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Measurements of CO_2, lactic acid and sodium bicarbonate secreted by cultured cells using a flow-through type pH/CO_2 sensor system based on ISFET

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Abstract

We developed a pH/CO_2 sensor system using two ion-sensitive field effect transistors (ISFETs) to evaluate the metabolic activity of cultured cells. One ISFET measures total pH changes and the other ISFET, covered with a gas-permeable silicon tube, measures pH changes due to CO_2. These ISFETs were placed in a flow-through chamber with a window to be attached to a glass plate on which cells were cultured. Measurements were performed with a baseline solution of low buffer capacity. In the quantitative estimation of cellular products, we assumed that cells secreted CO_2, lactic acid and sodium bicarbonate ($NaHCO_3$), and then lactic acid and $NaHCO_3$ proceeded to the neutralization reaction to form sodium lactate and additional CO_2. We applied this system to evaluate the metabolic activity of bovine aortic endothelial cells (BAEC) and rabbit aortic smooth muscle cells (RASMC). The production of CO_2 by RASMC was significantly higher than that by BAEC (204 ± 16 and $4.1 \pm 0.3 \times 10^8$ molecules/cell/s, respectively). More $NaHCO_3$ than lactic acid was produced in RASMC, whereas they were not significantly different in BAEC.
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Keywords: ISFET; Extracellular acidification; Carbon dioxide; Lactic acid; Sodium bicarbonate

1. Introduction

Measurements of extra cellular acidification (ECA) have been extensively reported, especially since Molecular Devices Corporation developed a system called the Cytosensor Microphysiometer which uses a light-addressable potentiometric sensor [1,2]. For example, Parce et al. [1] measured the ECA of human keratinocytes grown on a glass cover slip, and estimated the net rate of proton production of this cell at about $1 \times 10^8\ s^{-1}\ cell^{-1}$. They also measured the production of lactate by a spectrophotometric enzymatic assay and obtained the rate of $3.3 \times 10^7\ s^{-1}$. Thibodeau et al. [3] measured the ECA of isolated gastric glands, and found that ECA was stimulated by activating H^+-K^+-ATPase with 8-(4-chlorophenylthio) adenosine 3′,5′-cyclic monophosphate, while it was depressed by inhibiting the same enzyme with omeprazole. On the other hand, they found that inhibition of the Na^+/H^+ exchanger did not reduce ECA. Landwojtowicz et al. [4] observed that the ECA of pig kidney cells and mouse embryo fibroblasts was activated or inhibited by selective activators or inhibitors of the ATPase of P-glycoprotein, while treatment of cells with inhibitors of Na^+/H^+ or the Cl^-/HCO_3^- exchanger did not reduce the ECA.

Following these studies, Lehmann et al. [5] first reported the simultaneous measurement of ECA and oxygen consumption using a PO_2 sensor combined with an ion sensitive field effect transistor (ISFET)-based pH sensor. They treated colon adenocarcinoma cells with a glycolysis inhibitor, iodocetate and observed a decline of acidification and an unchanged O_2 consumption. These results showed the possibility of evaluating cellular metabolic production in more detail by using combined sensors.

It has been considered that the compounds causing ECA are mostly lactic acid and CO_2 formed during energy metabolism with glucose and glutamine as carbon sources [6]. However,

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it is reasonable to speculate that cells secrete not only acids but also alkaline components, such as $NaHCO_3$ [8]. In such a case, a conventional microphysiometer which includes a single pH sensor can measure only the overall pH around cells, and therefore it has been impossible to define the amount of CO_2 (p), lactic acid (q) and $NaHCO_3$ (r) secreted. We constructed a flow-through type pH/CO_2 sensor system using two ISFETs that enables the measurements of the overall pH change and the pH change due to CO_2 around the cells. It is impossible to measure CO_2, lactic acid and $NaHCO_3$ separately even by using this combined sensor system, but it was found that the amounts of $p+r$, $p+q$ and $r-q$ can be derived from the output of the two sensors. In the present paper, chemical meanings were given to $p+r$, $p+q$ and $r-q$, and estimations of these values for two kinds of cells will be demonstrated.

2. Experimental

2.1. Software

In the present experiments, two data were obtained as observed values. These are pH values read by the pH sensors placed outside and inside a gas-permeable silicone tube (pH_o and pH_i, respectively). On the other hand, there are three unknowns: the concentrations of secreted CO_2, secreted lactic acid and secreted sodium bicarbonate. Equations correlating observed proton concentrations to the concentrations of secreted acids and base were derived as follows.

