Direct Monitoring of Respiratory Activity of Cultured Cells by Potential-step Chronoamperometry

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A new method to determine oxygen uptake rate of living cells is described. Two kinds of cultured mammalian cells were incubated in a 2.0 ml vol cuvette, which was equipped with an oxygen electrode as specially designed to measure the oxygen concentration ($[O_2]$) in the culture medium. This new method allowed 1) instant determination of the respiratory activity of living cells, and 2) simultaneous monitoring of responses of cell against extracellular stimuli.

A variety of modulators related with cell activity have been reported, and the molecular mechanisms of those have been extensively studied, however, it is still unclear how these factors couple each other. As the result of output of these complex and accumulated processes, the respiration activity, i.e. the O_2 uptake, attracted our interests. The O_2 uptake is an essential activity of widely distributed aerobic eukaryotic cells such as insect, plant, and mammalian cells. An effective stimulation of these cells lead to more O_2 uptake that reflects accelerated processes of production of chemical energy to be used for subsequent cell proliferation or differentiation.[1] Therefore, the O_2 uptake rate will be closely related with overall activity of the cells under consideration. Because the O_2 uptake of the cells can be estimated from the consumption of extracellular oxygen, we can monitor the current activity of cells by simply determining $[O_2]$ in the culturing medium without any serious damage of the cells.

Platinum electrode is widely used for determining $[O_2]$. We employed a specially designed potentiostatic O_2 electrode with naked working Pt electrode terminals fabricated by Daikin Industries Ltd., Japan.[2] The electrode was settled on a cuvette placed on an aluminum heat block maintaining 37℃ during monitoring the O_2 consumption. The electrode holding part of the cuvette has a hole for a long needle injector to reach the culture medium for occasional addition of extracellular modulators. Diameter of the hole fits to the needle to avoid unexpected gas exchange. We did not stir the sample solution to avoid irregular O_2 flow near the electrode surface.

Two kinds of cultured cells, mouse fibroblastoma cells (L929) and rat T-lymphocytes, were used in this work. The adhesive L929 cells were used for the study of quantitative relationship between the cell population and the respiratory activity. T-lymphocytes were chosen for monitoring the cell respiration as related with the immunogenecity of the cell because the activity is significantly changed on immunochemical stimulations. L929 cells were maintained in Eagle's MEM supplemented with 10 % FBS and 0.03 % glutamine.[3] The cells being in their growth phase were lightly trypsinized and resuspended to be 10^5 cells/ml. The cell suspension was seeded in another medium in the presence of 0.5 %(w/w) of cytodex 3 beads (cross-linked dextran beads of a diameter of 175 μm, coated with pig skin collagen, at the density of 1.04 g/ml, Pharmacia Biotech, Uppsala, Sweden), to obtain cultured L929 cells supported on the beads at the density of 2.5×10^4 cells/cm^2 after incubation for 24 h at 37 ℃ under 5 % CO_2. The concentration of L929 cells were adjusted to 5.0×10^6 cells/ml.

Rat T-lymphocytes were freshly obtained from the mesenteric lymph nodes of Wister male rats[4] (5 weeks old, Shimizu Co. Ltd.). The concentration of the lymphocytes was adjusted to 1.0×10^7 cells/ml. The T/B cell ratio was approximately 99.3/0.7.

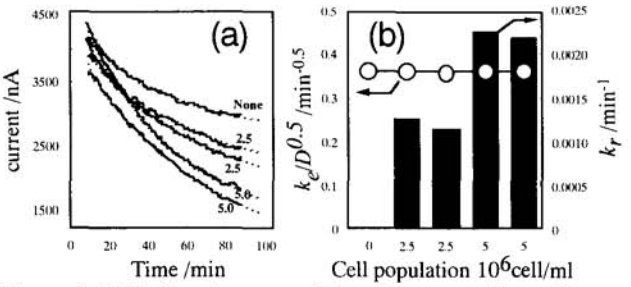


Figure 1. (a) Nonlinear least square fitting of O_2 consumption profile to eq. 2. Cell population (10^6 cell/ml) was indicated for every profile. (b) Estimated parameters $k_e/D^{0.5}$ and k_r for increasing populations of L929 cells.

