Influences of Ionic Environments on ACh-induced Secretory Responses in Isolated Perfused Pancreas of Rats

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Abstract The influence of extracellular Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_o\), on the secretory response to acetylcholine (ACh) was analyzed in isolated perfused rat pancreas. The decrease of \([\text{Ca}^{2+}]_o\) strongly diminished the amylase output and pancreatic juice flow in response to continuous stimulation with \(5 \times 10^{-8} \text{ M ACh}\). A quantitative relation was found between the amount of amylase released by \(5 \times 10^{-8} \text{ M ACh}\) and the \([\text{Ca}^{2+}]_o\) over a range of 0.1–2.5 mM. The partial replacement of NaCl with LiCl produced a diminution in both amylase output and pancreatic juice flow. A quantitative relation existed between the amount of ACh-induced amylase release and the \([\text{Na}^+]_o\) over a range of 86–157 mM. The partial replacement of KCl with NaCl produced falls in both amylase output and pancreatic juice flow. Again, a quantitative relation existed between ACh-induced amylase release and \([\text{K}^+]_o\) over a range of 1.0–5.6 mM. These results are compatible with the view that both the amylase output and the juice flow induced by \(5 \times 10^{-8} \text{ M ACh}\) are proportional to the amount of carrier-Ca complex and that the inward movement of the complex may be linked closely to the activation of Na pumps on the pancreatic acinar cell.

A dose-response relation was found between the concentration of ACh and the amylase output. The relation was shifted to the left when 1 mU/ml cholecystokinin-pancreozymin (CCK-PZ) was added. A similar shift was observed when 1 mU/ml secretin was added. These results support the view that ACh, CCK-PZ, and secretin may activate the common cellular process in stimulus-secretion coupling, although these secretagogues may severally act on the different receptor sites.

Cholecystokinin-pancreozymin (CCK-PZ) and acetylcholine (ACh) are the physiological secretagogues to which the pancreatic acinar cells respond by releasing their zymogen granules. The effect of these secretagogues is believed to be exerted by a rise in the cytoplasmic concentration of free, diffusible Ca\(^{2+}\), although no direct measurement of intracellular free Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_i\),
has yet been made in pancreatic acinar cells (see Case, 1978). Two views have been proposed concerning the nature of the process responsible for the secretagogue-induced rise in \([\text{Ca}^{2+}]_i\). One is that the rise may be due to a release of \(\text{Ca}^{2+}\) from the cell membrane or from an intracellular Ca store (see Case, 1978; Chandler, 1978). The other is that the rise may be due to an increase in \(\text{Ca}^{2+}\) entry into the pancreatic acinar cell from the extracellular space. The former view has been proposed from the results of experiments in which the pancreas was stimulated by a relatively high concentration of secretagogues in isolated preparations of acinar cells or acinus (Case, 1974; Chandler and Williams, 1974; Gardner et al., 1975; Nishiyama and Petersen, 1975; Shelby et al., 1976; Argent et al., 1976; Poulsen and Williams, 1977; Petersen and Ueda, 1976a, b; Ueda and Petersen, 1977; Iwatsuki and Petersen, 1977a, b, c). On the other hand, the second view has been supported mainly by results obtained from experiments with a physiological concentration of secretagogues used to stimulate the isolated perfused pancreas (Kanno, 1972, 1975; Kanno and Nishimura, 1976; Kanno and Saito, 1976; Kanno and Yamamoto, 1977; Kanno et al., 1977). Kanno and Habara (1980) have recently reported that pancreatic enzyme release depended mainly upon extracellular Ca when the isolated perfused rat pancreas was stimulated by ACh of low concentration \((5 \times 10^{-8} \text{M})\), whereas the release induced by ACh with a higher concentration \((10^{-8} \text{M} \text{ or more})\) was not inhibited by a Ca-deficient environment. The preliminary results of the present experiments show that there is a quantitative relation between \([\text{Ca}^{2+}]_o\) and the amount of enzyme released by \(5 \times 10^{-8} \text{M} \text{ ACh}\).

Moreover, the inhibitory effects of lowering \([\text{Na}^+]_o\) and \([\text{K}^+]_o\) on the CCK-PZ-induced secretory responses have been reported by Kanno et al. (1977), and Kanno and Saito (1978). The quantitative analysis of these inhibitory influences is compatible with the view that CCK-PZ may activate a carrier molecule bearing Ca and Na atoms through which extracellular \(\text{Ca}^{2+}\) can enter the acinar cell, and that the inward movement of the carrier may be linked closely to the activation of the Na pump on the cell membrane. This view was again supported in the present experiments in which the inhibitory effects of lowering \([\text{Na}^+]_o\), or \([\text{K}^+]_o\), on the ACh-induced secretory responses were analyzed.