In the present experiments, a solution composed of 15 mM D-glucose, 140 mM NaCl, 5 mM KCl, 0.9 mM $MgCl_2$, 0.34 mM $NaHCO_3$ and 0.028 mM HEPES (*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethane-sulfonic acid) was used as the baseline solution. $NaHCO_3$ was added to this baseline solution to adjust the pH of the final solution to 7.4. Because all components other than $NaHCO_3$ and HEPES had a neutral pH, it was assumed that the pH of the baseline solution was determined by the initial concentrations of $NaHCO_3$ (α) and HEPES (β), and the concentrations of CO_2 (p), lactic acid (q) and $NaHCO_3$ (r) secreted by cells. The effects of other neutral components, such as D-glucose, NaCl, KCl and $MgCl_2$ on the acid–base equilibrium were neglected in the following discussions.

Thus, the chemical equations responsible for determining the pH of the baseline solution are the following three equations. The dissociation of bicarbonate ion to carbonate ion was assumed to be negligible.

$$CO_2 + H_2O = H^+ + HCO_3^- \quad (1)$$

$$RCOOH = H^+ + RCOO^- \quad (2)$$

$$H_2O = H^+ + OH^- \quad (3)$$

Here, RCOOH and $RCOO^-$ mean lactic acid and lactate anion, respectively. The equilibrium constant for each equation is defined by the following equations:

$$K_1 = \frac{(H^+)(HCO_3^-)}{CO_2} = 10^{-6.3525} \quad (4)$$

$$K_2 = \frac{(H^+)(RCOO^-)}{RCOOH} = 10^{-3.858} \quad (5)$$

$$K_w = (H^+)(OH^-) = 10^{-13.997} \quad (6)$$

HEPES is assumed to be dissociated completely to proton and HEPES anion ($HEPES^-$) because it is a strong acid.

In the baseline solution surrounding the pH_o, the positive ions are the Na^+ of the original and secreted sodium bicarbonate and proton, and the anions are bicarbonate ion, hydroxide ion, lactate ion and HEPES anion. Due to the neutral charge condition, one can obtain the following equation:

$$(Na^+)_o + (H^+)_o = (HCO_3^-)_o + (OH^-)_o + (RCOO^-)_o + (HEPES^-) \quad (7)$$

Here, subscript 'o' in each term means that these values are for the solution outside the silicone tube. Because the concentrations of HEPES anion outside and inside the silicone tube are identical, ($HEPES^-$) has no suffix. The sum of CO_2 and bicarbonate ion should be equal to $\alpha+p+r$, giving Eq. (8).

$$(CO_2)_o + (HCO_3^-)_o = \alpha + p + r \quad (8)$$

For lactic acid,

$$(RCOOH)_o + (RCOO^-)_o = q \quad (9)$$

and for HEPES ion,

$$(HEPES^-) = \beta \quad (10)$$

From (4) and (8), Eq. (11) can be obtained.

$$(HCO_3^-)_o = \frac{\alpha + p + r}{1 + h_o/K_1} \quad (11)$$

Here, h_o is $(H^+)_o$. Eq. (12) is also obtained.

$$(RCOO^-)_o = \frac{q}{1 + h_o/K_2} \quad (12)$$

Because $(Na^+)_o = \alpha + r$, one can obtain Eq. (13) from Eqs. (7)–(12).

$$\alpha + r + h_o = \frac{\alpha + p + r}{1 + h_o/K_1} + \frac{K_w}{h_o} + \frac{q}{1 + h_o/K_2} + \beta \quad (13)$$

Because lactic acid and lactate ion cannot permeate the silicone tube, the neutral charge condition for the baseline solution inside the silicone tube is Eq. (14).

$$(Na^+)_i + (H^+)_i = (HCO_3^-)_i + (OH^-)_i + (HEPES^-) \quad (14)$$

Here, subscript 'i' means that the value is for the baseline solution inside the silicone tube. Because $(Na^+)_i$ is α, Eq. (15) can be derived from Eq. (14), using Eqs. (4)–(6).

$$\alpha + h_i = \frac{K_1 (CO_2)_i}{h_i} + \frac{K_w}{h_i} + \beta \quad (15)$$

Here, h_i is $(H^+)_i$. It is assumed that the concentration of molecular CO_2 is identical on both sides of the silicone tube in the

equilibrium state, resulting in Eq. (16).

