DOSE-DEPENDENT EFFECTS OF ACETYLCHOLINE STIMULATING RESPIRATORY CHAIN AND SECRETION OF ISOLATED PERFUSED RAT PANCREAS

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ABSTRACT

Redox states of cytochromes and nicotinamides, nucleotides, oxygen consumption, and secretory responses were recorded simultaneously and continuously in the isolated perfused rat pancreas. Continuous stimulation with 3×10^{-8} M acetylcholine (ACh) reduced cytochromes, $a(a_3)$, b , and $c + c_1$ to 20% of their respective maximum control levels; increased NAD(P)H-fluorescence to 20% greater than control level; increased oxygen consumption; and increased secretory response (pancreatic protein output). Continuous stimulation with ACh at higher concentrations induced greater respiratory and secretory responses. The dose-response curve of ACh-induced oxygen consumption paralleled that of NAD(P)H-fluorescence, suggesting that the NAD(P)H/NAD(P) ratio and the ATP biosynthesis are increased by ACh. In contrast to this relationship, continuous stimulation with ACh induced a dose-dependent decrease in ATP level in the isolated perfused rat pancreas, suggesting that the rate of hydrolysis of ATP is also increased by ACh. The dose-response relation of ACh-induced reduction of cytochromes resembled that of ACh-induced NAD(P)H-fluorescence except that there was an excessively large reduction with 10^{-7} M ACh. The activity-linked reduction of electron transfer system seems to coincide with the mitochondrial ATP biosynthesis and Ca^{2+} uptake. The mitochondrial Ca^{2+} uptake, in turn, maintains intracellular Ca^{2+} concentration within a narrow range that is known to be most effective for the stimulus-secretion coupling in the pancreatic acinar cell.

Cholecystokinin-pancreozymin (CCK) and acetylcholine (ACh) are the physiological secretagogues to which the pancreatic acinar cells respond by releasing their contents of zymogen granules. ACh is known to act like CCK, although via a specific receptor, and it is believed that both these secretagogues bring about their effects by increasing the cytoplasmic concentration of free, diffusible Ca^{2+} . The process of stimulus-secretion coupling requires metabolic energy (9), most of which may be supplied from mitochondria (1). With the aid of a scanning organ spectrophotometer and a fluorometer, a previous study in our laboratory

showed that CCK acts, in physiological concentration, on the acinar cells to reduce cytochromes, $a(a_3)$, b , and $c + c_1$, and to increase NAD(P)H-fluorescence several minutes prior to the initiation of enzyme release (11). Furthermore, that study provided evidence that there was a dose-dependent change in the redox state since continuous stimulation with 10^{-10} M CCK induced immediate reduction of cytochromes, almost no change in NAD(P)H-fluorescence, and a gradual increase in protein output; whereas continuous stimulation with 10^{-9} M CCK induced immediate marked reduction of cytochromes, a small increase in NAD(P)H-

fluorescence, and a large rapid increase in protein output followed by a gradual return to the resting level. A dose-dependent difference in stimulus-secretion coupling was also shown when the isolated perfused rat pancreas was stimulated with ACh since continuous stimulation with 10^{-8} M ACh induced an increase in secretory response, which was significantly decreased in a Ca-deficient environment, and was not associated with an increase in NAD(P)H-fluorescence, but continuous stimulation with 10^{-6} M ACh induced a small increase in secretory response, which was further enhanced in the Ca-deficient environment, and was associated with an increase in NAD(P)H-fluorescence (10). In the present study, the dose-dependent difference in metabolic energy supply was further studied by recording reduction of cytochromes, NAD(P)H-fluorescence, oxygen consumption, and ATP content in the pancreas before and during continuous stimulation with ACh at different concentrations.

MATERIALS AND METHODS

Isolation and Perfusion of the Pancreas

Wistar 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 (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 flow was kept constant at 2 ml/min by a roller pump. The hepatic end and the duodenal end of the pancreatic duct were also cannulated, and the duct was perfused with 160 mM NaCl solution containing phosphate buffer (pH 7.1) at a flow rate of 2 ml/min. The perfusate of the duct was collected from the duodenal outlet cannula. 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 modified Krebs-Henseleit solution, maintained at 37°C.

