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The Buffering Power in the Human Atrial Myocardium

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The quantification of intracellular buffering power is essential for calculating sarcolemmal acid-equivalent fluxes from intracellular pH (pH_i)-recordings. However, it is not well established in the human atrial myocardium. The buffering power (β_{tot}) in general has two components: the intrinsic buffering power (β_i) and the buffering capacity (β_{CO2}). Thus, we calculate the buffering power by microspectrofluorimetry with a fluorescence probe 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Experiments are performed under conditions free of Na^+, Cl^- and high K^+ to prevent the operation of pH regulators. Small stepwise reductions of external NH_4Cl (from 30 to 0 mM) result in stepwise reductions of pH_i. Similar procedures are performed in two kinds of solutions which are buffered with the CO_2/HCO_3^- - condition or the HEPES- condition. Results show that, in the CO_2/HCO_3^--condition, the values of β_{tot} can be described as β_{tot} = -1633.3 pH_i+12544.9 (R^2=0.86) in the pHi ranges of 7.1~7.5. The values of β_{tot} increase while pH_i decreasing. In HEPES-condition, the values of β_i can be described as β_i=-212.1 pH_i +1931.9 (R^2=0.026) in the pH_i ranges of 7.1~7.5. Note that there is not a significant correlation between β_i and pH_i. Moreover, between β_{tot} and β_i, there is a significant difference in the pH_i ranges of 7.1~7.3 ($p<0.01$; n=4, n=15, respectively). This means that the factor of β_{CO2} becomes more important while in the acidic conditions. Also, the magnitude of intracellular β_{CO2}, derived from β_{tot} - β_i , has been described as β_{CO2}=3670.7 pH_i^2-54082.7 pH_i +199255.4 (R^2=0.95) in the pH_i ranges of 7.1~7.5 ($p<0.01$; n=12). The result supports that CO_2-permeation and CO_2-hydration/dehydration reaction are not rapid enough to behave as an open system for CO_2 in human atrial myocardium. In conclusion, in our present study, for the first time, we quantify the buffering power in human atrial myocardium.

Key words: buffering power, human atrial myocardium, intracellular pH, fluorescence probe, microspectrofluorimetry, BCECF

INTRODUCTION

Intracellular pH (pH_i) exerts considerable influence on cellular functions, such as contractility and rhythm of cardiomyocytes. In the heart, pH_i is kept within a narrow range (7.0~7.2) through the combined operation of H^+ transporters and the intracellular buffering capacity.[1,2] Intracellular buffering capacity minimizes immediate changes of intracellular pH, either in the acid or alkaline direction. The total intracellular buffering power (β_{tot}) has two components: the intrinsic buffering power of the cell (β_i) and the buffering capacity caused by intracellular CO_2/HCO_3^- (β_{CO2}). β_i is principally due to physicochemical buffers, such as the weak acid/base moieties of cytoplasmic proteins. In the absence of CO_2, β_{tot} is simply equal to the β_i. In the presence of CO_2/HCO_3^-, β_{tot} equals the sum of β_i and β_{CO2}.

The magnitude of intracellular β_{CO2} depends on whether a cell behaves as an open or a closed system to CO_2. Koppel & Spiro initially suggested that cellular CO_2/HCO_3^- could serve as a buffer in a closed CO_2- system.[3] This would mean that all reactions occur within a single compartment with no exchange of the buffer species (CO_2) with the surroundings. In such a system, the total buffer concentration always remains constant and

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exerts its maximum buffering power at a pH equal in value to the pK_a of the buffer. In contrast, Van Slyke demonstrated that the contribution of CO_2/HCO_3^- to the buffering of blood tends to operate as an open CO_2 system.[4] In an open system, it is assumed that two compartments exist, with the outer compartment acting as a reservoir of uncharged buffer species (CO_2) that freely enters and equilibrates with the inner compartment.

The Na^+/H^+ exchanger (NHE) have been sought to regulate the equivalent of H^+ ions. Several mechanisms have been suggested to mediate the activation of Na^+/H^+ exchanger, especially in NHE-1 by ATP, such as (1) the catalytic phosphorylation of the NHE-1 protein, (2) free energy of ATP hydrolysis, (3) direct ATP binding to the exchanger, and (4) redistribution of phospholipids across the plasma membrane as reported in Na^+/Ca^{2+} exchange.[5,6] Besides, the predominant effects of ATP depletion on NHE-1 are to alter the sensitivity of the exchanger to intracellular H^+ level and to reduce the maximal rate of transport. In our present experiment, in order to prevent the effects of NHE-1, Na^+-free condition is used and the mechanisms activated by ATP and through intracellular Ca^{2+} are ruled.[7]

Systematic measurements of intracellular β_{tot} have not so far been made in human atrial myocardium. Knowledge of β_{tot} is essential in order to calculate transporter-mediated membrane fluxes of acid-equivalents. Our aim is to estimate β_{tot}, β_{CO2} and β_i in human atrial myocardium.

