Dependence of Cell pH and Buffer Capacity on the Extracellular Acid-Base Change in the Skeletal Muscle of Bullfrog

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Abstract Intracellular pH was measured with single- or double-barreled liquid ion-exchanger microelectrodes in the bullfrog sartorius muscle perfused in vitro. A neutral carrier ligand was used for pH sensor of microelectrodes. Average slopes of the single-barreled microelectrodes were $-56.4 \pm 1.34$ mV/pH ($n = 30$) and the double-barreled $-52.6 \pm 1.34$ mV/pH ($n = 65$). While changing acid-base parameters of bathing media (pHe from 6.7 to 8.4, $P_{CO_2}$ from 3.7 to 37 mmHg, and $HCO_3^-$ concentrations from 5 to 75 mM), paired muscle cell pH (pHi) and membrane potential ($E_M$) values were determined at 23°C. In control conditions (pHe = 7.6, $HCO_3^-$ = 15 mM, $P_{CO_2}$ = 11 mmHg), pHi and $E_M$ ($n = 20$) averaged $6.99 \pm 0.04$ (S.E.) and $-69.2 \pm 2.2$ mV, respectively. A negative correlation was observed between pHi and $E_M$ (correlation coefficient $r = -0.564$, $p < 0.002$). The change in $E_M$ per unit pH change was approximately $-30$ mV, indicating that the H$^+$ distribution across the cell membrane only incompletely obeys the Donnan rule. The pHi varied more or less with pHe. Namely, changes in pHe and $P_{CO_2}$ at constant $HCO_3^-$ produced relatively large changes in pHi, but elevation of pHe and $HCO_3^-$ at constant $P_{CO_2}$ produced relatively minor rise in pHi. The stability of pHi or the size of buffer capacity were proportional to external $HCO_3^-$ concentrations. These data suggested that a transmembrane distribution of buffer pairs depends largely on non-ionic diffusion of $CO_2-HCO_3^-$ buffer system and partly on ion fluxes of $HCO_3^-$ or H$^+$.

Key words: cell pH, muscle pH, pH-microelectrode, buffer capacity, non-ionic diffusion.

The intracellular studies of acid-base status have been carried out with various methods in many biological systems. For determination of intracellular pH (pHi), two methods are currently used: 1) the direct method to read the pH value by means
of a pH meter; 2) the indirect method (indicator method) to discriminate the change of color tone produced by dissociation of indicator. In view of the remarkable progress of both techniques, the selection suitable for practical application is important. For quantitative analysis and continuous observation of pH change, the direct access with pH-microelectrodes is more useful than the indirect indicator methods, although it inevitably causes cell damages.

The materials used for those pH-microelectrodes were usually pH-sensitive glass (Caldwell, 1954; Carter et al., 1967; Thomas, 1978) and liquid ion-exchanger (abbreviated herein as LIX; for its nature, see general reviews edited by Nuccitelli and Deamer, 1982; Kessler et al., 1985). As regards the latter, Ammann et al. (1981) first reported an intracellular pH-microelectrode with a neutral carrier as H⁺ ligand. Comparing with natural antibiotics, such as nigericin and monensin (Matsumura et al., 1981, 1985), the neutral carrier has high ion selectivities and good stability. Now, this material is used for a wide variety of studies with the pH-microelectrode (for example, Fujimoto et al., 1983; Sasaki and Berry, 1984; Yoshitomi and Froemter, 1984; Saito et al., 1988). However, mainly because of technical difficulty, studies on pH and intracellular buffering are still not common.

To advance knowledge of acid-base regulation of intracellular fluid, we used both single- and double-barreled microelectrodes with a neutral carrier. The present study for regulatory mechanism of H⁺ within the cell of bullfrog sartorius muscle revealed that the extracellular buffer concentration and pH affect not only pH, but also the buffer capacity of the cell.

To be specific, the aim of the present study is 3-fold: 1) how much does external pH influence the intracellular pH; 2) whether or not the H⁺ distribution across the membrane obeys the non-ionic diffusion of CO₂-HCO₃⁻ system; and 3) what are the regulatory factors in the intracellular pH.

METHODS AND MATERIALS

a. Fabrication of microelectrodes. Pyrex glass capillaries with 1.0 mm outer diameter (o.d.) and 0.6 mm inner diameter (i.d.) were used in this study. The individual glass capillary had a fine solid glass filament of 0.08–0.10 mm o.d. inside the lumen (filamented glass tubing). They were immersed in chromic acid and rinsed repeatedly with redistilled water and dried completely before used. Two capillaries were mounted in parallel on a horizontal micropipette puller (Narishige, PD-5). The middle part of the capillaries was heated with a platinum ribbon over 5 mm in width for 30 s and gently rotated 180° along its longitudinal axis, and pulled in two changeable steps until the tip diameter was less than 1 µm. The length of the shank was about 1.5 cm.