METHODS

Isolation and perfusion of the pancreas. Wistar strain male rats weighing about 200 g were fasted, but allowed to access water, for 24 hr before the experiments. The isolated and perfused pancreases were prepared by the method of Kanno et al. (1976). Under ether anesthesia, the vascular system was cannulated and perfused through the superior mesenteric and coeliac arteries with the portal vein used as the outlet. The rate of vascular flow was kept constant at 1.3 ml/min by a roller pump. The hepatic end of the common duct was ligated, and the

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pancreatic juice was collected from the duodenal end of the duct 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 then placed in a Lucite chamber containing 20 ml of a modified Krebs-Henseleit solution maintained at 37°C.

Hormones, drugs and solutions. The composition of the standard Krebs-Henseleit solution used for perfusing and bathing the preparation was as follows (mM): NaCl, 131; KCl, 5.6; CaCl₂, 2.5; MgCl₂, 1.0; NaHCO₃, 25; NaH₂PO₄, 1.0; glucose, 5.0. The standard solution was modified in a number of ways. In the low-Ca solutions, CaCl₂ was replaced with osmotically equivalent amounts of NaCl. In the low-Na solutions, NaCl was replaced with equimolar LiCl. In the low-K solutions, KCl was replaced with equimolar NaCl. The other modifications are the same as those given in papers by KANNO et al. (1977), and by KANNO and SAITO (1978). Dextran T-70 (Pharmacia, Uppsala) was added to the vascular perfusing solution at a final concentration of 5% (w/v). The solution was equilibrated with 5% CO₂ in O₂ and had a pH of about 7.4. Pure natural CCK-PZ (3500 U/mg, GIH Research Unit, Karolinska Institute, Stockholm), or pure natural secretin (75 U/mg including cystein and HCl, GIH Research Unit, Karolinska Institute, Stockholm), were infused through a cannula into the inlets of the vascular perfusion. The concentration of CCK-PZ and secretin was expressed in Ivy dog units (IVY and JANECEK, 1959) and clinical units, respectively. ACh (Sigma, Saint Louis) was also injected through the cannula.

Estimation of digestive enzyme and flow of pancreatic juice. The estimation of the flow rate of the pancreatic juice was made as follows: a calibrated tube made of silicon-rubber (about 0.5 mm O.D.) was attached to the free end of the pancreatic duct cannula. At 5- or 10-min intervals the tube was replaced, and the rate of pancreatic juice flow down the tube was measured. The collected juice samples were then diluted with 10 ml of 6.7 mM NaCl containing phosphate buffer (pH 7.1). Amylase was assayed by the modified method of BERNFELD (1955) after appropriate dilution of samples, as described by KANNO (1975). One unit of amylase activity was defined as the amount of enzyme which produced 1 mg maltose during a 5-min incubation period at 37°C.

Statistics. The results were expressed as the mean ± S.E. of several experiments (n), and were analyzed by Student’s t test.

RESULTS

Relation between secretory response and concentration of ACh

At first, determination of the concentration of ACh which produced the most stable and prolonged secretory responses in the isolated and perfused rat pancreases was carried out. In previous papers it was shown that 5 mU/ml CCK-PZ caused the highest and most prolonged amylase output (SAITO and KANNO, 1973; KANNO,
1975; Kanno et al., 1977), and continuous stimulation with $5 \times 10^{-8}$ M ACh induced stable and prolonged secretory responses (Kanno and Habara, 1980). As previously noted, the secretory responses to ACh varied markedly with its

![Graph A](image)

![Graph B](image)

Fig. 1. Time course of pancreatic juice flow (A) and amylase output (B) in response to perfusion with media containing different concentrations of ACh as indicated. Each value represents the mean of 5 min flow and amylase output from several experiments as indicated. The stippled horizontal bar indicates the period of ACh perfusion.
concentration, but the following findings were obtained: (1) continuous stimulation with $8 \times 10^{-8}$ M ACh or lower caused prolonged secretory responses; (2) continuous stimulation with $3 \times 10^{-7}$ M ACh evoked a maximum amylase output followed by a gradual decrease of output; and (3) continuous stimulation with $10^{-6}$ M ACh or higher induced small and transient responses. The secretory responses induced by $3 \times 10^{-7}$ M ACh were completely inhibited by $5 \times 10^{-4}$ M atropine which was added to the perfusing solution 10 min prior to continuous stimulation with ACh. A similar relation between the juice flow and the concentration of ACh was observed (Fig. 1A and B).

