THE EFFECT OF ACETYLCHOLINE CONCENTRATION ON MEMBRANE POTENTIAL, REDOX STATE AND SECRETION OF THE PANCREATIC ACINAR CELL

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ABSTRACT

Continuous stimulation of the isolated perfused rat pancreas with acetylcholine (ACh) at a low concentration (5×10^{-8} M) induced flat increases in secretory responses, which were decreased to values of about 30 per cent of control obtained in the standard medium when calcium was omitted from the perfusing and bathing solution. The secretory responses were associated with little or no change in the membrane potential and the redox state of the acinar cells.

Continuous stimulation with ACh at a moderate concentration (3×10^{-7} M) induced secretory responses which consisted of the following three phases; initial rise, second plateau and the final decline. When calcium was removed from the perfusing and bathing solution, the initial phase was decreased to about a half of the control obtained in the standard medium, and the second and the final phase were strongly inhibited. Continuous stimulation with 3×10^{-7} M ACh caused only a transient small depolarization of the acinar cell, and a gradual reduction in the redox state.

Continuous stimulation with ACh at a higher concentration (10^{-6} M) induced a rather small flat increase in amylase output, which was further enhanced when calcium was removed from the perfusing and bathing solution. Continuous stimulation with 10^{-6} M ACh caused a transient oxidation followed by a gradual reduction in the redox state.

KEY WORDS acetylcholine / membrane potential / redox state / secretion / pancreatic acinar cell

Cholecystokinin-pancreozymin (CCK-PZ) and acetylcholine (ACh) are the physiological secretagogues to which the pancreatic acinar cells respond by releasing their contents of zymogen granules. It is commonly believed that the effect of these secretagogues is exerted by increasing the cytoplasmic concentration of free, diffusible Ca, although no direct measurement of intracellular free Ca concentration, $[Ca]_i$, has yet been made in pancreatic acinar cells (2). Two views have been proposed on the nature

of the process responsible for the secretagogue-induced rise in $[Ca]_i$. One view is that the rise may be produced by an increase in Ca entry into the pancreatic acinar cell, and the second view is that the rise may be due to a release of Ca from an intracellular Ca store (2, 5). A previous study in our laboratory provided evidence that the amylase output induced by 5 mU/ml CCK-PZ was inhibited by the addition of Mn, an inhibitor of the Ca influx (10). The present experiments showed that the secretory response induced by ACh at a low concentration (5×10^{-8} M) was strongly reduced in a Ca-defi-

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cient environment, while the response induced by ACh at a higher concentration (10^{-6} M) was not inhibited but rather enhanced by the Ca removal.

Continuous stimulation with ACh at the low concentration induced neither depolarization nor an increase in fluorescence of the pyridine nucleotides, whereas continuous stimulation with ACh at the higher concentration caused transient depolarization and gradual increase in fluorescence of the pyridine nucleotides. These observations show that the cellular processes in stimulus-secretion coupling induced by ACh at the low concentration may differ from those induced by ACh at higher concentrations.

METHODS

Isolation and Perfusion of the Pancreas

Male rats of Wistar strain weighing about 200 g were fasted but allowed water for 24 hr before the experiments. The isolated and perfused pancreas was prepared as reported previously (13). 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 1.3 ml/min by a roller pump in the experiments demonstrating the effects of ACh on the secretory response and membrane potential, and 2 ml/min in the experiments demonstrating the effects of ACh on the secretory response and the redox state. The hepatic end of the common duct was ligated and the pancreatic juice was collected from the duodenal end of the duct following cannulation with a stainless-steel tube (the draining preparation). The flushing preparation (13) was used in the experiments recording simultaneously the secretory response and the membrane potential or the redox state. 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 Solution

The composition of the standard Krebs-Henseleit solution used for perfusing and bathing the preparation was as follows (final concentrations, mM): NaCl, 131; KCl, 5.6; CaCl_2 , 1.0; NaHCO_3 , 25; MgCl_2 , 1.0; NaH_2PO_4 , 1.0; glucose, 5.0. In the low Ca and the Ca-deficient

solutions, CaCl_2 was replaced with isotonic amounts of NaCl. Dextran T-70 (Pharmacia, Uppsala) 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 about 7.4. Highly purified natural CCK-PZ (3500 U/ml, GIH Research Unit, Karolinska Institute, Stockholm) was added into the perfusing solution. The concentration of CCK-PZ was expressed in Ivy dog units (7). ACh (Sigma, St. Louis) was also added into the perfusing solution.