One side of the double-barreled tip was prepared so as to measure voltage of chemical potential of H⁺ (E₄); thus, it is referred to as “ionic limb” or “ionic barrel.” The output signal of ionic limb always gives the E₄ superimposed on the
potential difference (PD) between the tip and the external reference. Another limb, referred to an "PD limb" or "PD barrel," was used just for measuring that PD. Therefore, the differential output of both limbs represents the signal of net pH changes.

Technically, the ionic limb must be hydrophobic so that H⁺ LIX (liquid ion-exchanger) can remain in place within a limited length of the tip, whereas the PD limb must be kept hydrophilic so that the aqueous internal solution can easily flow down into the tip. For the former purpose, pure acetone is manually injected from the open end of the stem toward the tip with an injection needle (0.2 x 100 mm, long needle, Taguchi, Tokyo) connected to a syringe. The entire tip of the double-barreled micropipette is dipped for 30 s into a fresh solution of 0.2-0.3 vol% silicone oil (Shin-Etsu Chemical Co., KF-96 1,000 CS) diluted with trichloroethylene. To assure siliconization, the micropipette is then backed on a hot plate at 300°C for about 40 min (FUJIMOTO and KUBOTA, 1976).

For the ionic barrel, LIX was filled with a filament-like polyethylene tube, which had been pulled from FR No. 5 tubing (Hibiki, Tokyo) upon heating. The exchanger consists of 10% tri-n-dodecylamine, 0.7% sodium tetraphenyl-borate, and 89.3% o-nitrophenyloctyl ether and was equilibrated with 100% CO₂ gas before use. It is now commercially available as WPI H⁺ LIX (IE-010). After insuring LIX of the tip, the back of LIX column was filled with a mixture of 0.02 M NaCl and 0.1 M KCl, adjusted to pH 7.5 with 0.1 M Tris-HCl buffer, with another injection needle for the internal reference solution (FUJIMOTO and MORIMOTO, 1986).

The PD barrel was filled with 0.5 M KCl from the open end of the stem to the tip throughout the total length of micropipette with a similar technique. The microelectrodes thus fabricated were stored vertically with their tips down in a moist-chamber (WPI microelectrode-receptacle) until use.

For standard calibration of pH-microelectrodes, we used a series of Tris-HCl buffers (Sigma Chem.) or phosphate buffers upon adjusting their ionic strength to 0.12 and examining their pH by means of a glass pH-macroelectrode (Toa Digital pH meter, HM-15A).

Electrical measurements of the electromotive forces (EMF) of the pH-microelectrode were performed with a high input-impedance electrometer (10¹⁵ Ω; FD-223, WPI). In the case of double-barreled pH-microelectrodes, pHᵢ was calculated by the following formula:

\[ pΗᵢ = pΗₛ + \frac{(Eₛ - Eᵢ)}{α}, \]  

where the subscripts i and s stand for the intracellular fluid and standard solution in the extracellular surface, E represents EMF, and α is the slope of that electrode. In this study, the slope (EMF change per unit pH) of the calibration curve averaged \(-56.4±1.34\) (S.E.) mV/pH \(n=30\) for single-barreled and \(-52.6±0.78\) mV/pH \(n=65\) for double-barreled pH-microelectrodes.

Electric resistance of ionic barrels averaged \(6×10¹¹\) Ω for single-barreled and \(4×10¹²\) Ω for double-barreled microelectrodes. The time required for 95% of full
response of EMF in test solution was less than 10 (usually 3–9) and 20 (7–15) s, respectively. Their reproducibility and stability were less than 2 mV (0.03 pH unit) and 1.0 mV/h.

In the case of single-barreled pH microelectrodes, their EMF reflects ionic activity plus PD ($E_M$ in the case of biological measurements); therefore, the $E_M$ had to be separately measured and subtracted in order to arrive at a value for cell pH.

b. Animal experiments. Micropuncture studies were performed in frog sartorius muscle with both single- and double-barreled pH-microelectrodes. Bullfrogs (*Rana catesbeiana*), weighing from 100 to 300 g, were used for this study. Each animal was anesthetized by immersion in 3% urethane solution for 10–15 min. Sartorius muscles were isolated and placed in a plastic chamber (5.0 x 6.0 x 1.0 cm). Perfusion fluids were kept in glass reservoirs (300 ml graduated cylinders) at 50 cm H$_2$O above the level of muscle. The chamber was continuously perfused at a rate of 1.5 ml/min throughout the experiment at room temperatures (22–24°C).