Influence of $[\text{Ca}^{2+}]_0$ on secretory responses

The inhibitory effect of lowering $[\text{Ca}^{2+}]_0$ was observed by changing $[\text{Ca}^{2+}]_0$ from 0.1 to 2.5 mm before, during and after continuous stimulation with $5 \times 10^{-8}$ M ACh (Fig. 2). In the control medium which contained 2.5 mM Ca$^{2+}$, continuous stimulation with $5 \times 10^{-8}$ M ACh induced a stable and prolonged amylase output ($1,633.0 \pm 427.5$ U/40 min during the stimulation), which was reduced to about one-half ($879.1 \pm 105.8$ U/40 min) in a low-Ca medium containing 0.1 mM Ca$^{2+}$. In order to analyze further the influence of $[\text{Ca}^{2+}]_0$, the amylase output evoked by $5 \times 10^{-8}$ M ACh was plotted against the linear scale of $[\text{Ca}^{2+}]_0$ (Fig. 3A). The numbers shown in Fig. 3A were replotted in Fig. 3B to show the relation between the reciprocal of the amylase output and the reciprocal of $[\text{Ca}^{2+}]_0$. Extrapolation of the line drawn in Fig. 3B intersected the abscissa and yielded a Michaelis constant of 0.1. A similar relation was observed between $[\text{Ca}^{2+}]_0$ and the pan-

![Fig. 2. Time course of pancreatic juice flow and amylase output in response to $5 \times 10^{-8}$ M ACh with media containing different $[\text{Ca}^{2+}]_0$ as indicated. Each value represents the mean of 10 min measurements from several preparations as indicated. The stippled horizontal bar indicates the period of ACh perfusion.](image)
Fig. 3. Relation between the amylase output in response to $5 \times 10^{-8}$ M ACh and the $[\text{Ca}^{2+}]_o$.

A: each symbol is the mean value depicted in Fig. 2. The amylase output is the total amount of amylase in a sample collected for 40 min during the continuous stimulation.

B: relation between reciprocal of the mean of amylase output shown in Fig. 3 A and reciprocal of the $[\text{Ca}^{2+}]_o$. Note that the standard error bars are not symmetrical because of the reciprocal scale on the ordinate.

Fig. 4. Time course of pancreatic juice flow and amylase output in response to $5 \times 10^{-8}$ M ACh with media containing different concentrations of $[\text{Na}^+]$ as indicated. Each value represents the mean of a 10 min measurements from several preparations as indicated. The stippled horizontal bar indicates the period of ACh perfusion.
creatic juice flow induced by $5 \times 10^{-8}$ m ACh.

**Influence of $[Na^+]_o$ on secretory responses**

Kanno et al. (1977) showed that the amylase output and the pancreatic juice flow induced by 5 mU/ml CCK-PZ were strongly influenced by $[Na^+]_o$. The inhibitory effect of lowering $[Na^+]_o$ in the perfusing and bathing solution was examined in the present experiments by measuring simultaneously both the amylase output and the pancreatic juice flow during perfusion with different $[Na^+]_o$ before, during and after continuous stimulation with $5 \times 10^{-8}$ m ACh (Fig. 4). Partial replacement with equimolar amounts of LiCl produced a diminution in both the amylase output and the pancreatic juice flow induced by ACh. When the $[Na^+]_o$ was reduced to 86 mm at 20 min prior to continuous ACh stimulation, the amylase output was almost completely inhibited. The pancreatic amylase output increased with a rise in $[Na^+]_o$. The amylase output induced by $5 \times 10^{-8}$ m ACh in a medium containing 157 mm Na and 2.5 mm Ca was 1,800.3 ± 356.3 U/40 min. A similar relation was observed between the pancreatic juice flow induced by $5 \times 10^{-8}$ m ACh and $[Na^+]_o$.

**Influence of $[K^+]_o$ on secretory responses**

Kanno and Saito (1978) showed that the amylase output and the pancreatic juice flow induced by 5 mU/ml CCK-PZ were also influenced markedly by $[K^+]_o$.

![Graph of pancreatic juice flow and amylase output in response to ACh](image)

Fig. 5. Time course of pancreatic juice flow and amylase output in response to $5 \times 10^{-8}$ m ACh during perfusion with media containing different concentrations of $[K^+]_o$ as indicated. Each value represents the mean of 10 min measurements from several preparations as indicated. The stippled horizontal bar indicates the period of ACh perfusion. Note that the response in the media containing 5.6 mM K is the same as the control experiments (157 mm Na) in Fig. 4.