Estimation of Digestive Enzyme and Flow of Pancreatic Juice

The estimation of the flow rate of pancreatic juice was made as follows: a calibrated tube made of silicon-rubber (about 0.5 mm in outer diameter) was attached to the free end of the pancreatic duct cannula. At specified intervals the tube was replaced and the rate of pancreatic juice flow down the tube was measured. The collected juice sample was then diluted with 160 mM NaCl solution containing phosphate-buffer (pH 7.1). Amylase in appropriately diluted samples was assayed by the method of Bernfeld (1) as modified previously (9). One unit of amylase activity is defined as the amount of enzyme which produced 1 mg maltose during a 5 min incubation at 37°C.

Recording of Membrane Potentials

Intracellular recordings were made from the pancreatic acinar cells by manually advancing KCl-filled microelectrodes under direct visual control with the aid of a micromanipulator driven by oil pressure (Narishige, MO 102 R, Tokyo). Resting potentials were observed on the screen of a cathode ray oscilloscope (Nihon Kodens, ATAC-250, Tokyo) and simultaneously recorded on a direct visual oscillograph (Yokogawa, type 2915, Tokyo) with a frequency response of 5 kHz. Current pulses of 50 msec duration were passed through the recording electrode in order to polarize the cell membrane. A full description of the procedure has been given in previous papers (6, 8).

Recording of Redox State

Redox state of the isolated and perfused rat pancreas was recorded with a redoximeter (Tateishi Institute of Life Sciences, type HEF-4, Kyoto). The mechanical and electrical details of

Fig. 1 Time course of changes in pancreatic juice flow and amylase output during continuous perfusion of the isolated pancreas with 5×10^{-8} M ACh in the standard medium (A). In Fig. 1B, the perfusion medium was changed from the standard medium to a Ca-deficient medium in the course of continuous stimulation. Each value represents the mean of several experiments as indicated. The stippled horizontal bar indicates the period of ACh perfusion.

this instrument were described by Kobayashi (15). Briefly, the instrument is a modification of the microfluorometer introduced by Chance, Cohen, Jobsis and Schoener (4) that records blue fluorescence emitted from the microscopic field of tissues upon the 366 nm excitation-light. The fluorescence intensity is a function of the amount of reduced form of the pyridine nucleotides (nicotinamide adenine dinucleotide, NADH, and nicotinamide adenine dinucleotide phosphate, NADPH), and a direct and continuous measurement of the relative value of the intracellular redox state can thus be attained.

Statistics

Results are expressed as the mean $\pm$ S. E. of several experiments (*n*), and are analyzed by Student's *t*-test.

RESULTS

Influence of Ca Removal on Secretory

Responses Induced by ACh at Different Concentrations

Continuous stimulation with ACh at a low concentration (5×10^{-8} M) induced flat increases in amylase output and pancreatic juice flow (Fig. 1A). The total amylase output in the initial 40 min of continuous stimulation in the standard medium was 1633.0 ± 427.5 U. The secretory responses were strongly reduced when Ca was removed from the perfusing and bathing solution (Fig. 1B). The total amount of ACh-induced amylase output into the common duct collected in the Ca-deficient environments was 555.0 ± 112.0 U per 40 min. Analysis of these two results showed that the diminution of ACh-induced amylase output in Ca-deficient environments was significant ($P < 0.05$). Residual Ca released from the isolated pancreas into the vascular perfusate was measured with an atomic absorption spectrophotometer (Hitachi-Perkin-Elmer, model 303). About 0.02 mM Ca still remained in the perfusate even after 40 min of perfusion with the Ca-deficient solution. The

secretory responses that remained in the Ca-deficient environment could thus be due to the presence of a small amount of Ca in the extracellular environment surrounding the acinar parenchyma.