Control solution had a measured osmolality of 210 mOsm, and had the following composition (in mM): NaCl, 100; KCl, 3.5; MgCl$_2$, 1.0; CaCl$_2$, 1.8; NaHCO$_3$, 15; NaH$_2$PO$_4$, 0.5; glucose, 5.0; glycine, 1.0; alanine, 0.5; and polyvinylpyrrolidone, 20 g/l. $P_{CO_2}$ was adjusted to 11 mmHg and pH to 7.65 (7.6–7.7), by continuously bubbling with a mixture of 1.5% CO$_2$ and 98.5% O$_2$ gas. In experiments, $P_{CO_2}$ was changed from 11 to 3.7 or from 11 to 37 mmHg, and HCO$_3^-$ to 5, 45, and 75 mM with complementary changes of Cl$^-$. The intra- and extra-cellular HCO$_3^-$ concentration ([HCO$_3^-$]$_i$ and [HCO$_3^-$]$_e$) were estimated by measuring cell pH and perfusate pH after equilibrating with a known $P_{CO_2}$, using the Henderson-Hasselbalch equation, as shown below:

$$pH = pK + \log \frac{[HCO_3^-] \cdot \gamma}{S \cdot P_{CO_2}},$$  

where pK is 6.32 as the first dissociation exponent of carbonic acid, which is determined thermodynamically at 23°C, S stands for the absorption coefficient 0.045 mM/mmHg $P_{CO_2}$, and $\gamma$ represents the activity coefficient of HCO$_3^-$, as proposed for amphibian body fluids by KAJINO et al. (1982).

The magnitude of Cl$^-$ permeability of muscle cell membrane was predicted from the transport number (transference number) of Cl$^-$, assuming that only changes in Cl$^-$ concentrations are responsible for the observed change in $E_M$ (HODGKIN and HORWICZ, 1959). By definition,

$$t_{Cl} = \frac{\Delta E_M}{\Delta E_{Cl}},$$

where $\Delta E_M$ stands for the change of membrane potential ($E_M$) in ion replacement and $\Delta E_{Cl}$ represents the change of the equilibrium potential of Cl$^-$ across the cell membrane:

$$\Delta E_{Cl} = 58 \log [Cl_{e2}/Cl_{e1}].$$

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In practice, when the external Cl⁻ is changed from Clₑ₁ to Clₑ₂, ΔEₐ is calculated by Eq. (4). The underlying assumption here is that the intracellular Cl⁻ concentration remains unchanged at the moment the external Cl⁻ concentration is changed. Based on the experimental data (such as those in Fig. 3), transport number of Cl⁻ (t(Cl⁻)) was calculated from the electrical responses to changes in the external Cl⁻ concentrations from 49 to 119 mM.

For observation of bicarbonate effect on the membrane potential (Eₘ) of muscle cell, effects of Cl⁻ had to be excluded. For this purpose, we used gluconate⁻ for Cl⁻ substitute with supplemental addition of Ca-gluconate until free [Ca²⁺] was 1.2 mM, as monitored with Ca²⁺-macroelectrode (Toa, Ca-135). An Ag-AgCl electrode (Toa, HS-205C, Tokyo) was connected to the tissue surface through a 3 M KCl agar bridge. The digital and analog voltages were recorded with digital multimeters (Keithley, 160), in combination with a data-logger (Fluke, 2240A) and a dual-trace pen recorder (Rikadenki, B-281L). In micropuncture experiments, electrical data were sampled after 8 to 10 min equilibration with different levels of [HCO₃⁻]ₑ and Pco₂. After each experiment, calibration of used pH-macroelectrode was carried out in situ with the extracellular standard buffers which were perfused on the surface of muscle cells.

The results of measurements were expressed as mean ± S.E. (standard error) with the number of observations, n, unless otherwise stated. Regression equation was calculated by the least squares method. Statistical significance was tested by Student’s t-test on paired or unpaired data according to the sort of samples.

RESULTS

1. Measurements of pH_i and Eₘ

Carrying out micropuncture experiments with these microelectrodes, we obtained pH_i values of about 7 for the in vitro sartorius muscle cell in steady state conditions.

Figure 1 shows an example of direct cellular impalement, in which normal Ringer solution was perfused as control bathing fluid (pH 7.65; Pco₂, 11 mmHg). The output of the double-barreled microelectrode advanced into a muscle cell gave a pH_i of 7.00 and a PD (Eₘ) of −75 mV, simultaneously. As soon as the cellular impalement was successful, the microelectrode was withdrawn and then calibrated with two buffer solutions (A: pH 8.04; and B: 7.17) in situ.

Figure 2 summarizes the data of pH_i and Eₘ obtained in 20 similar experiments. The average pH_i was 6.99 ± 0.04; and Eₘ was −69.2 ± 2.2 mV. This result is essentially in agreement with the data reported by MATSUMURA et al. (1980) with double-barreled antimony microelectrodes. As a whole, a significant correlation was found (the correlation coefficient: r = −0.564, p < 0.002). The change of Eₘ per unit change of pH_i was about −30 mV. All plots were scattered in the area far above the Nernst potential of H⁺, as indicated in the dotted line for the equilibrium potential for H⁺ (E_H) (in mV at 23°C, E_H = −58 × [pHₑ − pH_i]). This suggests that the
distribution of H⁺ in the muscle does not strictly obey the Donnan rule, but it is influenced either by $E_M$ or by a potential close to the Donnan potential for other ions, such as K⁺ or Cl⁻ across the membrane. Since H⁺ is extruded actively from the cell interior to the interstitium by a pump mechanism, while it leads passively from the interstitium to the cell interior with the electrochemical gradient across the membrane, a "pump-and-leak" mechanism might operate in regulation of H⁺ in the cell (BORON, 1980).