$$(CO_2)_i = (CO_2)_o = \frac{(H^+)_o(HCO_3^-)}{K_1} \quad (16)$$

Then using Eqs. (16) and (11), Eq. (17) can be obtained.

$$\alpha + h_i = \frac{(h_o/h_i)(\alpha + p + r)}{1 + h_o/K_1} + \frac{K_w}{h_i} + \beta \quad (17)$$

From this one can obtain the following equation:

$$p + r = \left(\alpha - \beta + h_i - \frac{K_w}{h_i}\right)\left(\frac{h_i}{h_o}\right) = \left(1 + \frac{h_o}{K_1}\right) - \alpha \quad (18)$$

Because h_o is in the order of 10^{-6} to 10^{-7} and K_2 is about 10^{-4}, h_o/K_2 is of the order of 10^{-2} to 10^{-3}. Therefore $1 + h_o/K_2$ is nearly equal to 1. Then from Eqs. (13) and (18), one can obtain the following equation:

$$q - r = \frac{(\alpha - \beta + h_o + h_i)(h_o - h_i)}{h_o} \quad (19)$$

From (18) and (19), one can obtain (20).

$$p + q = \left(\alpha - \beta + h_i - \frac{K_w}{h_i}\right)\left(\frac{h_i}{K_1}\right) + h_o - \frac{K_w}{h_o} - \beta \quad (20)$$

Thus, one can obtain the values of $p+q$, $p+r$ and $q-r$ from observed values of h_o and h_i, because α, β, K_1 and K_w are known values.

Let us speculate about the chemical meanings of $p+q$, $p+r$ and $q-r$. It is supposed that after lactic acid and $NaHCO_3$ are secreted from cells they are neutralized by the following reaction:

$$RCOOH + NaHCO_3 = RCOONa + CO_2 + H_2O \quad (21)$$

By definition, q and r represent the concentration of RCOOH and $NaHCO_3$ just after their secretion from cells (before neutralization).

Case 1: When the secreted molar concentration of lactic acid (q) is larger than that of $NaHCO_3$ (r), $q-r$ will represent the final concentration of the lactic acid. In this case, "final" means remaining after neutralization. On the other hand, the neutralization reaction (21) will bring the formation of CO_2 in addition to the secreted CO_2 (p). The concentration of CO_2 due to reaction (21) will be same to that of $NaHCO_3$ (r). Therefore, the final CO_2 concentration after the neutralization reaction will be $p+r$.

Case 2: When the secreted concentration of $NaHCO_3$ (r) is larger than that of lactic acid (q), $r-q$ will represent the final $NaHCO_3$ concentration. The final CO_2 concentration after the neutralization reaction will be $p+q$.

Case 3: When lactic acid and $NaHCO_3$ are secreted in equimolar concentrations, the final lactic acid and final $NaHCO_3$ will be zero, and the final CO_2 after neutralization will be $p+q$.

In summary, we can obtain the final concentration of lactic acid ($q-r$) and CO_2 ($p+r$) in Case 1, final $NaHCO_3$ ($r-q$) and CO_2 ($p+q$) in Case 2 and final CO_2 ($p+q$) in Case 3 by calculating the two measured values after the neutralization reaction (21).

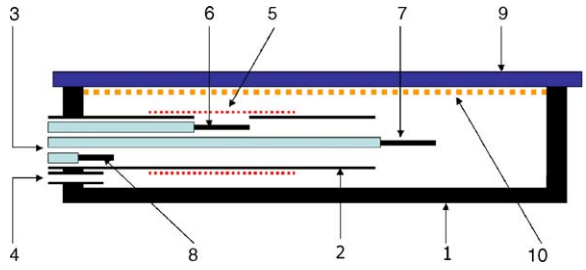


Fig. 1. A schematic representation of the pH/CO_2 sensor system for measurement of extracellular acidification of cells on a glass plate. (1) Measurement box, (2) 1.2 mm inner diameter polyethylene tube, (3) inlet of baseline solution, (4) outlet of baseline solution, (5) gas-permeable silicone tube, (6) ISFET for measuring acidification due to CO_2 ($ISFET_i$), (7) ISFET for measuring total acidification ($ISFET_o$), (8) reference electrode (Ag/AgCl), (9) glass plate and (10) cells.

