above 2×10^{-9} M may cause pathological effects: the effects were much smaller than the physiological and pharmacological effects (5), and coincided with uncoupling between adjacent acinar cells, leading to leakage of pancreatic enzyme and zymogen from the acinar lumen to the portal vein (20).

$[Ca^{2+}]_o$ on the Secretory Responses

The present experiments provide direct evidence that CCK requires extracellular Ca^{2+} in order to induce secretory effects in the isolated perfused rat pancreas. The requirement of extracellular Ca^{2+} was most evident when the pancreas was continuously stimulated with 2×10^{-11} M CCK, a possible physiological concentration. The requirement of extracellular Ca^{2+} became gradually less as the CCK concentration used was increased: the initial phase of secretory responses to 3×10^{-10} M CCK remained but the plateau phase was almost completely abolished in a Ca^{2+} -deficient environment (Fig. 2). These results lead us to conclude that 1) influx of extracellular Ca^{2+} is the dominant source of increase in $[Ca^{2+}]_i$ when the pancreatic acinar cell is stimulated with CCK at a physiological concentration, and that 2) in addition to Ca^{2+} influx, Ca^{2+} is released from intracellular Ca^{2+} stores immediately after the cell is stimulated with CCK at a pharmacological concentration or at the upper limit of physiological concentration while the influx of extracellular Ca^{2+} is continuously increased. The present conclusion is consistent with the previous conclusion obtained from the study on the Ca^{2+} -dependent secretory responses to ACh (6). The second conclusion corresponds to the view that the initial secretagogue action is to release intracellular Ca^{2+} , that the resulting increase in $[Ca^{2+}]_i$ triggers exocytosis of zymogen granules, and that, in the sustaining phase of secretory response, influx of extracellular Ca^{2+} is important as the source of $[Ca^{2+}]_i$ (16, 22).

The pathological responses of the pancreatic acinar cell to CCK at a high concentration may be due to an extreme rise in $[Ca^{2+}]_i$, which may

inhibit secretagogue-induced secretory responses (15), and cause uncoupling between adjacent cells (20).

In the context of the present study, it is interesting that the depolarizing effect of secretagogues has been demonstrated at pharmacological and pathological concentrations (13, 17), and the hyperpolarizing effect has been recorded at a physiological concentration (5, 8).

$[Cl^-]_o$ and Organic Acids on the Secretory Responses

The ionic mechanism of the depolarizing effect of ACh has been studied by replacing the extracellular ions. The Cl^- permeability was estimated by comparing the ACh-evoked depolarization in a Cl^- -rich solution and that in a solution in which Cl^- was replaced with SO_4^{2-} (14). In the Cl^- -free solution, the caerulein-evoked amylase and fluid secretion were strongly inhibited (18). Although Petersen and Ueda (18) used 10 mM $CaSO_4$ to compensate for the low $[Ca^{2+}]_o$ that exists in the presence of sulfate, this procedure may partially reduce the $[Ca^{2+}]_o$, and may decrease the secretory responses of the pancreatic acinar cell to caerulein. A same explanation can be applied to the present results shown in Fig. 3. The previous study showed that the replacement of NaCl with NaBr has slight and insignificant influence on the CCK-induced secretory responses, whereas the replacement with Na-isothionate or with Na-pyruvate abolished the secretory responses (9). A similar result was obtained in the present experiments (Fig. 4). These results may be explained by the possibility that the hydrated isothionate and pyruvate ion do not readily cross the membrane whereas Cl^- and Br^- do. A similar explanation was given for the difference in the effects of isothionate, Cl^- , and Br^- on the post-tetanic hyperpolarization in mammalian non-myelinated nerve fibers (19). There is another possibility that the organic acids including amino acids and sugars *per se* may inhibit the secretagogue-induced secretory responses. The present study showed that the CCK-induced secretory responses were inhibited when 4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate were added to the perfusing solution (Fig. 5). A previous study in our laboratory provided evidence that an increase in sugar (glucose, xylose, and galactose) concentration in the perfusing solution inhibited the CCK-induced secretory responses (21). The depolarizing effect of secretagogues has usually been

recorded from the pancreatic fragments superfused with a solution containing 4.9 mM Na-pyruvate, 2.7 mM Na-fumarate, and 4.9 mM Na-glutamate (13, 14, 17), and the secretory responses of the acinar cells in the fragment may, at least in part, be inhibited.

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