2. Effects of $\text{HCO}_3^-$ on $E_M$

The effect of $\text{HCO}_3^-$ on the membrane potential ($E_M$) was examined by changing external $\text{HCO}_3^-$ concentration ($[\text{HCO}_3^-]$ₐ) (Fig. 3). The upper half of Fig. 3 demonstrates an actual trace of an example, in which Cl⁻ in perfusates was replaced with $\text{HCO}_3^-$ ($P_{\text{CO}_2}$ was maintained at 11mmHg, but pH changed concomitantly). Alkaline perfusion of high $\text{HCO}_3^-$ with low Cl⁻ caused a transient depolarization of $E_M$ followed by a slow recovery to the original level, and a

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*Fig. 1. A trace of cellular impalement by a LIX pH-microelectrode in the sartorius muscle of bullfrog.*

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biphasic change of pH shifted in alkaline direction, as it was affected partly by the extracellular alkalosis and partly by the depolarization caused by the perfusion. Perfusion of low HCO₃⁻ + high Cl⁻ solution produced an opposite effect both on EM and pH; i.e., hyperpolarization and cell acidosis.

Since EM of the muscle is known to undergo effects of altered Cl⁻ concentration in the perfusate (Hodgkin and Horowicz, 1959), similar experiments to exclude Cl⁻ effects were performed in low Cl⁻ conditions, in which Cl⁻ was replaced by gluconate⁻ as balancing ion of variable HCO₃⁻ concentrations (Fig. 3, lower half). The result showed that EM, as well as pH; was largely affected by external Cl⁻ concentrations ([Cl⁻]e: changed from 109.1 to 9.1 mM, and vice versa). But EM remained unchanged even in various HCO₃⁻ concentrations from 15 to 75 mM, while pH; fluctuated significantly. Comparing two traces shown in Fig. 3, it is clear that the EM was not affected by altered concentrations of HCO₃⁻ but mainly by Cl⁻ concentrations.

Fig. 2. Relationship between pH; and EM of frog sartorius muscle under normal conditions.

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Fig. 3. Changes in pH, and $E_M$ of frog sartorius muscle in perfusions with different [HCO$_3^-$]$_e$ substituted for [Cl$^-$_]$_e$. Upper half: changes in pH, and $E_M$ in the presence of Cl$^-$. Lower half: changes in pH, and $E_M$ in low Cl$^-$ conditions (substituted for Cl$^-$ with gluconate$^-$.)

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Figure 4 summarizes the results of $E_M$-$\text{HCO}_3^-$ relationships in three different $P_{CO_2}$ levels (3.7, 11, and 37 mmHg). The samples were collected after 10 min equilibration with the new perfusates with various $\text{HCO}_3^-$ and $\text{Cl}^-$ concentrations. The result indicates that the $E_M$ remained virtually unaffected by $[\text{HCO}_3^-]_e$ in the range of 5 to 75 mM. The only exception was observed in one experiment, in which the induced depolarization did not recover well in low $P_{CO_2}$ and high $\text{HCO}_3^-$ perfusate within 10 min. Such a prolonged depolarization might be somehow related with the low concentration of molecular CO$_2$ and $\text{Cl}^-$ in the system: i.e., the anion lack would possibly prolong the change of $E_M$. In fact, the magnitude of transient depolarizations could be predicted as the change of $E_M$ with a transport number of $\text{Cl}^-$ ($t_{Cl}$) of about 0.7 (for reference, the changes in $E_M$ at $t_{Cl}$ of 0.5, 0.6, and 0.7 are shown in a dotted, a broken, and a chain line, respectively). Unless ionic abnormality exists, the transient change of $E_M$ would recover soon to an appropriate level (see Fig. 3).

### 3. Effects of $P_{CO_2}$ on $pH_e$

On the other hand, effects of $P_{CO_2}$ on $pH_e$ were tested in conditions of constant $\text{HCO}_3^-$ concentration (15 mM) with various $pH_e$ (7.13–8.13) and $P_{CO_2}$ (3.7–37 mmHg).

Figure 5 demonstrates that changes of $P_{CO_2}$ and $pH_e$ produced remarkable changes in $pH_e$ without changing $E_M$. Some graded responses of $pH_e$ (6.86, 7.08, and 7.20) were observed in stepwise changes in $pH_e$ as 7.13, 7.65, and 8.13, respectively. Figure 6 illustrates the data of $pH_i$ and $pH_e$ in 4 paired muscle specimens, when...
P<sub>CO₂</sub> was altered from 11 mmHg (control: bubbling with 1.5% CO₂ gas) to either 3.7 mmHg (low P<sub>CO₂</sub> bubbling with 0.5% CO₂ gas) or 37 mmHg (high P<sub>CO₂</sub> bubbling with 5% CO₂ gas) while the external HCO<sub>3</sub><sup>-</sup> is maintained at 15 mM. It is obvious that the size of changes in pHe is far larger than that of changes in pH<sub>i</sub>.