Solution and Drugs

The composition of the control Krebs-Henseleit solution used for perfusing and bathing the preparation was as follows (final concentrations in mM): NaCl, 131; KCl, 5.6; CaCl_2 , 2.5; NaHCO_3 , 25; MgCl_2 , 1; NaH_2PO_4 , 1; glucose,

5. 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. ACh (Sigma, St. Louis) was added into the perfusing solution.

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.* (20), with bovine serum albumin as a standard.

Recording of Oxidation-Reduction of Cytochromes

Oxidation-reduction of cytochromes in the isolated perfused rat pancreas was recorded with a scanning organ spectrophotometer (Tateisi Institute of Life Sciences, Kyoto). The mechanical and electrical details of this instrument have already been described in detail (17). Briefly, the instrument consists of a light source, wavelength scan, light guide, photodetector, and data processor. Characteristics of each component are as follows: The light source is a 300 W xenon arc lamp. The wavelength scan is performed by vibration of a grating (Jobin Yvon, M27R; 600 grooves/mm). Scanning time over the range 400–650 nm is 1 sec. Each wavelength is read with a rotary encoder. A two-branched light guide made of quartz fiber receives the separated lights from the monochromator leading them to a sample and a reference. The reflected light rays are collected and lead to the respective photomultipliers. The photoelectric signals from the photomultipliers are amplified and converted to digital signals. The data processing and control of the instrument are carried out with a microprocessor, which contains computing programs for absolute spectrum, difference spectrum, time course of difference between two points in the spectrum, and mean value of data.

Recording of NAD(P)H-fluorescence

Change in NAD(P)H-fluorescence of the isolated perfused rat pancreas was recorded with a fluorometer (Redoximeter, Tateisi Institute of Life Sciences, type HER-4, Kyoto, Japan). The mechanical and electrical details of this instrument were originally described by Koba-

yashi *et al.* (16). Briefly, the instrument is a modification of the microfluorometer introduced by Chance *et al.* (2) that records field of tissues upon the 366 nm excitation-light and 460 nm emission. The fluorescence intensity is a function of the amount of reduced form of the nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), so that a direct and continuous measurement of the relative value of the intracellular redox state can be attained.

Measurements of O₂ Tension and ATP Content

Polarographic measurements of relative changes in local tissue oxygen tension were made with a Clark-style electrode (Model 125/05; Intech Laboratories, Philadelphia, U.S.A.) which was connected to a chemical microsensor (Model 1201; Transidyne General Corporation, Ann Arbor, MI, U.S.A.). ATP content was assayed by use of an ATP-test kit (Boehringer-Mannheim, Mannheim, Germany) after freezing and homogenizing the isolated perfused pancreas as reported previously (13).

Statistics

Results are expressed as the mean $\pm$ SE of several experiments, and are analyzed by Student's *t* test.

RESULTS

Cytochrome Reduction and Secretory Response of the Isolated Pancreas to Continuous Stimulation with ACh

The isolated pancreas was first perfused with standard solution for 20 min to reach steady levels, after which, the absorption spectrum of the cytochromes was measured. The tip of the light guide of the scanning organ spectrophotometer was placed on the surface of the isolated perfused rat pancreas. The average of 16 scannings of absorption spectra was computed within 16 sec and stored in the built-in microprocessor. The differences between the stored initial average and the subsequent average were continuously computed, and were displayed as the difference spectra every 16 sec throughout the experiments. The standard solution was changed to the solution containing 3×10^{-8} M ACh, and the same measurements were continued for an additional 30 min. The ACh solution was replaced with the standard solution