The electrode reaction in a closed vessel is modeled as the diffusion equation with minor modification.

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} - k_e(c - c_e)\delta(x) - k_r(c - c_r) \quad (1)$$

The term $\partial c/\partial t$ is a change rate of c ($[O_2]$) at a given distance from the electrode surface. The equation $\partial c/\partial t = D\partial^2 c/\partial x^2$ comprises a diffusion equation with diffusion constant D of oxygen. This diffusion equation is modified with the terms $-k_e(c-c_e)\delta(x)$ and $-k_r(c-c_r)$ for integrating oxygen consumption. The term $-k_e(c-c_e)\delta(x)$ represents the consumption at the electrode surface with the rate constant k_e, where $\delta(x)$ is hyperfunction delta, employed for the convenience to obtain a more general solution of the equation ($\delta(x)$ gives an expression equivalent to a combination of equations using O_2 flow and a specific boundary condition that O_2 is consumed by electrode only at the surface, x = 0). Adjustment in $-k_e(c-c_e)\delta(x)$ was made by placing $-c_e$ in order to integrate the minimum chemical potential for the electrode reaction. It is represented by dimension of concentration because it easily reminds of that the electrode reaction proceeds if the $[O_2]$ is higher than c_e. The term $-k_r(c-c_r)$ represents O_2 uptake by cells with the rate constant of k_r, assuming that the cells are equally distributed in the cuvette and show an identical respiratory activity. Another adjustment was made also in this term to integrate minimum $[O_2]$ to uptake, $-c_r$. Initially, the electrode voltage was kept low enough (actually zero) so that no electric current was observed. After applying -400 mV as the electrode voltage, the electric current was recorded for 180 min under the applied voltage kept constant. The obtained current

change was analyzed as a function of time according to the above model.

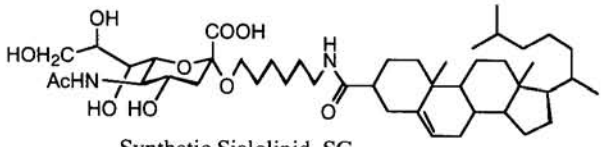
Reliability of the respiratory activity was first examined for L929 cells. The solution of eq. 1 is given as follows with constant and uniform respiratory coefficient k_r ($c_0 = [O_2]$ at $t = 0$).

$$c(t) = \left(c_0 - \frac{c_r v_r^2 - c_e v_e^2}{v_r^2 - v_e^2}\right) Erfc\left(v_e\sqrt{t}\right) e^{-(v_r^2 - v_e^2)t} + \frac{c_r v_r - c_e v_e}{v_r - v_e} + \frac{(c_r - c_e) v_r v_e}{v_r^2 - v_e^2} Erfc\left(v_r\sqrt{t}\right)$$

$$v_r = \sqrt{k_r},\ v_e = \frac{k_e}{2\sqrt{D}},\ Erfc(t) = 1 - \frac{2}{\sqrt{\pi}}\int_0^t e^{-s^2} ds \quad (2)$$

According to the eq. 2, successful estimation of cellular O_2 uptake rate was achieved for different populations of L929 cells by nonlinear least square fitting (Figure 1a). The characteristic parameter $k_e/D^{0.5}$ was nearly constant, indicating the electrode reaction and O_2 diffusion were reasonably stable. The O_2 consumption was proportional to the population of cells because the respiratory coefficient showed a linear dependency to the cell population (Figure 1b).