Figure 7 shows individual pH<sub>i</sub> data plotted against pH<sub>e</sub> at three different levels of P<sub>CO₂</sub> (37 mmHg with pH<sub>e</sub> 7.0–7.2, 11 mmHg with pH<sub>e</sub> 7.65–7.75, and 3.7 mmHg with pH<sub>e</sub> 8.13–8.20). The P<sub>CO₂</sub> in each level was maintained at constant as far as possible. The overall average of pH<sub>e</sub> was 7.646 ± 0.145 (n=12) and that of pH<sub>i</sub> 7.110 ± 0.060, with a correlation coefficient (r) of +0.617 (significant at p < 0.05 on paired samples) and the regression equation, [pH<sub>i</sub>] = 0.264 [pH<sub>e</sub>] + 5.092. This finding indicates that the pH<sub>e</sub> with varied P<sub>CO₂</sub> significantly affects the pH<sub>i</sub> roughly in proportion to the acid-base derangement, and the size of pH<sub>i</sub> change could be predicted as approximately a quarter of pH<sub>e</sub> change.

The above data suggested that pH<sub>i</sub> changes would be induced mainly by that of titration of CO₂ passing through the cell membrane in molecular form, and might not be affected by permeation of flux of HCO<sub>3</sub><sup>-</sup> as such, through the membrane. To confirm this possibility, a series of experiments were performed on the sartorius muscle in vitro by systematically changing P<sub>CO₂</sub> (from 11 to 37 mmHg) and pH<sub>e</sub> (from 7.1 to 8.3) at different levels of HCO<sub>3</sub><sup>-</sup> (from 5, 11, 45, 75 mM) in the bathing media.

Figure 8 illustrates the changes of pH<sub>i</sub> (in upper half) and calculated concentration of cellular bicarbonate, [HCO<sub>3</sub><sup>-</sup>]<sub>i</sub> (in lower half), plotted on [HCO<sub>3</sub><sup>-</sup>]<sub>e</sub>. The open circles represent the control data of P<sub>CO₂</sub>, whereas the closed circles the data of high P<sub>CO₂</sub>. The result indicates that when [HCO<sub>3</sub><sup>-</sup>]<sub>e</sub> was elevated,
Fig. 6. Changes of pH\textsubscript{i} and pH\textsubscript{e} in response to 3 different levels of P\textsubscript{CO}\textsubscript{2} at constant [HCO\textsubscript{3}\textsuperscript{-}]\textsubscript{e}. The open circles give plots of pH\textsubscript{e} and the closed circles those of pH\textsubscript{i}.

Fig. 7. Changes of pH\textsubscript{i} in response to pH\textsubscript{e} at constant [HCO\textsubscript{3}\textsuperscript{-}]\textsubscript{e}.
the size of change in pH, became smaller and the change in calculated [HCO₃⁻], became larger. It is expected, therefore, that when [HCO₃⁻]ₑ is raised, the buffer capacity, which is estimated by d[HCO₃⁻]/d pH, becomes larger.

Figure 9 gives the relationship between the pH gradient across the cell membrane and [HCO₃⁻]ₑ. The open circles represent the data of 11 mmHg P₇CO₂ experiment and the closed circles those of high P₇CO₂ experiment of 37 mmHg. The dotted lines indicate the theoretical relation predicted from Eq. (5), based on non-ionic diffusion (see DISCUSSION). Although the slope of data was only slightly less, the data of 11 mmHg P₇CO₂ were on the theoretical line. But those of 37 mmHg P₇CO₂ had by far a milder slope of {pH gradient/[HCO₃⁻]ₑ} relationship, which was different from the theoretical prediction. This finding would suggest, therefore, that a mechanism of [HCO₃⁻]ₑ change other than the simple non-ionic diffusion of CO₂ would play a part in the regulation of pH, especially in high P₇CO₂ conditions.

Figure 10 demonstrates the relationship of mean ΔpH, and P₇CO₂ change in different levels of [HCO₃⁻]ₑ (75, 45, 15, and 5 mM, n = 5 for each group), indicating that [HCO₃⁻]ₑ affects the stability of pH in response to a unit change of P₇CO₂. In

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**Fig. 8.** Changes in pH, and calculated [HCO₃⁻], at various levels of [HCO₃⁻]ₑ. Changes in pH, were induced by elevating P₇CO₂ from 11 to 37 mmHg in sartorius muscle. Values are given as mean ± S.D. Solid lines represent regression lines.
bicarbonate-rich media, pH$_i$ is more stabilized than in bicarbonate-poor media.

All these results indicate that pH$_i$ is more or less affected by pH$_e$, and the degree of pH$_i$ change strongly depends on the extracellular buffer concentration and $P_{CO_2}$ level of the system.