and a third set of measurements were made. The standard solution was finally replaced with the anoxic (N₂-gassed) solution. After perfusing with the anoxic solution for 5 min, the perfusion was stopped, and a final set of measurements was obtained. The steady levels of the final set of measurements were considered to be the maximum reduction levels for the cytochromes. At the maximum reduction levels, the difference between optical density at 575 nm and that of cytochrome *a*(*a*₃) (605 nm); at 575 nm and that of cytochrome *b* (562 nm); or at 540 nm and that of cytochrome *c*+*c*₁ (550 nm) was taken as 100% reduction of the respective cytochromes. The changes in redox states of cytochromes were expressed in terms of percent reduction, and displayed on the C.R.T. (cathode ray tube) console, and recorded on a line printer. Fig. 1 shows an example of the time course of the redox states of the cytochromes and difference spectra taken before, during, and after the perfusion with the solution containing 3×10^{-8} M ACh. Reduction of cytochromes, *a*(*a*₃), *b*, and *c*+*c*₁, was detected in the difference spectrum obtained 2 min after the start of perfusion with the ACh solution. Reduction of these cytochromes reached peak levels of about 20% of respective maximum levels about 6 min after the start of perfusion with ACh solution. Redox state of these cytochromes returned very slowly to resting levels when the ACh solution was replaced with the standard solution. While measurements of redox states of cytochromes were being made, perfusates were collected from the common duct and subjected to analysis for pancreatic digestive protein. Correlation among reduction of cytochromes and secretory response of exocrine pancreas is clearly shown in Fig. 2. The protein output gradually increased to reach the maximum level 6 min after initiating the continuous stimulation with 3×10^{-8} M ACh and then gradually decreased toward the resting level.

When the isolated perfused rat pancreas was continuously stimulated with 10^{-7} M ACh, the reduction of cytochromes was potentiated to reach peak levels of about 50% of respective maximum levels about 6 min after the start of perfusion with ACh solution (Fig. 3). The peak levels of cytochrome reduction were maintained during the continuous stimulation, and returned slowly to respective resting levels after cessation of the stimulation. Comparing the time course of the reduction of cytochromes with that of the secretory response showed that these two responses ascended simultaneously

Fig. 1 Examples of records of cytochrome reduction in the isolated perfused rat pancreas. *Left*: Records obtained 6 min after beginning of continuous stimulation with 3×10^{-8} M ACh. *Top*, time course of difference spectrum of cytochrome $c + c_1$. *Middle*, time course of difference spectrum of cytochrome b. *Bottom*, difference spectrum of cytochromes, $a(a_3)$, b and $c + c_1$, are indicated on the spectrum. *Right*: Records obtained at the maximum reduction levels 5 min after beginning of flow stop followed by 5 min perfusion with the anoxic (N_2 -gassed) solution. Horizontal bars above the top record indicate the period of perfusion with 3×10^{-8} M ACh, and of anoxic treatment. N, period of perfusion with N_2 -gassed solution followed by flow stop (S). Records on top, middle and bottom, and other symbols are as in the left.

but the plateau phase of cytochrome reduction occurred during the descending phase of secretion (Fig. 3). Further increase in ACh concentration to 10^{-6} M caused reduction of cytochromes, which reached peak level of about 40% of respective maximum level about 6 min after the start of stimulation (Fig. 4). The peak levels of cytochrome reduction were maintained during the continuous stimulation. Continuous stimulation with 10^{-6} M ACh caused a slow rise in secretory response to reach a peak level about 8 min after the start of stimulation. The peak level of secretory response was about half of that produced with 10^{-7} M ACh, and declined to the resting level during the stimulation (Fig. 4). Stimulation of the isolated perfused rat pancreas with 10^{-5} M ACh, the highest concentration used in the present experiments, caused (a) an immediate reduction of cytochromes to reach peak level of about 50% of respective maximum level within 2 min after the start of stimulation, and (b) a very slow rise in secretory response to reach a peak level about 18 min after the start of stimulation (Fig. 5). The peak levels of cytochrome reduction were maintained but the secretory response decreased

gradually to the resting level during the stimulation.