2.2. Sensor system

A pH/CO_2 sensor system of a flow-through type, illustrated in Fig. 1, was constructed by using two ISFETs and an Ag/AgCl type reference electrode. The ISFET used for this system was described in a previous paper [7]. The ISFET is 0.2 mm thick, 0.45 mm wide and 5.5 mm long. The gate region is coated with a pH-sensitive Ta_2O_5 membrane deposited by a sputtering method. This ISFET has a pH–response time on the order of millisecond [9]. The pH sensitivity of the ISFET was 58 mV/pH at 37 °C. As illustrated in Fig. 1, a polyethylene tube of 1.2 mm inner diameter was used as a flow pass of the baseline solution. In the middle of the tube, a side hole was opened and the hole was covered by a gas-permeable silicone tube. Two ISFETs and a reference electrode were introduced into the tube so that one ISFET ($ISFET_i$) was placed at the hole site and another one ($ISFET_o$) projected out of the tube. Then this polyethylene tube was fixed in the measuring chamber, which has an open window of 5 mm × 20 mm, and an outlet for the baseline solution. A glass plate with cultured cells on it was attached to the measuring chamber to close the window tightly, and the whole box was put into a 37 °C water bath. The baseline solution was air-bubbled before use and introduced into the measuring chamber for 3 min at a rate of 2.9 ml/min; the flow was stopped for the next 40 min during which the pH changes were measured by the two ISFETs. The total dead volume of the measuring chamber was 0.25 ml.

2.3. Response of sensor system to solutions containing known amount of lactic acid and/or $NaHCO_3$

The following measurements were carried out in order to evaluate the quantitative reliability of the present sensor system. The sensor without a glass plate was placed in a solution containing fresh baseline solution at 37 °C. The pump was turned on for 3 min and just after the pump was stopped the sensor was transferred into another baseline solution at the same temperature containing known amount of lactic acid and/or $NaHCO_3$

with or without bubbling 5% CO_2. The concentration of lactic acid and/or $NaHCO_3$ was changed from 0 to 0.3 mM. The value of $q - r$ was calculated by Eq. (19) with $\alpha = 0.34$ mM and $\beta = 0.028$ mM from the h_i and h_o measured with $ISFET_i$ and $ISFET_o$, respectively.

2.4. Measuring ECA of bovine aortic endothelial cells (BAEC) or rabbit aortic smooth muscle cells (RASMC) cultured on a glass plate

Bovine aortic endothelial cells (Cell System, Kirkland, WA, USA) or rabbit aortic smooth muscle cells (Cell Applications, San Diego, CA, USA) were cultured on a glass plate. The glass plate was taken out of the culture solution, put into the baseline solution to replace the culture medium, and attached to the window of the measurement chamber. The ECA measurements were repeatedly carried out by 3 min of pump-on and 40 min of pump-off.

After measurement, the cells on the glass plate were fixed with formaldehyde and stained with hematoxylin–eosin, and the density of cells on the plate was obtained by microscopy. The final concentrations of CO_2 and $NaHCO_3$ or lactic acid in the measuring chamber were calculated from Eqs. (18)–(20) using h_o and h_i at 40 min of pump-off time. These concentrations were converted into the numbers of molecules secreted per cell using the measured cell density, the inner volume of the measuring chamber (0.25 ml) and the window surface area (100 mm^2).

3. Results

3.1. Response curves of sensors to solutions containing known amounts of lactic acid and/or $NaHCO_3$

Fig. 2A shows the response curves after the sensors were immersed in the baseline solution equilibrated to 5% CO_2. In this case, the component that causes the pH change in the baseline solution was only CO_2, which can permeate the silicone tube. Therefore, the same final pH change ($\Delta pH_o = \Delta pH_i = -1.60$) was observed for $ISFET_i$ and $ISFET_o$, although the response was a little slower in $ISFET_i$ because the CO_2 gas has to diffuse through the silicone tube.

Fig. 2B and C show the response curves for the baseline solution saturated with 5% CO_2 and containing 0.3 mM $NaHCO_3$ or 0.156 mM lactic acid, respectively. In the solution containing $NaHCO_3$, the pH_o ($\Delta pH_o = -1.22$) was less acidic than pH_i ($\Delta pH_i = -1.61$) due to bicarbonate ions, which cannot permeate the silicone tube. On the contrary, in the solution containing lactic acid, the pH_o ($\Delta pH_o = -2.04$) was more acidic than pH_i ($\Delta pH_i = -1.68$), because lactic acid cannot permeate the silicone tube.