**DISCUSSION**

Intracellular application of pH-sensitive microelectrodes to the study of acid-base balance has a number of merits. They will give almost immediate result of H$^+$ activities in cytoplasm of single cell in vivo or in vitro continuously for a certain period of time. Taking advantage of these merits, we conducted a series of micropuncture experiments on the sartorius muscle perfused in known concentration of acid-base parameters.

*Relationship between $E_M$ and pH$_i$ in skeletal muscle.* In this report, $E_M$ and pH$_i$ were studied on the frog sartorius muscle fiber perfused in vitro by changing two out of three acid-base parameters of perfusing media (pH$_e$, [HCO$_3^-$]$_e$, and $P_{CO_2}$), while
the remaining one was kept constant. Under the control conditions, we obtained an average pH of 7.0 at EM of -70 mV. Similar pH values have been reported by many workers with various methods (see review by Roos and Boron, 1981). In earlier studies of pH in frog skeletal muscle, Kostyuk and Sorokina (1961) found a pH to be in the range of 7.1 with respect to the pH of 7.36, as estimated with glass pH-microelectrodes. The value of normal amphibian pH had been believed to be about 7.4 at that time (the same as mammalian species), but to our present knowledge, the normal values of pH and PCO₂ in the frog are known to be 7.65 and 11.5 mmHg at 22°C, respectively (Howell et al., 1970).

The EM values in the present experiments (-70 mV) were lower than the reported values (-80 mV or more) by Hodgkin and Horowicz (1959) and Bolton and Vaughan-Jones (1977). This would be due to a difference of K⁺ concentration of perfusing media: i.e., we employed 3.5 mM, whereas the other workers used 2.5 mM. The latter would cause a hyperpolarization of the resting potential by some -8 mV with respect to the former conditions.

Further, we recognized a significant correlation between EM and pH (Fig. 2), indicating that the cytosol is more acidic in hyperpolarized cells, and it is more alkalotic in depolarized cells. Assuming that the H⁺ generated in the cell would

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Fig. 10. Changes in pH in perfusion with various [HCO₃⁻]e, plotted with the level of different PCO₂.
equilibrate with the interstitium by passive diffusion along the electrical gradient across the cell membrane, the hyperpolarization would favor a gain of positively charged H⁺ in the cell and the membrane depolarization would facilitate a loss of cell H⁺. This was only partially true (Figs. 2 and 3). However, if H⁺ would obey the Donnan rule, one would expect that the pHᵢ should have to be about 6.4 at the normal $E_M$ of -70 mV (see Fig. 2, Nernst line). CARTER et al. (1967) and PAILLARD (1972), who used pH-sensitive glass microelectrodes on the rat skeletal muscle and crab muscle, reported an even lower value (pHᵢ: 5.6-6.6). But this is never the case in a variety of acid-base abnormalities in our study (Figs. 2-10). Therefore, we consider that H⁺ obeys the Donnan rule only incompletely, as is in agreement with the conclusion from the study of crab muscle by HINKE and MENARD (1976). The concept is now established that cell pH regulation is attained by the balance of active extrusion of intracellular H⁺ to the interstitium and passive leak of extracellular H⁺ to the cell interior across the cell membrane.

Effect of [HCO₃⁻]ₑ on $E_M$. In the present study, we demonstrated that $E_M$ was not affected by changing [HCO₃⁻]ₑ, but was markedly affected by changing [Cl⁻]ₑ. This is explained by high permeability to Cl⁻ in the membrane of skeletal muscle (HODGKIN and HOROWICZ, 1959), while that to HCO₃⁻ is negligibly small, as inferred from the data of Fig. 3. In HCO₃⁻ replacement with Cl⁻, initial transient changes in $E_M$ were predictable by postulating the transport number of Cl⁻ ($t_{Cl}$) as 0.7 or so, irrespectively of the subsequent recovery from those initial changes (Fig. 4).

In low HCO₃⁻ experiments in Fig. 4 (shown toward upper left corner), a deviation of $E_M$ by less than 2 mV appeared between actual change of $E_M$ (presented by open symbols) and the change predicted by $t_{Cl}$ (presented by discontinuous lines), assuming that [Cl⁻]ᵢ would remain constant at 7 mM (from our own data in normal conditions: not published). BOLTON and VAUGHAN-JONES (1977) pointed out that equilibrium potential of Cl⁻ ($E_{Cl}$) in normal conditions is close enough to $E_M$, but in acidosis, a discrepancy occurred between $E_M$ and $E_{Cl}$, so that Cl⁻ conductance was reduced and intracellular Cl⁻ might be accumulated by some active process. If this is the case, a minor change of $E_M$, as was observed during ion replacement in Fig. 4, might be ascribed to some changes of ion environment in the cell in external acid-base abnormalities. In fact, in barnacle muscle, intracellular concentrations of Na⁺ and K⁺ were decreased and Cl⁻ was increased in the external acidosis (HINKE et al., 1973).