Continuous stimulation with ACh at a moderate concentration (3×10^{-7} M) induced secretory responses which consisted of the following three phases; initial rising, second plateau and the final decline (Fig. 2A). The total amylase output in the initial 40 min of continuous stimulation in the standard medium was 3328.0 ± 535.2 U. When Ca was removed from the perfusing and bathing solution, the initial phase was decreased to about a half of the control obtained in the standard medium, and the second and the final phase were strongly inhibited. The total amount of ACh-induced amylase output into the common duct collected in the Ca-deficient environments was 1199.7 ± 235.0 U per 40 min. Analysis of these two results showed that the diminution of ACh-induced amylase output in Ca-deficient environments was significant ($P < 0.01$).

Continuous stimulation with ACh at a higher concentration (10^{-6} M) induced a rather small

flat increase in amylase output into the common duct associated with a gradual increase in output into the portal vein (Fig. 3A). The total amount of ACh-induced amylase output into the common duct in the initial 40 min of continuous stimulation in the standard medium was 888.7 ± 127.5 U. A previous study (20) showed that continuous stimulation with pancreaticozymine at a higher concentration induced rather a small amylase output into the common duct and a large output into the portal vein. When Ca was removed from the perfusing and bathing solution, the initial phase of ACh-induced amylase output was rather enhanced (Fig. 3B). The total amount of ACh-induced amylase output into the common duct collected in the Ca-deficient environment was 797.1 ± 164.7 U per 40 min. Analysis of these two results showed that the diminution of ACh-induced amylase output in Ca-deficient environments was not significant ($P > 0.6$).

Membrane Potential and Amylase Output

Fig. 4 shows a typical recording of intracellular membrane potential and effective membrane

Fig. 2 Time course of changes in pancreatic juice flow and amylase output during continuous perfusion of the isolated pancreas with 3×10^{-7} M ACh in the standard medium (A). The perfusion medium was changed from the standard medium to a Ca-deficient medium in the course of continuous stimulation (B). Each value represents the mean of several experiments as indicated. The stippled horizontal bar indicates the period of ACh perfusion.

Fig. 3 Time course of changes in pancreatic juice flow and amylase output during continuous perfusion of the isolated pancreas with 10^{-6} M ACh in the standard medium (A). The perfusion medium was changed from the standard medium to a Ca-deficient medium in the course of continuous stimulation (B). Each value represents the mean of several experiments as indicated. The thin stepped line represents output into the portal vein. The stippled horizontal bar indicates the period of ACh perfusion.

resistance from an acinar cell of the perfused pancreas. Perfusion with the standard solution containing 5×10^{-8} M ACh resulted in a minute depolarization followed by gradual recovery towards the resting level. The depolarization was associated with a burst of miniature depolarizations and these changes coincided with a definite increase in amylase output into the common duct (Fig. 4).

One minute stimulation with 10^{-5} M ACh was enough to cause a slow and large depolarization of an acinar cell which coincided with a small increase in amylase output into the common duct (Fig. 5). The regain of output immediately after the initial small increase induced by 10^{-5} M ACh may be due to a response for diluted ACh, since continuous stimulation with 10^{-5} M ACh caused only a small transient increase in amylase output into the common duct (Y. Habara, unpublished data) (Fig. 5). ACh, 10^{-7} M, evoked a small transient depolarization of the same acinar cell, and a definite increase in amylase output (Fig. 5).