NAD(P)H-fluorescence Induced by Continuous Stimulation with ACh

The tip of the light guide of a fluorometer (Redoximeter) was placed on the surface of the isolated perfused rat pancreas. The isolated pancreas was first perfused with the control solution for 20 min to allow the response to reach steady levels. After this period, fluorescence of NAD(P)H was recorded for 10 min to determine the 'resting' levels. The vascular perfusion was then switched from the control solution to the solution containing ACh, and the fluorescence measurement was continued for a further 30 min. The perfusing solution was replaced by the drug-free control solution, and a third set of measurements was obtained. The perfusing solution was finally replaced with the anoxic N_2 -gassed solution, and a fourth set of measurement was made. After perfusion with the anoxic solution for 5 min, the perfusion was stopped, and a final set of measurements was obtained. The resting level of fluorescence

Fig. 2 Time course of cytochrome reduction and protein output before, during, and after continuous perfusion of isolated perfused rat pancreas with Krebs-Henseleit solution containing 3×10^{-8} M ACh followed by anoxic treatment. The horizontal bars indicate the period ACh was present, the period of perfusing with a N_2 -gassed (N), and the period of stopping perfusion after perfusing with N_2 -gassed solution (S). Each value represents the mean ($\pm$ SE) of five experiments.

Fig. 3 Time course of cytochrome reduction and pancreatic protein output before, during, and after the continuous perfusion of isolated rat pancreas with a solution containing 10^{-7} M ACh followed by anoxic treatments. Symbols in the horizontal bars are as in Fig. 2. Each value represents the mean ($\pm$ SE) of five experiments.

was taken as zero level, and the steady levels of the final set of measurement was taken as maximum 100% level for NAD(P)H-fluorescence. An example of recording of NAD(P)H-fluorescence is shown in Fig. 6. Perfusion with the standard solution containing 10^{-6} M ACh induced an increase in NAD(P)H-fluorescence, which reached the peak level of about 30% of the maximum level. In this case, the time course of protein output induced by stimulation with 10^{-6} M ACh was similar to that depicted in Fig. 4. Similar results were obtained in other 4 experiments. Continuous stimulation with ACh at a low concentration (3×10^{-8} M) induced small flat increase in protein output associated with slight increase in NAD(P)H-fluorescence. Continuous stimulation with ACh at a high concentration (10^{-6} to 10^{-5} M) induced a dose-dependent increase in NAD(P)H-fluorescence, whereas the secretory response to ACh at high concentrations was significantly smaller than that to 10^{-7} M ACh (Fig. 7).

Fig. 4 Time course of cytochrome reduction and pancreatic protein output before, during, and after the continuous perfusion of isolated rat pancreas with a solution containing 10^{-6} M ACh followed by anoxic treatments. Symbols in the horizontal bars are as in Fig. 2. Each value represents the mean ($\pm$ SE) of five experiments.

Fig. 5 Time course of cytochrome reduction and pancreatic protein output before, during, and after the continuous perfusion of isolated rat pancreas with a solution containing 10^{-5} M ACh followed by anoxic treatments. Symbols in the horizontal bars are as in Fig. 2. Each value represents the mean ($\pm$ SE) of five experiments.

Increase in Oxygen Consumption Induced by Continuous Stimulation with ACh

Oxygen tension (pO_2) obtained with Clark-style oxygen electrode placed into the inlets of the vascular perfusion in 10 preparations ranged from 450 to 500 mmHg (mean 470 ± 5 mmHg). pO_2 obtained with the oxygen electrode placed into the outlet of the vascular perfusion in 20 preparations ranged from 150 to 200 mmHg

(mean 170 ± 5 mmHg) in the resting state, and it decreased when the isolated pancreas was perfused with the control solution containing ACh. Fig. 7 shows examples of the response to ACh at the concentrations ranging from 3×10^{-8} M to 10^{-5} M. A dose-response relation was obtained for ACh-induced increase in oxygen consumption (Figs. 7 and 8).

