MARKED REDUCTION OF THE CYTOCHROMES INDUCED IN BULLFROG BRAIN BY PERFUSING WITH A CALCIUM-DEFICIENT OR A HIGH POTASSIUM SOLUTION

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ABSTRACT
Redox states of cytochromes, a(a3), b, and c+c1, were recorded simultaneously and continuously in the perfused bullfrog brain with the aid of a scanning spectrophotometer. The tip of the light guide of a spectrophotometer was placed on the surface of cerebral hemispheres. Continuous perfusion with a Ca-deficient solution induced immediate marked reduction of cytochromes without any additional procedure in some preparations. In other preparations, the cytochrome reduction induced by the Ca-deficient solution was observed after the procedure of reversible osmotic opening of the blood-brain barrier. Continuous perfusion with solution containing high KCl also induced immediate reduction of cytochromes. The results show that decrease in Ca or increase in K in the blood vessels of the brain acts on the respiratory chain in mitochondria of cells in the cerebral hemispheres to induce immediate reduction of cytochromes. The reduction induced by these changes in ionic environments does not appear to be due to hypoxia.

KEY WORDS cytochromes / brain / Ca-deficient solution / high-K solution / mitochondria / blood-brain barrier

The respiratory chain in mitochondria is the avenue for supplying free energy for almost all living cells to maintain their functions. This holds true especially for neurons and pancreatic acinar cells, in both of which the respiratory chain in mitochondria supply more than 90% of free energy. A fresh clue to the redox states of the respiratory chain in mitochondria of the cells in the perfused organ has recently been provided by scanning spectrophotometry (3-5). With the aid of a scanning spectrophotometer and a fluorometer, the previous study in our laboratory has shown that cholecystokinin, a physiological stimulus to the pancreatic acinar cell, acts on the respiratory chain in mitochondria of pancreatic acinar cells to induce immediate reduction of cytochromes prior to the initiation of enzyme output (3). We have now applied the scanning organ spectrophotometry to the brain of bullfrog, and recorded unexpected large reduction of cytochromes in the cerebral hemispheres during perfusion with a Ca-deficient solution, as well as with a high K solution.

MATERIALS AND METHODS

Perfusion of the Bullfrog Brain
Male and female bullfrogs (Rana Catesbiana) weighing about 600 g were used in the present experiments. The perfused brain in situ was prepared as follows. The animal was anesthetized by packing in ice until reflex responsiveness was lost. The caracocavicular nerves, brachial nerves and the seventh to tenth spinal nerves were cut to prevent movement of the animal after recovery from the anesthesia. The external carotid arteries were ligated. The inlets for vascular perfusion were the internal carotid
arteries, into which a plastic cannula, filled with perfusion solution, was inserted; advanced into the artery so that its tip was near the origin of the previously ligated external carotid artery; and tied in place. The outlets were the internal jugular veins. The rate of vascular flow was kept constant at 2.0 ml/min with the aid of roller pump. After the skin and muscles of the head were removed, the skull was opened to expose the cerebral hemispheres, onto which the tip of the light guide was placed (Fig. 1). The composition of the standard solution used for perfusing the brain was as follows (mM): NaCl, 110; KCl, 2.0; CaCl₂, 1.8; NaHCO₃, 2.6; glucose, 1.0; HEPES, 10. In the Ca²⁺ deficient solution, CaCl₂ was replaced with osmotically equivalent amounts of NaCl. In the high K solution, NaCl was replaced with equimolar KCl. Solutions were equilibrated with O₂ and had a pH of 7.4.

![Fig. 1 Schematic representation of the dorsal surface of bullfrog brain and the location of the tip of light guides.](image)

1. olfactory nerve; 2. olfactory lobe; 3. cerebral hemisphere; 4. parapophysis; 5. epiphysis; 6. optic lobe; 7. cerebellum; 8. trochelear nerve; 9. trigeminal nerve; 10. facial nerve; 11. auditory nerve