Redox State and Amylase Output

A clue to the mode of action of mitochondria

in an intact cell has been provided by studies made with the measurement of the NAD(P)H-fluorescence (4). Fig. 6 shows a typical recording of the NAD(P)H-fluorescence from the isolated and perfused pancreas. Perfusion with the standard solution containing 5×10^{-8} M ACh produced almost no change in the fluorescence except a small transient deflection immediately after initiation and termination of the continuous stimulation, and this change coincided with a transient rise in amylase output followed by a prolonged output into the common duct. Continuous stimulation with 3×10^{-7} M ACh in the standard medium induced an increase in NAD(P)H-fluorescence (reduction) towards a maximum of 20% of the resting level (Fig. 7). This change coincided with the initial rise in amylase output followed by gradually declining phase of output into the common duct (Fig. 7).

Continuous stimulation with 10^{-6} M ACh in the standard medium induced a transient decrease followed by a gradual increase in NAD(P)H-fluorescence towards a maximum of 20% of the resting level. This change coincided with a slight increase in amylase output into the common duct (Fig. 8).

Fig. 4 An example of a recording of membrane potential with superimposed displacements resulting from the passage of current pulses (50 msec duration) through the recording electrode. The bar above the current recording indicates the period of perfusion with the standard solution containing $5 \times 10^{-8} \text{ M}$ ACh. The top records are the parts of continuous recording (shown by the dashed arrows) at high magnitude in the interest of distinguishing the burst of miniature depolarizations during the continuous stimulation. The time course of amylase output of this flushing preparation is shown by the stepped line. The stippled horizontal bar indicates the period during which ACh was present.

DISCUSSION

$[Ca]_o$ on the Secretory Response

The present experiments provide evidence that ACh requires external Ca in the perfusing solution in order to induce amylase output in the isolated and perfused rat pancreas. The requirement of external Ca was clearly demonstrated when the pancreas was stimulated with $5 \times 10^{-8} \text{ M}$ ACh. This is in agreement with the result obtained in the secretory response induced by 5 mU/ml CCK-PZ (10, 12, 14).

Membrane Potential and Secretory Responses

It has been suggested that there are two components in the effect of CCK-PZ on the membrane potential of the acinar cells: transient depolarization which coincides with an increase in Na permeability in the initial phase, and continuous hyperpolarization due to an electrogenic Na pump which conceals the transient depolarizing phase when the pump is dominant (9). This view was supported by the fact that

CCK-PZ at 5 mU/ml hyperpolarized the pancreatic acinar cell, and the hyperpolarization was inhibited in the environments that excluded the contribution of the electrogenic Na pump to the membrane potentials of nerve and muscle cells (9). It seems possible that the initial phase corresponds to the inward movement of the complex composed of a carrier molecule bearing Ca and Na ions, and the latter hyperpolarizing phase is due to the activity of the Na pump (11, 12). A stable counterbalance between the hyperpolarizing and depolarizing effect may be an explanation for the present result that $5 \times 10^{-8} \text{ M}$ ACh caused almost no change in membrane potential coinciding with a sustained amylase output. The counterbalance, however, seems to reflect on the burst of miniature depolarizations recorded during the continuous stimulation with $5 \times 10^{-8} \text{ M}$ ACh.

Redox State and Secretory Response

Mitochondria of animal tissues are very active in respiration-coupled inward transport of cytoplasmic free Ca (for reference, see 16), which

Fig. 5 An example of a recording of membrane potential with superimposed displacements resulting from the passage of current pulses (50 msec duration) through the recording electrode. The bars above the current recording indicates the period of perfusion with the solution containing 10^{-5} M ACh and 10^{-7} M ACh, respectively. The top records are the parts of continuous recording (shown by the dashed arrows) at high magnitude in the interest of distinguishing the change in membrane potential. The time course of amylase output of this preparation is shown by the stepped line. The stippled horizontal bar indicates the period during which ACh was present.