3.2. Quantification of final concentration of lactic acid or $NaHCO_3$

Fig. 3 shows the correlation between true (Y) and calculated (X) values of $p - q$. The correlation equation was $Y = 1.07X - 2.2$ and the correlation coefficient R^2 was 0.974.

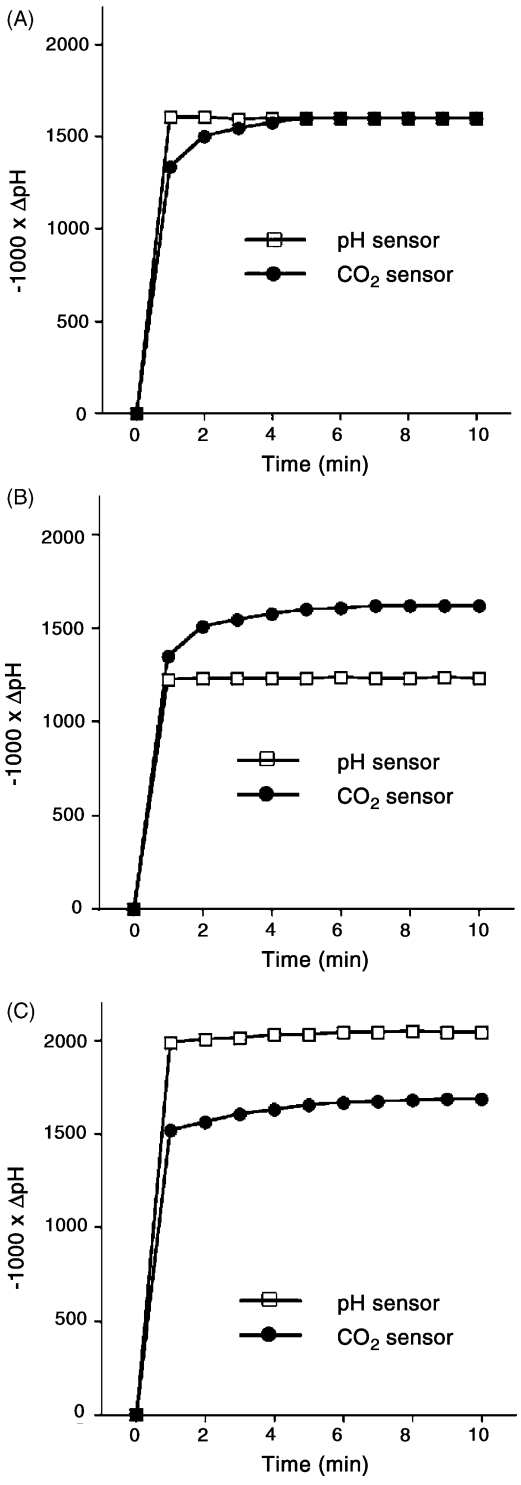


Fig. 2. (A) Response curve of the pH/CO_2 sensor to a baseline solution saturated with 5% CO_2. Square: $ISFET_o$ (pH sensor), circle: $ISFET_i$ (CO_2 sensor). (B) Response curve of the pH/CO_2 sensor to a baseline solution saturated with 5% CO_2 and containing 0.3 mM $NaHCO_3$. Square: $ISFET_o$ (pH sensor); circle: $ISFET_i$ (CO_2 sensor). (C) Response curve of the pH/CO_2 sensor to a baseline solution saturated with 5% CO_2 and containing 0.156 mM lactic acid. Square: $ISFET_o$ (pH sensor); circle: $ISFET_i$ (CO_2 sensor).

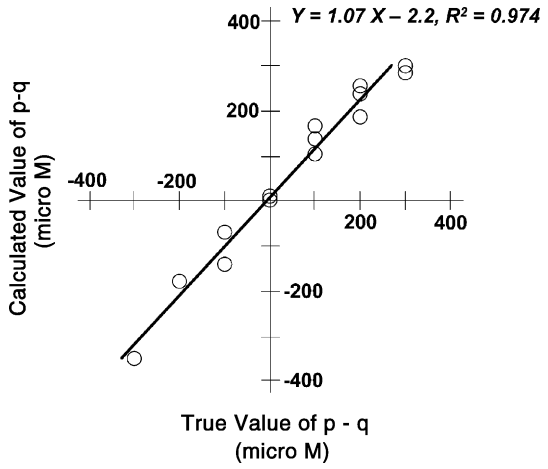


Fig. 3. Relationship between calculated (Y) and true (X) values of $p-q$. The correlation equation was $Y=1.07X-2.2$ and the correlation coefficient was $R^2=0.974$.