**Recording Oxidation-Reduction of Cytochromes**

Oxidation-reduction of cytochromes in the perfused bullfrog brain was recorded with a modified scanning organ spectrophotometer (Tateisi Institute of Life Sciences, Kyoto). The mechanical and electrical components of the modified type are essentially identical to those of the prototype, the characteristics of which have already been reported (3–5). Briefly, the instrument consists of the light source, the wavelength scan, the light guide, the photodetection, and the data processing. The light source is a 300 W xenon arc lamp. The wavelength scan is performed with vibration of a grating (Jobin Yvon, M27R; 600 grooves/nm) and the scanning time over the range 400–650 nm region 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 monochromater leading them to a sample and a reference. The reflected lights are collected and lead to the respective photomultipliers. The photoelectric signals from the photomultipliers are amplified for logarithmic operation and converted to digital with a high speed analog-digital converter to 12 bit signals (photodetection). The data processing and control of the instrument are carried out with a microprocessor. The data input interval corresponds to 0.25 nm for each wavelength. A floppy disc is utilized as an external storage device. The modified type is equipped with a microprocessor which contains computing programs of absolute spectrum, difference spectrum, time course of difference between two points in the spectrum, and mean value of data.

**RESULTS**

The bullfrog brain was first perfused with the standard solution for 10 min to reach steady levels, after which, the absorption spectrum of the cytochromes was measured. The tip of the light guide of the scanning spectrophotometer was placed on the surface of the cerebral hemispheres. The average of 16 scannings of absorption spectra was computed and stored in the built-in microprocessor. The differences between the stored initial average and the subsequent averages were continuously computed, and were displayed as the difference spectra every 16 sec throughout the experiments. The resting levels of difference spectra obtained during perfusion with the standard solution for
about 10 min usually showed cyclical fluctuations of minute amplitude. The vascular perfusion was then switched from the standard solution to the Ca-deficient solution, and these measurements were continued for a further 5 min. The perfusing solution was again replaced by the standard solution, and the measurements were continued for about 15 min, i.e. until the difference spectrum returned to the resting level. Two additional sets of measurements were obtained by intermittent perfusion with the Ca-deficient solution for 5 min. The perfusing solution was finally replaced by the anoxic N2-gassed solution. After perfusion with the anoxic solution for 5 min, the perfusion was stopped, and a final set of measurements was obtained. The steady levels of these final sets of measurements were considered to be the maximum reduction levels for the cytochromes. The maximum reduction levels were similar to the complete reduction levels that were achieved when the brain was perfused with the anoxic solution saturated with Na2S2O4, a strong reducing agent. At the maximum reduction level, the difference between optical density at 575 nm and that of cytochrome a(a3) (605 nm); at 575 nm and that of cytochrome b (562 nm); or at 540 nm and that of cytochrome c+c1 (550 nm) was taken as 100 percent reduction of the respective cytochromes. The changes in redox states of cytochromes during the entire course of the experiments were expressed in terms of percent reduction, and displayed on the C.R.T. console, and recorded on a line printer.

Fig. 2 shows an example of the time course of the redox states of the cytochromes and difference spectra taken before, during, and after the initial perfusion with the Ca-deficient solution. Reduction of cytochromes, a(a3), b, and c+c1, was detected in the difference spectrum obtained 1 min after the start of perfusion with the Ca-deficient solution. Reduction of these cytochromes reached peak levels of 80% of respective maximum reduction levels about 3 min after the initiation of the perfusion. Redox states of cytochromes returned to the resting levels when the Ca-deficient perfusing solution was replaced with the standard solution. The second and the third perfusions with the Ca-deficient solution also caused reduction of the cytochromes, but

![Graph showing time courses of redox states of cytochromes](image-url)

Fig. 2 Time courses of redox states of cytochromes (cyt), a(a3), b, and c+c1, recorded before, during and after perfusing with the Ca-deficient (Ca-def) solution on the left side; numbers and dots on top line (cyt a(a3)) correspond to time when difference spectra on left were recorded; open horizontal bars indicate the period of perfusion with the Ca-deficient solution. N, period perfusion with anoxic N2-gassed solution followed by flow stop (S)
the rate of change and the peak level of the reduction were smaller than those of the initial reduction. There are, however, large variations among individuals in the amplitude of reduction of cytochromes induced by perfusion with the Ca-deficient solution: reduction of cytochromes in many preparations did not always rise to such extent that was depicted in Fig. 2. A possible mechanism for this individual variation is in variation in individual development of the blood-brain barrier. In fact, such individual variation is demonstrated in the rat brain (8). The blood-brain barrier can be made permeable, in man and animals, by perfusing a hypertonic solution of a water-soluble nonelectrolyte such as urea, lactamide, mannitol, or arabinose into the carotid circulation (8). In other experiment, the effect of perfusion with the Ca-deficient solution was examined after the preceding perfusion with a solution containing various mannitol concentration (Fig. 3). The experiment shows that there is an optimum concentration of mannitol to induce osmotic opening of the blood-brain barrier in the bullfrog. The amplitude of the reduction was the largest after preceding perfusion with the solution containing 500 mM mannitol. In other four experiments, we further confirmed the effect of reversible osmotic opening of the blood-brain barrier on the reduction of cytochromes induced by perfusion with the Ca-deficient solution.