Effects of changes in pHₑ on pHᵢ. KOSTYUK and SOROKINA (1961), observed that varying $P_{CO₂}$ (pHₑ: 5-10) of the medium by bubbling 1 atm CO₂ gas caused a considerable change in pHᵢ with a minor change of $E_M$. Further, they showed that changes in $E_M$ by external K⁺ alterations at constant pHₑ did not produce noticeable change in pHᵢ. This observation was done by changing $P_{CO₂}$ of the system over a wide range of pH (bubbling with 1 atm CO₂ gas and varying pHₑ between 5 and 10).

In our study, pHₑ was kept within the physiological range between 6.7 and 8.8,
even with the ion replacements of Cl⁻ with gluconate⁻. We demonstrated how intracellular acid-base balance of the skeletal muscle fiber did change in response to the acid-base balance in the extracellular fluid (ECF). Namely, changes in [HCO₃⁻]ₑ and pHₑ at constant P⁰₂ induced only minor effect on pHᵢ, while changes in P⁰₂ with pHₑ at constant [HCO₃⁻]ₑ caused by far a large effect. Since the conductance of HCO₃⁻ as such was shown to be negligible (Fig. 3) and the permeability to CO₂ was demonstrated to be high (Fig. 5), it can be considered that the response of pHᵢ to the change of pHₑ accompanied by changes of P⁰₂ or [HCO₃⁻]ₑ may be accounted for by non-ionic diffusion (MILNE et al., 1958) in CO₂-HCO₃⁻ system in the skeletal muscle.

In non-ionic diffusion, assuming that P⁰₂ and the dissociation exponent of carbonic acid in both ECF and the cell interior are common, the Henderson-Hasselbalch equation predicts the following relation, as

\[ \text{pHₑ} - \text{pHᵢ} = \log \frac{[\text{HCO₃⁻}]ₑ}{[\text{HCO₃⁻}]ᵢ}. \tag{5} \]

The view that CO₂ in the skeletal muscle in resting state is in complete equilibrium with its surrounding medium has been verified, as demonstrated directly by studies of ion-selective microelectrodes for (HCO₃⁻) and pHᵢ (KHURI et al., 1974; KAJINO et al., 1982). The reported value for [HCO₃⁻]ᵢ in frog skeletal muscle was 4.4 mM at pHₑ = 7.38, P⁰₂ = 22.7 mmHg, and [HCO₃⁻]ₑ = 11.8 mM (KHURI et al., 1974). In Fig. 6 of the present report, the [HCO₃⁻]ᵢ values calculated from the measured pHᵢ (= 7.19) averaged 4.9 ± 0.3 at pHₑ = 7.65, P⁰₂ = 11 mmHg, and [HCO₃⁻]ₑ = 15 mM. The agreement of calculated value of [HCO₃⁻]ᵢ with the actually measured value seems to support the concept of non-ionic diffusion of CO₂-HCO₃⁻ system across the cell membrane.

The above equation (Eq. (5)) predicts that [HCO₃⁻]ᵢ is a function of 1) the pH gradient across the cell membrane, and 2) the level of [HCO₃⁻]ₑ under CO₂ equilibrium. With the increase of [HCO₃⁻]ₑ, the transmembrane pH gradient becomes larger (Fig. 9) and calculated [HCO₃⁻]ᵢ becomes also larger (lower half of Fig. 8). On inspection of Fig. 9, one can realize that when 11 mmHg P⁰₂ was administered, the plots of measured pH gradient against [HCO₃⁻]ₑ were overlapped on the predicted line from Eq. (5) which was derived from the non-ionic diffusion equation, but when 37 mmHg P⁰₂ was administered, a difference in the slopes of their regression lines between the predicted and the measured values became manifest (Fig. 9). This might be due to some direct transmembrane flux of HCO₃⁻ in high P⁰₂ conditions. Accordingly, the intracellular HCO₃⁻ concentration is determined by:

\[ [\text{HCO₃⁻}]ᵢ = [\text{HCO₃⁻}]₄ + [\text{HCO₃⁻}]₇, \tag{6} \]

where [HCO₃⁻]₄ gives the increment of [HCO₃⁻]ᵢ due to the non-ionic diffusion of CO₂ and [HCO₃⁻]₇ represents the increment of [HCO₃⁻]ᵢ due to the transmembrane flux of HCO₃⁻ (or H⁺), including amount of diffusion of HCO₃⁻ as such or
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HCO₃⁻/Cl⁻ exchange (or indirectly via Na⁺/H⁺ exchange) across the membrane during the experiment with different P₇CO₂ levels.