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The inhibitory effect of lowering \([K^+]_o\) in the perfusing and bathing solution was examined by measuring simultaneously both the amylase output and the pancreatic juice flow during perfusion with a different \([K^+]_o\), before, during and after continuous stimulation with \(5 \times 10^{-8}\) M ACh (Fig. 5). Partial replacement of KCl with equimolar amounts of NaCl produced a diminution in both the amylase output and the pancreatic juice flow evoked by ACh. Both the amylase output and the juice flow were almost completely inhibited when \([K^+]_o\) was reduced to 1.0 mM at 30 min prior to the initiation of stimulation.

Relation between amylase output and concentration of ACh in the presence or absence of CCK-PZ or secretin

The preceding experiments provided evidence that the influences of \([Ca^{2+}]_o\), \([Na^+]_o\) and \([K^+]_o\) on the ACh-induced secretory responses were essentially similar to the influences of ions on the CCK-PZ-induced responses. These results could indicate that these two secretagogues may cause secretory responses via a common cellular process. This view was examined in the following experiment in which a dose-response relation for the ACh-induced secretory responses was observed in the presence or absence of a low concentration of CCK-PZ (1 mU/ml) (Fig. 6A and B). The dose-response relation in the presence of 1 mU/ml CCK-PZ shifted to the left, though 1 mU/ml CCK-PZ per se evoked only a small secretory response. The maximum amylase output induced by ACh in the presence of CCK-PZ was almost equal to that induced by ACh stimulation alone (Fig. 8).

In addition to a significant action on the HCO\(_3\)-dependent flow of the pancreatic juice, a small but definite increase in the amylase output was induced by secretin and this output was also inhibited by the Ca-deficient environment (Kanno and Yamamoto, 1977). This result suggests that secretin may also have some influence on the ACh-induced amylase output. The correlation between the action of ACh and secretin was analyzed in the following experiments in which the relation between the concentration of ACh in the presence of secretin and the secretory responses was examined (Fig. 7A and B). The maximum response (3,428.4 ± 190.8 U/40 min) was obtained at a concentration of \(1.5 \times 10^{-7}\) M ACh in the presence of a low dose (1 mU/ml) of secretin, which induced only a small amount of amylase output (476.4 U/40 min). The dose-response relation obtained in the presence of secretin also moved to the left in parallel with the relation obtained without secretin. The maximum response, however, was almost equal to that induced by ACh stimulation alone (Fig. 8).

DISCUSSION

Similarity between the ACh-induced and CCK-PZ-induced secretory responses

\([Ca^{2+}]_o\) on the secretory responses. The results of the present experiments show that ACh requires external Ca\(^{2+}\) (in the perfusing solution) for its effect on
Fig. 6. Time course of pancreatic juice flow (A) and amylase output (B) in response to perfusion with media containing 1 mU/ml CCK-PZ and different concentrations of ACh as indicated. Each value represents the mean of 5 min flow and amylase output from several experiments as indicated. The striped horizontal bar and the stippled horizontal bar indicate the period of CCK-PZ and ACh perfusion, respectively.

Amylase output in the isolated and perfused rat pancreas. A quantitative relation was found between the amount of amylase released by $5 \times 10^{-8}$ M ACh and $[Ca^{2+}]_o$ over a range of 0.1–2.5 mM. A double reciprocal plot for the data also shows that
Fig. 7. Time course of pancreatic juice flow (A) and amylase output (B) in response to perfusion with media containing 1 mU/ml secretin and different concentrations of ACh as indicated. Each value represents the mean of 5 min flow and amylase output from several experiments as indicated. The filled horizontal bar and the stippled horizontal bar indicate the period of secretin and ACh perfusion, respectively.

The pancreatic enzyme secretion induced by ACh linearly depends on the extracellular Ca²⁺ concentration. DEL CASTILLO and KATZ (1954a, b) suggested that
calcium combines with an active molecule X, which then releases a quantum of ACh by "carrying" it across the membrane of the nerve terminal. Similarly, KANNO (1972) has suggested that there is probably a Ca carrier X on the pancreatic acinar cell membrane, from quantitative experiments on pancreatic response to CCK-PZ. His basic assumption was that the following reaction took place on the surface of the membrane of the acinar cell:

\[ \text{Ca} + X \rightleftharpoons \text{CaX} \]  