Table 1
The pH changes of the baseline solution inside (ΔpH_i) and outside (pH_o) of the silicone tube after 40 min of pump-off period, and the calculated amounts of final CO_2 and $NaHCO_3$ molecules produced by one cell in 1 s

Repetition cycle number	ΔpH_o	ΔpH_i	Final CO_2 (10^8 molecules/cell/s)	Final $NaHCO_3$ (10^8 molecules/cell/s)
BAEC (cell density: 131 cells/mm^2)				
1	−0.174	−0.179	4.31	0.17
2	−0.162	−0.179	4.31	0.60
3	−0.169	−0.173	4.24	0.14
4	−0.127	−0.131	3.72	0.14
RASMC (cell density: 2.3 cells/mm^2)				
1	−0.102	−0.131	211	58.8
2	−0.096	−0.137	216	84.1
3	−0.092	−0.125	208	67.0
4	−0.057	−0.082	181	50.4

3.3. ECA measurement of BAEC or RASMC cultured on a glass plate

Typical response curves of the present sensor for BAEC and RASMC cultured on a glass plate are shown in Fig. 4A and B. Both pH sensor and CO_2 sensor showed mostly linear decreases of pH. The BAEC cells were almost confluent on the glass plate and the cell density was 131 cells/mm^2. RASMC cells were not confluent, and the cell density was 2.3 cells/mm^2. In Table 1, the pH changes of the baseline solution inside (ΔpH_i) and outside (ΔpH_o) the silicone tube during the 40-min pump-off period and the calculated amounts of final CO_2 and $NaHCO_3$ molecules produced by one cell in 1 s are summarized.

4. Discussion

The characteristic feature of the present sensor system is that two ISFETs are placed in a stream of the same baseline solution containing D-glucose and bicarbonate ion. The only difference between the two ISFETs is that $ISFET_i$ is surrounded by a gas-permeable silicone tube. By flowing the baseline solution for 3 min, the solution surrounding the two ISFETs is replaced by

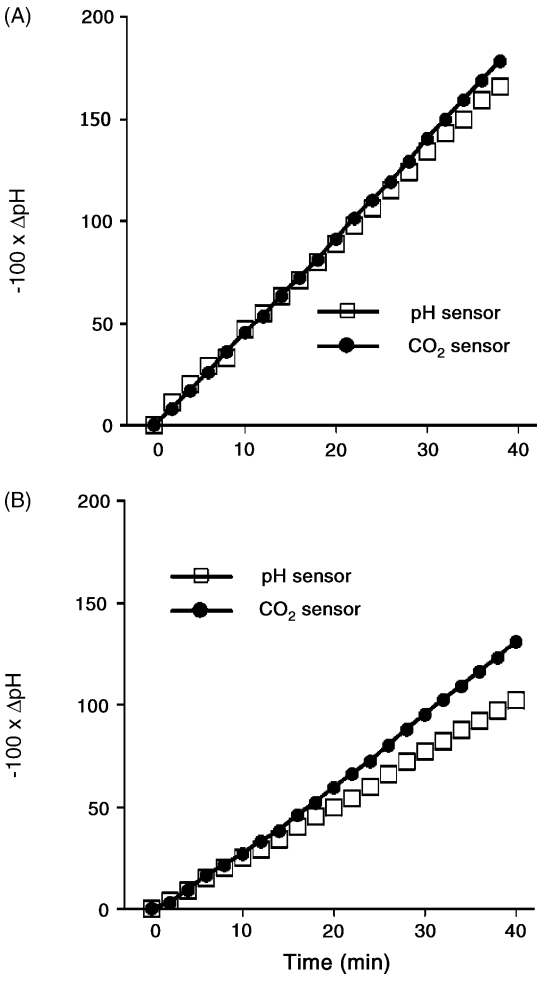


Fig. 4. (A) An example of the response curve of the present sensor to a BAEC/glass plate with the cell density of 131 cells/mm^2. Square: pH sensor ($ISFET_o$); circle: CO_2 sensor ($ISFET_i$). (B) Example of the response curve of the present sensor to a RASMC/glass plate with the cell density of 2.3 cells/mm^2. Square: pH sensor ($ISFET_o$); circle: CO_2 sensor ($ISFET_i$).