Change in the respiratory activity of cultured T-lymphocytes by the addition of sialolipids, or activity modulators, was successfully determined from the O_2 consumption profile. Rat T-lymphocytes were subjected to examine effects of activity modulators, naturally occuring ganglioside (GT_{1b})[4,5]- or a synthetic sialolipid (SC_6)[6]-reconstituted liposome (Figure 2a) both showed a fast increase in the intracellular Ca^{2+} ion release.[6]



Synthetic Sialolipid, SC_6

The solution of eq. 1 of changing respiratory coefficient $k_r(t)$ is given by the following equation:

$$c(t) = c_e + (c_0 - c_e) Erfc\left(v_e\sqrt{t}\right) e^{-w(t)} + (c_r - c_e) e^{-w(t)} \int_0^t k_r(s) Erfc\left(v_e\sqrt{t-s}\right) e^{w(s)} ds$$

$$w(t) = \int_0^t k_r(u) du - v_e^2 t \quad (3)$$

Some short manipulation of eq. 3 yields a Volterra's integral equation of the second kind for the first derivative of $e^{w(t)}$. Several times of iterative refinement of the solution gave a profile of $k_r(t)$ over the observed time span (Figure 2b).

By the addition of activity modulator, the respiratory coefficient rapidly increased, followed by a slower decrease. Interestingly, experience of the activation caused an alteration of the status of cells with a relatively high respiratory activity, which remained even after completion of the deactivation process. Thus, the cells were in an initial resting stage (R_1) before the stimulation and reached a highly activated stage (T) subsequently followed by the recover to another resting stage (R_2). These two processes, the first activation (R_1 to T) and the second deactivation (T to R_2), are characterized by the activation and the deactivation rate constants, k_a and k_d. Table 1 shows these parameters affected by two different modulators, GT_{1b} and SC_6. The SC_6-reconstituted liposome showed a fast activation with T-lymphocytes, but the effect did not continue longer. In contrast, the cells were gradually activated by the GT_{1b}-reconstituted liposome and the lifetime of activated stage was 13.2 min, which was 3 times longer than the case of SC_6-reconstituted liposome.

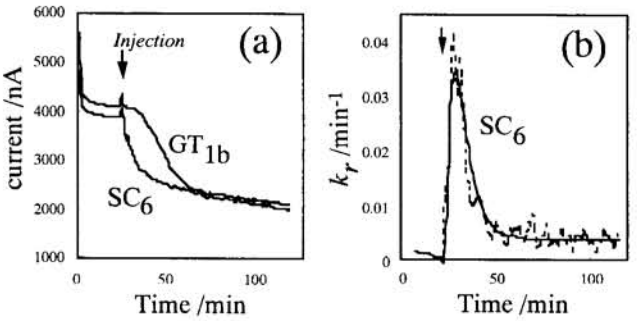


Figure 2. Oxygen consumption profile (a) and the derived time course of respiratory coefficient change (b) of rat T-lymphocyte cells. 1.0×10^7 cell/ml of T-lymphocyte cells were suspended in RPMI medium and placed in the cuvette. (a) Arrow indicates the addition of 100 μl of ganglioside-reconstituted DPPC liposome (0.37 mM as lipid concentration, suspended in PBS(-) buffer, 100 nm diameter, containing 14 mol% cholesterol and 5.4 mol% of ganglioside on the outer surface of liposome). (b) Broken line, derived respiratory coefficient; solid line, curve fitting to double exponential activation-deactivation model.

Table 1. Activation and deactivation rate constants of GT_{1b}- and SC_6-reconstituted liposomes

sialolipid	k_a /min^{-1}	k_d /min^{-1}
SC_6	0.23±0.008	0.22±0.009
GT_{1b}	0.079±0.012	0.076±0.012

This new method is basically according to a free oxygen diffusion and local oxygen consumption model. Potential-step approach enables to follow the transient decay of the oxygen concentration consumed by electrode and living cells. Because the effect of cells' consumption at far from the electrode surface propagates by the rate as defined by the diffusion constant, D, the obtained current profile can be interpreted by an appropriate model as proposed here. Though the oxygen consumption rate at the electrode surface and the diffusion constant are not able to be independently determined, we succeeded in this work to determine a time course of respiratory activity change of cells by using the proposed method more quantitatively than previous analyses.[7] Moreover, the sensitivity of the cells to foreign stimuli and responding rate constants for the sensitizer are determined without any perturbation of cell's activity. Further applications for simultaneous monitoring of cell responses against chemical and physical stimuli are in progress.

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