(with dissociation constant \( K \))

where CaX was the complex necessary for amylase release. The overall reaction could be described by kinetic equations of the type of MICHAELIS and MENTEN (1913). Similar kinetic equations were found to be applicable to the effects of \([\text{Ca}^{2+}]_o\) on the release of pancreatic enzyme evoked by \(5 \times 10^{-8} \text{ M ACh}\). Thus, the present results are interpreted as follows: the pancreatic enzyme release evoked by ACh is mediated by a Ca carrier, and the mode of the \(\text{Ca}^{2+}\) influx is a facilitated diffusion: \(i.e.,\) the major contributor to the rise in \([\text{Ca}^{2+}]_i\) is the \(\text{Ca}^{2+}\) influx from the extracellular space. An identical view has already been proposed to explain the quantitative relation between the amount of amylase released by 5 \(\mu\text{U/ml}\) CCK-PZ and the \([\text{Ca}^{2+}]_o\) (KANNO and NISHIMURA, 1976; KANNO et al., 1977).

\([\text{Na}^+], \text{ and } [\text{K}^+]_o\) on the secretory responses. The mechanism of the parallel increases in pancreatic enzyme and juice flow remains to be fully defined (CASE, 1979). One possible model to explain the parallel increases induced by CCK-PZ has been proposed by KANNO et al. (1977) and KANNO and SAITO (1978). The
model states: (1) Ca may be transported by a carrier which also transports Na atoms concomitantly, (2) a rise in [Ca\(^{2+}\)]\(_i\) in turn causes exocytosis of zymogen granules, and (3) the resultant increase in [Na\(^+\)]\(_i\), may be reduced to the resting level by activation of Na pumps on the basal and apical membrane of the acinar cell. When the pancreas was stimulated by CCK-PZ, a quantitative relation was found between the amount of amylase and [Na\(^+\)]\(_o\) over a range of 26–157 mM (KANNO et al., 1977). In the present experiments a similar quantitative relation was found between the amount of amylase released by 5 × 10\(^{-8}\) M ACh and [Na\(^+\)]\(_o\) over a range of 86–157 mM.

A quantitative relation was also found between the amount of amylase released by 5 mU/ml CCK-PZ and [K\(^+\)]\(_o\) over a range of 1.0–5.6 mM (KANNO and SAITO, 1978). A similar quantitative relation was also obtained between the amount of amylase released by 5 × 10\(^{-8}\) M ACh and [K\(^+\)]\(_o\).

It may thus be argued that the cellular model proposed by KANNO et al. (1977) and KANNO and SAITO (1978) can also explain the present results.

**Interaction between the ACh-induced and the CCK-PZ-induced or secretin-induced secretory responses**

The present experiments showed that the influences of [Ca\(^{2+}\)]\(_o\), [Na\(^+\)]\(_o\) and [K\(^+\)]\(_o\) on the secretory responses induced by 5 × 10\(^{-8}\) M ACh were essentially similar to those of the responses induced by 5 mU/ml CCK-PZ. The dose-response relation for ACh-induced amylase output in the presence of 1 mU/ml CCK-PZ was almost parallel to that for ACh-induced output in the absence of CCK-PZ; the former was about 3 times the latter, but the maximal response in the former relation was almost equal to the latter, as shown in Fig. 8. These results show that there may be a common cellular process in the stimulus-secretion coupling initiated by ACh and CCK-PZ. As discussed above, a possible common cellular process may be the mechanism of Ca influx mediated by a Ca carrier or a Ca ionophore. The fact that Ca ionophore A23187 induced a Ca-dependent amylase output, which was inhibited in a low-Na medium in the isolated and perfused rat pancreas (KANNO et al., 1977), and in a low-K medium (KANNO and SAITO, 1978) supports this interpretation. On the other hand, it has been well established that the ACh-induced secretory response is inhibited by atropine, but that the CCK-PZ-induced response is not influenced. This fact suggests that a receptor on the membrane of the pancreatic acinar cell consists of two subunits; a receptor subunit, and a carrier (or ionophore) subunit. The receptor subunit may be specific for ACh and for CCK-PZ, respectively, but the activation of these two different receptor subunits may activate the Ca carrier subunit which is common to the two receptors. An essentially identical view was put forth recently by CASE (1979) in his review of recent advances in this field. Case suggested that the overall membrane perturbation (receptor stimulation) produced by CCK-PZ and acetylcholine appears to be the same, and that there is a difference only in the nature of
the outwardly facing part of the receptor complex. A similar explanation may also be possible for the interaction between ACh and secretin which causes a Ca-dependent amylase output in addition to the HCO3-dependent pancreatic juice flow.

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