From Eqs. (5) and (6), we obtain Eq. (7), as:

$$\text{pH}_e - \text{pH}_i = \log \frac{[\text{HCO}_3^-]_e}{[\text{HCO}_3^-]_d + [\text{HCO}_3^-]_f}. \quad (7)$$

If the fraction of the ion flux ([HCO₃⁻]₇) would increase, the slope of the relationship would become lower. By inspection of [HCO₃⁻]₇ levels at pHₑ = pHᵢ in Fig. 9 (intersections with horizontal dashed line), one can estimate [HCO₃⁻]ₑ as the sum of [HCO₃⁻]₇ + [HCO₃⁻]₉. For instance, in 37 mmHg P₇CO₂ experiment, we obtain 10 mM for the theoretical value for [HCO₃⁻]ₑ in Eq. (5) and 8 mM for the sum of [HCO₃⁻]₇ + [HCO₃⁻]₉. Thus, the value of [HCO₃⁻]ₑ is -2 mM, or 20% of [HCO₃⁻]ₑ is the fraction due to the transmembrane efflux of HCO₃⁻ as such or influx of H⁺. Thus, non-ionic diffusion is not the sole mechanism of these cellular events. Although Bolton and Vaughan-Jones (1977) suggested a possibility of stimulated Cl⁻ uptake in high P₇CO₂ conditions, whether or not the above change of [HCO₃⁻]ₑ is coupled to the change of this Cl⁻ flux is presently unknown.

Adler et al. (1965a, b), who used the indicator method for measuring pHᵢ, pointed out that the pHᵢ of muscle cells is readily influenced by [HCO₃⁻]ₑ as well as by CO₂ tension. Similarly, Izutsu (1972) stated that changes in pHᵢ were caused by both CO₂ titration and ionic flux of H⁺ or HCO₃⁻. In support of this view, the perfusion with high P₇CO₂ produced a large change in [HCO₃⁻]ₑ (Fig. 8 in the present report), the slope of {calculated [HCO₃⁻]ₑ/[HCO₃⁻]₇} being about 7 times that in low CO₂ media. This indicates that in high P₇CO₂, changes in [HCO₃⁻]ₑ appear more remarkably in response to changes in [HCO₃⁻]ₑ.

Intracellular buffer capacity. The buffer capacity of CO₂-HCO₃⁻ system (or buffering power, buffer value: defined as Δ[HCO₃⁻]/ΔpH) is a measure of resistance to pH changes pertaining to carbonic acid buffer system in response to added acid or alkali. The amount of acid or alkali produced in our experiments can be calculated as change in [HCO₃⁻]ₑ by applying the measured pHᵢ and external P₇CO₂ to the Henderson-Hasselbalch equation.

A wide variety of data on the intracellular buffer capacity have reported it to be from 9 to 118 mEq/(pH·l) by a number of authors (see review by Roos and Boron, 1981). With a technique of CO₂ application to the skeletal muscle and measurements of pHᵢ with glass pH-microelectrodes, Aickin and Thomas (1977a, b) and Bolton and Vaughan-Jones (1977) reported that the buffer capacity of mouse soleus and frog sartorius muscle was 43 and 35 mM/(pH·l), respectively.

In the present paper, the ratio of changes in [HCO₃⁻]ₑ and pHᵢ in application of CO₂ in the frog sartorius muscle gives a result of intracellular buffer capacity of 28.9 mEq/(pH·l) for paired samples (derived from the pHᵢ data of Figs. 5 and 7 and the calculated changes of [HCO₃⁻]ₑ) and 38.2 mEq/(pH·l) for unpaired samples (Fig. 8) at [HCO₃⁻]ₑ = 15 mM. Since CO₂ is able to cross the cell membrane with ease and hydrate immediately, the cell accumulates HCO₃⁻ in CO₂ application,
thus increasing the cellular buffer capacity greatly.

It is now worthwhile mentioning that variations in acid-base parameters in ECF cause changes of buffer capacity in the cell. In Fig. 8, one can estimate various values of intracellular buffer capacity corresponding to various $[\text{HCO}_3^-]_e$, such as 7.5, 38.2, 53.1, and more than 300 mEq/(pH·l) for $[\text{HCO}_3^-]_e$ of 5, 15, 45, and 75 mM, respectively. The wide variation of reported values of intracellular buffer capacity from 9 to 118 mEq/(pH·l), as stated above, might be ascribed to this fact: i.e., a combined effect of CO$_2$ titration and flux change of H$^+$ or buffer anions.

Further, the magnitude of pH$_i$ change per unit change of $P_{CO_2}$ is also a function of $[\text{HCO}_3^-]_e$ (Fig. 10). This means that the acid-base status with a higher $[\text{HCO}_3^-]$ is more stable against $P_{CO_2}$ changes. Thus, CO$_2$ exerts an important effect upon $[\text{HCO}_3^-]$ not only in the external media but also in the cell. When $[\text{HCO}_3^-]_e$ or CO$_2$ is increased, it provides a large source of HCO$_3^-$ or CO$_2$ to the cell interior, and the intracellular buffer capacity can be increased remarkably.

We conclude, therefore, that 1) the stability of cell acidity is influenced by $[\text{HCO}_3^-]_e$ as well as by external $P_{CO_2}$, and 2) under normal conditions non-ionic diffusion through CO$_2$ should play a major role in regulating the intracellular pH, but under abnormal conditions, other mechanism can also participate in the cellular pH regulation.

Further studies are needed especially on the transient change of pH$_i$ induced by Cl$^-$ replacement and on the mechanism of transport of HCO$_3^-$ as such across the membrane.

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