ATP Content of Isolated Perfused Pancreas before and after Stimulation with ACh

The ATP content of homogenate of the isolated pancreas was measured by means of the ATP-test kit (Boehringer-Mannheim, Mannheim, Germany) after perfusing the pancreas for 10 min with the control solution and after perfusion for 10 min with the solutions containing ACh. As shown in Table 1, ATP content was decreased during the ACh perfusion in a dose related way.

DISCUSSION

Correlation among Cytochrome Reduction, NAD(P)H-fluorescence and Secretory Response

Fig. 7 shows dose-response relation of ACh-induced oxygen consumption, NAD(P)H-fluorescence, reduction of cytochrome $c+c_1$, and secretory response (pancreatic protein output) in the isolated perfused rat pancreas. The dose-response curve of ACh-induced oxygen consumption paralleled that of NAD(P)H-fluorescence. These two curves show that increase in ACh concentration induces a smooth and gradual increase in the responses. These two curves resemble the dose-response relation of cerulein-induced uptake of $[^3H]2$ -deoxy-D-

Fig. 6 An example of recording of NAD(P)H-fluorescence obtained before, during, and after the continuous perfusion of isolated rat pancreas with a solution containing 10^{-6} M ACh followed by anoxic treatments. Symbols in the horizontal bars are as in Fig. 2.

Fig. 7 Comparison of oxygen consumption, NAD(P)H-fluorescence, reduction of cytochrome $c+c_1$, and pancreatic protein output in isolated rat pancreas perfused with a solution containing different concentrations of ACh. Values of oxygen consumption represent the mean of a 10 min measurement in three preparations. Values of NAD(P)H-fluorescence represent the mean of a 30 min measurement in five experiments. Values of reduction of cytochrome $c+c_1$ and protein output represent the means of a 30 min measurement as indicated in Figs. 2, 3, 4, and 5. Values shown by filled circles represent the corresponding resting values before stimulation with ACh.

glucose in isolated mouse pancreatic acini (18). These results strongly suggest that the NAD(P)H/NAD(P) $^+$ ratio and ATP biosynthesis may concomitantly be increased in accordance with an increase in ACh concentration. In contrast to these dose-dependent increases in oxygen consumption and NAD(P)H-fluorescence, continuous stimulation with ACh induced a dose-dependent decrease in ATP content in the isolated perfused rat pancreas (Table 1). This result is compatible with that of Baudin *et al.* (1), who showed that stimulation of pancreatic enzyme secretion with carbamylcholine diminished pancreatic ATP level. However, Williams *et al.* (25) reported that there was no effect of secretagogue (carbacol or caerulein) concentrations maximally effective for amylase release on

Fig. 8 Examples of recordings of oxygen consumption obtained before and during continuous perfusion of isolated rat pancreas with a solution containing different concentrations of ACh as indicated. The horizontal bar indicates the period ACh was present.

ATP content whereas supramaximal secretagogue concentrations actually increased ATP levels. It is highly possible that continuous stimulation with ACh induces bidirectional changes, ATP biosynthesis and hydrolysis; and the latter may exceed the former in the isolated perfused rat pancreas. The dose-response rela-

Table 1 Effect of Continuous Stimulation with ACh at Various Concentration on ATP Content of the Rat Pancreas

Concentration of ACh	ATP content ($\mu\text{mol/g}$ wet weight)
Control (4)	0.81 ± 0.07
3×10^{-8} M (4)	0.65 ± 0.11
1×10^{-6} M (5)	$0.44 \pm 0.13^*$
1×10^{-5} M (4)	$0.38 \pm 0.06^{**}$

ATP content was measured 10 min after beginning of the continuous stimulation. All values are the mean $\pm$ SE of the number of observations in parentheses. * $P < 0.05$, ** $P < 0.01$

tion of ACh-induced reduction of cytochrome $c+c_1$ resembles that of ACh-induced NAD(P)H-fluorescence, except for the distinct large reduction which occurred with 10^{-7} M ACh. Not only the reduction level of cytochrome $c+c_1$ but also that of cytochromes $a(a_3)$ and b induced with 10^{-7} M ACh are greater than the corresponding levels induced with 10^{-8} or 10^{-6} M ACh (Fig. 9). The cytochrome reduction induced with 10^{-7} M ACh may consist of two components; one coincides with a concomitant increase in oxygen consumption and in NAD(P)H-fluorescence, and the other does not. The latter component seems to correlate with the largest secretory response induced by continuous stimulation with 10^{-7} M ACh.