the fresh baseline solution of pH 7.4. When this sensor was placed in the baseline solution equilibrated by 5% CO_2, almost identical changes of pH ($\Delta pH_i=\Delta pH_o=-1.60$) were observed by the two ISFETs (Fig. 2A). When $NaHCO_3$ was added to this solution, $-\Delta H_o$ became smaller than $-dpH_i$ because the baseline solution outside the silicone tube was more alkaline than that inside the tube due to bicarbonate ion, which does not permeate the silicone tube (Fig. 2B). The opposite was true when lactic acid was added to the baseline solution (Fig. 2C). Therefore, one can distinguish the following three cases of ECA from the relative value of $-\Delta pH_i$ and $-\Delta pH_o$:

Case 1: $-\Delta pH_o > -\Delta pH_i$: secreted lactic acid > secreted $NaHCO_3$.
Case 2: $-\Delta pH_o < -\Delta pH_i$; secreted lactic acid < secreted $NaHCO_3$.
Case 3: $-\Delta pH_o = -\Delta pH_i$: secreted lactic acid = secreted $NaHCO_3$, or ECA is caused mostly by CO_2.

More quantitative evaluations of the final concentrations of CO_2, lactic acid and $NaHCO_3$ secreted by cells can be attained

by converting the values of ΔpH_i and ΔpH_o to $p+r$, $p+q$ and $q-r$ by Eqs. (18)–(20). As shown in Fig. 3, a good correlation was observed between the calculated and true values of $q-r$. This suggests that the present system is capable of quantification of the final concentration of CO_2, lactic acid and $NaHCO_3$ within the range of 0.3 mM.

Based on these preparations, the ECA of BAEC and RASMC on a glass plate was measured. BAEC were grown confluently on the glass plate and the cell density was 131 cells/mm^2. As shown in Fig. 4A, in the case of BAEC, $ISFET_o$ (total pH sensor) and $ISFET_i$ (CO_2 sensor) showed almost overlapped response curves, suggesting that the causative compound of ECA in BAEC was mostly CO_2, or CO_2 + lactic acid + $NaHCO_3$ with equal secretions of lactic acid and $NaHCO_3$ (Case 3). As shown in Table 1, similar acidification ($-\Delta pH_o = \sim 0.17$) was maintained during three repetitions, but the acidification decreased at the fourth repetition ($-\Delta pH_o = 0.13$), suggesting the weakening of cell activity. The production rate of the final CO_2 was estimated at 3.7–4.2 $\times 10^8$ molecules/cell/s. This value is comparable to the proton production rate of human keratinocytes (1×10^8) reported by Parce et al. [1].

In the case of RASMC, the proliferation of cells on the glass plate was poor, with a cell density of 2.3 cells/mm^2, but the same order of ECA was observed. Further, as shown in Fig. 4B, the response curves of $ISFET_o$ and $ISFET_i$ were clearly different. The acidification observed by $ISFET_i$ ($-\Delta pH_i = 0.13$) was greater than that observed by $ISFET_o$ ($-\Delta pH = 0.10$). This means that the outside of silicone tube was more alkaline than the inside, suggesting that $NaHCO_3$ was more abundantly secreted than lactic acid (Case 2). As shown in Table 1, a similar secretion pattern was observed during four repetitions, although an abrupt decay of activity was observed at fourth period, as in the case of BAEC. The production rate of the final CO_2 was 180–220 $\times 10^8$ molecules/cell/s. This value is larger than that of BAEC by two orders of magnitude. The production rate of final $NaHCO_3$ after the reaction (21) was 50–84 $\times 10^8$ molecules/cell/s. As far as present authors know, this is the first report of quantification of the cellular $NaHCO_3$ secretion rate.

As demonstrated above, the present sensor system can evaluate the cellar secretion rates of final CO_2, lactic acid and $NaHCO_3$ after the neutralization reaction (21) separately. This is an improvement over the conventional microphysiometer that is equipped with only a pH sensor corresponding to $ISFET_o$ in the present system and gives only an overall acidification. The reason for excess $NaHCO_3$ secretion over lactic acid in RASMC in the present study remains unclear. The present study, while focusing on the technique, suggested that there may be a wide variety of ECA both in its patterns (Cases 1–3) and its orders of magnitude. We expect that more detailed aspects of energy metabolism will be investigated by applying the present technique to a broad range of cells.

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