Fig. 6 An example of a long recording of NAD(P)H-fluorescence combined with a simultaneous measurement of amylase output. The stippled horizontal bar indicates the period during which 5×10^{-8} M ACh was present. Note that, in this experiment, the vascular flow was 2 ml/min, which was about 1.5-fold as much as the rate used in the preceding experiments.

may determine the rate of extrusion of zymogen granules. It is also known that the inward transport of cytoplasmic Ca coincides with inhibition of ATP reproduction in the mitochondria (16). The activity of mitochondria may reflect on the oxidation-reduction state of the pyridine nu-

cleotides (redox state). Simultaneous recordings of the redox state and amylase output showed that continuous stimulation with 5×10^{-8} M ACh induces a gradual and slight increase in NAD(P)H-fluorescence and a definite and continuous increase in amylase output. An

Fig. 7 An example of a long recording of NAD(P)H-fluorescence combined with a simultaneous measurement of amylase output. 3×10^{-7} M ACh was present. Note that the vascular flow was 2 ml/min in this experiment.

Fig. 8 An example of a long recording of NAD(P)H-fluorescence combined with a simultaneous measurement of amylase output. The stippled horizontal bar indicates the period during which 10^{-6} M ACh was present. Note that the vascular flow was 2 ml/min in this experiment.

interpretation of these results may be that the secretory response induced by 5×10^{-8} M ACh coincides with a counterbalance between the activity of mitochondria reproducing ATP and the inverse activity of transporting cytoplasmic Ca.

Response Induced by ACh at a Higher Concentration

The responses induced by 3×10^{-7} M ACh were different from those induced by 5×10^{-8} M ACh. In the standard medium, 3×10^{-7} M ACh induced (a) a rapid increase in amylase output followed by a rapid declining phase, (b) a small transient depolarization, and (c) a gradual increase in NAD(P)H-fluorescence. The present result that 3×10^{-7} M ACh caused no change in the membrane potential is in agreement with the result shown by Petersen and Matthews (18). In the Ca-deficient medium, ACh at the same concentration (3×10^{-7} M), induced a rapid increase in amylase output followed by a further acceleration of the declining phase.

The response induced by 10^{-6} M ACh was a small increase in amylase output which was not diminished in the Ca-deficient medium, and which was followed by a gradual increase in amylase output into the portal vein. Continuous stimulation with 10^{-6} M ACh caused a transient decrease in NAD(P)H-fluorescence (oxidation) followed by a gradual increase in the fluorescence. If these changes in fluorescence may, at least in part, be a reflection of the activity of mitochondrial pyridine nucleotides in the pancreatic acinar cells, the following mechanism demonstrated in isolated mitochondria (16) may also be applied to the acinar cells: a relatively reduced state of the mitochondrial pyridine nucleotides promotes uptake and retention of Ca and a relatively oxidized state promotes Ca release from the mitochondria. The increase in $[Ca]_i$ may in turn cause an increase in the rate of exocytosis of the zymogen granules. The responses induced by 10^{-5} M ACh were a small increase in amylase output which coincided with a definite transient depolarization.

These results lead us to conclude that the influx of external Ca is decreased and the release of Ca from an intracellular Ca store is increased when the pancreas is stimulated with ACh at the higher concentration. The present conclusion is consistent with the results reported in previous papers as follows. Case and Clausen (3) showed that amylase released by 10^{-5} M

ACh or 50 mU/ml CCK-PZ was not affected by a 25-fold change (from 2.54 to 0.1 mM) in the external concentration of Ca in the isolated rat pancreas. Williams and Chandler (21) showed that the dose-response relation for bethanechol stimulation of pancreatic amylase release in a Ca-deficient medium was shifted downward by about 50%, but was strikingly parallel to the relation obtained in 2.56 mM Ca. The relations, however, were obtained in doses of bethanechol over the range 3×10^{-7} M– 10^{-5} M. Renckens, Schrijen, Swarts, de Pont and Bonting (19) showed that 10^{-5} M carbachol induced pancreatic protein release from isolated rabbit pancreas even in a Ca-free medium.

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