Time Courses of Responses

Continuous stimulation with 10^{-7} M ACh induced the largest secretory response which consisted of the following two phases; initial sharp rise followed by a second plateau. The previous study (10) has shown that the second plateau phase induced with 3×10^{-7} M ACh was strongly inhibited when calcium was removed from the perfusing and bathing solution, whereas the first phase was still present in the calcium-deficient environment. The result is compatible with the view that the secretory response of the exocrine pancreas may consist of the following two phases; an initial phase evoked by increase in release of calcium from calcium stores in the acinar cell, and a second

phase resulting from calcium influx. Continuous stimulation with 3×10^{-8} M ACh induced a small monophasic secretory response, *i.e.*, a gradual rise followed by plateau. A major component of the response may be due to calcium influx, since the previous study (10) has shown that the response induced by 5×10^{-8} M ACh was decreased to values of about 30% of control when calcium was omitted from the perfusing and bathing solution. Continuous stimulation with 10^{-6} M or 10^{-5} M ACh induced a biphasic secretory response, *i.e.*, a markedly delayed rise followed by a gradual decline. A major component of the response may be due to release of calcium from calcium stores in the acinar cell, since the previous study (10) has shown that the response induced with 10^{-6} M ACh was changed to a fast sharp rise followed by a rapid decline when calcium was omitted from the perfusing solution.

These results show that the maximum secretory response induced by continuous stimulation with 10^{-7} M ACh is a transition from the response due to increase in calcium influx to that due to release of calcium from intracellular stores. The maximum secretory response may, thus, correspond to the largest cytochrome reduction.

Calcium and the Responses

It is commonly believed that the secretory effect of the secretagogues is exerted by increasing the cytoplasmic concentration of free, diffusible

Fig. 9 Mean peak level of cytochromes, $a(a_3)$, b , and $c+c_1$, calculated from the records obtained during continuous stimulation for 3 min with different concentration of ACh as indicated. The mean values were calculated from data depicted in Figs. 2, 3, 4, and 5. The values are shown as percent of the maximum reduction of corresponding cytochromes. Vertical lines are standard errors of the mean values.

calcium in the acinar cell. O'Doherty and Stark (22) has recently provided direct evidence supporting the concept that acinar cell secretion is triggered by increase in $[Ca^{2+}]_i$, which has ability to act as a primary intracellular mediator. They have also shown that Ca^{2+} , as an intracellular mediator during the secretory process, is most effective in the comparatively narrow range of calcium activities between 0.4 and 1 μM . Dormer and Williams (5) concluded that stimulation of isolated mouse pancreatic acini by carbachol results in the release of calcium from a microsomal compartment leading to a rise in cytoplasmic Ca^{2+} , increased exchange with granule and mitochondrial Ca^{2+} , and increased efflux of Ca^{2+} from the cell. They suggested that the most likely sites from which calcium release is stimulated by carbachol may be the endoplasmic reticulum and presumably elements of the Golgi apparatus. The study carried out in isolated dispersed rat pancreatic acini by Wakasugi *et al.* (24) suggested that there was both mitochondrial and nonmitochondrial ATP-dependent Ca^{2+} storage systems in pancreatic acini, and the latter was likely to be located in the rough endoplasmic reticulum. Dormer *et al.* (4) showed that antimycin A inhibited $^{45}Ca^{2+}$ uptake but stimulated washout, suggesting that the rate limiting process for calcium may be an ATP-dependent transport process, although there may also be a component of stimulated calcium efflux which requires energy. These and other studies support the view that influx, efflux and subcellular distribution of calcium may be, at least in part, ATP-dependent processes.