The effect of perfusion with the high K solution was examined in the next experiment. Fig. 4 shows an example of the time courses of redox states of cytochromes and difference spectra recorded before, during, and after perfusion with a solution containing various KCl concentration. Reduction of cytochromes was detected in the difference spectrum obtained 16 sec after the initiation of the perfusion with the high K solution. Reduction of these cytochromes reached peak levels of 50% of respective maximum reduction levels 1 min after the initiation of the perfusion. The reduction gradually returned to the resting level during the perfusion with high K solution, and returned to the resting level when the high K perfusing solution was replaced with the standard solution. Similar effect of high K perfusion on cytochromes was observed in the other two experiments.

DISCUSSION
Results of the present study showed that cytochromes, a(a3), b, and c+c1, were markedly and simultaneously reduced when the bullfrog

![Fig. 3](image-url)

Fig. 3 Time courses of cytochromes (cyt), a(a3), b, and c+c1, on the left recorded before, during, and after 30 sec perfusion with a hypertonic solution containing mannitol followed by perfusion with the Ca-deficient solution; numbers and dots on top (cyt a(a3)) correspond to time when difference spectra on left were recorded; horizontal bars indicate the period of perfusion with solutions containing high mannitol (filled bar) and the Ca-deficient solution (open bar) as indicated. Other symbols as in Fig. 2
brain was perfused with Ca-deficient or high K solution. The mechanism of this reduction remains unknown, but several possibilities may be considered. The present experiments show that there are large variations in development of blood-brain barrier among individual bullfrogs. In some bullfrogs such as depicted in Fig. 2, the development of the blood-brain barrier seems to be imperfect as in the newborn mammal, and changes in ionic composition of solution perfusing the blood vessels may cause a direct proportional change in ionic composition of the cerebrospinal fluid. In other bullfrogs such as depicted in Fig. 3, the blood-brain barrier may be as perfect as in the adult mammal, and the barrier can be made permeable by perfusing a hypertonic solution of a water-soluble non-electrolyte (8). In these preparations reduction of cytochromes became evident when the brain had previously been perfused with a hypertonic mannitol solution. The reduction of cytochromes seems to be due to decrease in Ca concentration in the ionic environment surrounding the cells in the bullfrog brain. In mammals, a severe state of tetany occurs when the Ca concentration of the cerebrospinal fluid is reduced sufficiently (7), and large increases in K ion in the cerebrospinal fluid was associated with a decrease in the frequency and increase in the amplitude of the EEG (1). It is worth noting that the K-Ca ratio, rather than either ion alone, in the cerebrospinal fluid is important in modifying the electrical activity of the brain since both high K and low Ca of the cerebrospinal fluid enhance the electrical activities of the brain (9). Thus it seems possible that perfusion of the bullfrog brain with the Ca-deficient or the high K solution may enhance the electrical activity. Moffett and Jöbsis (6) reported that when the isolated toad brain was bathed with a solution containing 30 mM K, a stimulant of brain respiration, the response was an immediate rapid reduction in all cytochromes, followed by a tendency to return to the initial levels. The question of how the reduction of cytochromes coincides an increase in respiration and electrical activity in the brain has been raised. This question cannot be answered if one holds the view that reduction of cytochromes always coincides with a reduction in respiration. In fact the previous study in our laboratory has shown that CCK-induced reduction of cytochromes precedes the initiation of enzyme output in the isolated perfused rat pancreas (3). Furthermore, Hersey and Jöbsis (2) presented the view that the reduction of cytochromes accom-
panying histamine addition in the isolated bullfrog gastric mucosa did not appear to be due to hypoxia, and Moffett and Jóbbis (6) suggested a tighter coupling between oxygen utilization and neuronal function than would be expected if mitochondrial redox states simply reflected changes in phosphate acceptor potential resulting from activity of \( \text{Na}^+/\text{K}^+ \) ATPase. This view is one possible explanation for question raised by the results in the present study.

Received for publication 12 October 1981

REFERENCES