Activity-linked Redox Changes

The activity-linked redox changes were initially described in the classical review by Chance and Williams (3) who proposed five respiratory states for isolated mitochondria. State 1 (fresh state) and state 4 (resting state) are both characterized by a slow rate of respiration which is limited by the availability of ADP. Both of these states are also characterized by a sequence of increasing reduction from cytochrome *c* to NADH (6). As differentiation of state 4 from state 1 is not easy in the intact tissue, both of these states are regarded as a resting respiratory state. In view of the correlation between oxidative metabolism and secretion, the most important aspect of the respiratory states concerns a transition between the resting state and state 3, the active state. The mitochondrial

transition from resting state to activated state should be characterized by an increase in respiration and by redox changes in the respiratory chain components, notably in oxidation of most of the components (3, 6, 21).

The essential features of these respiratory states in the isolated mitochondria have been regarded as models of those in the intact tissue. However, the present study carried out on the isolated perfused rat pancreas provided the evidence that cytochromes, $a(a_3)$, *b*, and $c+c_1$, were not oxidized but reduced when the pancreas was continuously stimulated with ACh. The activity-linked cytochrome reduction of respiratory chain components has already been demonstrated in the isolated gastric mucosa when acid secretion was evoked by histamine (8). The activity-linked reduction was also shown in the isolated perfused rat pancreas when pancreatic enzyme secretion was evoked by cholecystokinin (11), in the isolated perfused adrenal gland of dog when stimulated with ACh (23), in the perfused brain of bullfrog when stimulated with CCK (13) or high potassium (12). The activity-linked reduction in the isolated gastric mucosa has been interpreted 1) as being due to local anoxia (15); 2) as reflecting the properties of all the respiratory-chain components and not anoxia (7); and 3) as describing a mitochondrial transition from a substrate-depleted (state 2) to an active (state 3) state (6). The activity-linked reduction of respiratory chain components observed in the isolated perfused rat pancreas may hardly be interpreted as being due to local anoxia. The main argument for the first interpretation is that unstirred anaerobic layers may exist and serve to limit the supply of oxygen to the respiring tissue sufficiently to produce a core region of anoxia in the isolated gastric mucosa (15), but such anaerobic layers do not exist in the isolated perfused rat pancreas. Furthermore the first interpretation is contradictory to the following evidence: 1) the oxygen tension of the perfusing solution at the inlets of the vascular perfusion was about 470 mmHg and that of the perfusate was not lower than 150 mmHg. 2) The rate of vascular flow was kept constant at 2 ml/min; a rate twice that used in a previous study (14) to assure large and stable secretory responses to the secretagogues in the isolated pancreas. The second interpretation may be adequate for the activity-linked reduction, and is compatible with the present finding that stimulation with ACh induced parallel reduction of all of the cytochromes, $a(a_3)$, *b* and $c+c_1$.

(Fig. 9). If we hold the idea that the resting to active transition corresponds to a mitochondrial state transition, we must conclude that the resting condition is characterized by essentially substrate-starved mitochondria, *i.e.*, state 2 (6). The state 2 (starved state) is usually achieved under aerobic conditions at low temperature (10–12°C), and it does not correspond to the present experimental condition. Thus the activity-linked reduction of electron transfer system may hardly be interpreted as transition of state.

A novel explanation for the activity-linked reduction seems to be in mitochondrial Ca^{2+} uptake. Lehninger *et al.* (19) showed that the redox state of mitochondrial NAD^+ is a very important factor in the regulation of Ca^{2+} uptake and efflux from liver, heart, and tumor mitochondria: when the mitochondrial NAD^+ is in a largely reduced steady state (*i.e.*, when the NADH/NAD^+ ratio is high), the rate of efflux of Ca^{2+} is minimal and the mitochondria tend to retain accumulated Ca^{2+} . The activity-linked reduction of electron transfer system may correspond to the mitochondrial Ca^{2+} uptake, which in turn maintains intracellular Ca^{2+} concentration within the narrow range between 0.4 and 1 μM . The narrow range is known to be most effective for the stimulus-secretion coupling in the pancreatic acinar cell (22).

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