METHOD

Human atrium tissue

With the approval of the institutional review committee and with prior informed consent, specimens of right atrial appendages were obtained from 26 patients (15 females and 11 males) without apparent heart failure undergoing coronary artery bypass grafting. Right atrial trabecular tissue, 0.5~1 mm in diameter and 3-5 mm long, was removed, as described previously.[8,9] and immediately immersed in cold bicarbonate-containing Tyrode solution. The preparations were then perfused with oxygenated Tyrode solution, $100\%O_2$ for nominally bicarbonate-free Tyrode solution and $95\%O_2/5\%CO_2$ for bicarbonate-containing Tyrode solution, at 37^oC, pH 7.40 ± 0.02 for experiments. In other words, the oxygenation condition is changed from a pure oxygen condition to $5\%CO_2/95\%O_2$ upon the change of the perfusion medium from HEPES to bicarbonate Tyrode solution.

Measurement of the intracellular pH

Measurement of the pH_i has been described in detail elsewhere.[9,10] In brief, the pHi in the right human myocardium was measured using a pH-sensitive, dual excitation dual-emission fluorescent dye, BCECF-AM (Molecular Probes). The preparations were loaded with BCECF-AM (10 μM) by incubating them for 30 min at room temperature and were excited alternately with 490 and 440 nm wavelength light. The ratio of the 510 nm emission at 490 nm and 440 nm excitation (490/440) was calculated and converted to a linear pH using the following equation:

$$pH_i = pK_a + \log [(R_{max}-R)/(R-R_{min})] + \log (F_{440min}/F_{440max})$$

where R is the 510 nm emission at 490 nm excitation/510 emission at 440 nm excitation ratio, R_{max} and R_{min} are, respectively, the maximum and minimum ratio values from the calibration curve (data not shown), and pK is the dissociation constant for the dye, taken as 7.05. F_{440min}/F_{440max} is the ratio of the fluorescence measured at 440 nm of R_{min} and R_{max}. The overall sampling rate for the recorded fluorescent ratio (440nm /490 nm) was 0.5 Hz in the experiment. Throughout the whole experiment, the change of resting pHi induced by the tested drug was compared at the steady-state after treating the drug, unless otherwise stated.

The activities of NHE and NHS were represented by the slope following intracellular acid loading which was measured at the same pH_i. To make sure the preparations were in good condition, an intracellular acidosis was induced by an NH_4Cl prepulse (see below section for details) to test the acid extruding activity of the cell. Only those samples with a good response went through the designed protocols. Moreover, to prevent the possible influence of fluorescent dye-leakage on the pH_i recording, the strength of both wavelength of 510 nm emissions at 490 nm and 440 nm excitation were continuously monitored in the oscilloscope and computer. The background fluorescence and auto-fluorescence were small (< 5%).

In Situ Calibration Curves

Intracellular pH is estimated as a 490/440 ratio of fluorescence and calibrated as follows: human atrial myocardium is exposed to K^+-nigericin calibration solutions, which equilibrates the pH_i with the known extracellular pH. The 490/440 ratios are obtained during perfusion with five pH standard solutions (pH 5.5, pH 6.5, pH7.0, pH 7.5 and pH 8.5). Because the response ratio is linear in the pH range from 7.5 to 6.5, a simple transformation is performed to obtain the corresponding pH_i values

within the linear range.

Method for Determining β

In the present work, pre-pulsing a cell with a permeant weak base NH_4Cl is used to induce an intracellular acid load. The magnitude of the intracellular acid load, in combination with the size of the pH_i-change is used to compute intracellular buffering power. A detailed description of the use weak acids in estimating β is given by Roos and Boron.[11] In brief, β can be defined as:

$$\beta(mM)=[H^+]i / \Delta pH_i \quad (e.1)$$

where $[H^+]_i$ is the concentration of acid introduced to the cell and ΔpH_i is the resulting change in pH_i.

For experiments with the NH_4Cl prepulse technique, the application of NH_4Cl externally induces an intracellular alkalosis. This is due to the rapid diffusion of NH_3 into the cell and its subsequent hydration to form NH_4^+. Upon the removal of extracellular NH_4Cl, NH_4^+ exits the cell as an uncharged NH_3, leaving behind an equal concentration of H^+ and causing an intracellular acidosis. If $[H^+]_i$ is assumed to equal the intracellular concentration of NH_4^+ at the moment of their removal from the external solution, then equation 1 can be expressed as:

$$\beta(mM)=[NH_4^+]_i / \Delta pH_i \quad (e.2)$$

According to the Henderson-Hasselbalch equation, the relationship between internal and external NH_4^+ concentration is as follows:

$$pH_o\text{-}pH_i = \log([NH_4^+]_i / [NH_4^+]_o) \quad (e.3)$$

Equation 3 can then be re-arranged:

$$[NH_4^+]_i = [NH_4^+]_o \times 10^{(pHo-pHi)} \quad (e.4)$$

In the extracellular solution, $pH_o = pK_a + \log([NH_3]_o / [NH_4^+]_o)$ (Henderson-Hasselbalch equation). Therefore, re-arranging:

$$[NH_4^+]_o = C/(10^{(pHo-pK)} +1) \quad (e.5)$$

where C is the total extracellular concentration of the NH_4^+ and pK is the dissociation constant of the NH_4Cl. Combining equations 4 and 5, we can derive $[NH_4^+]_i$ at a given pH_i:

$$[NH_4^+]_i = [C/(10^{(pHo-pK)} +1)] \times 10^{(pHo-pHi)} \quad (e.6)$$

In an open system, the theoretical $\beta_{CO2}=2.3\times[HCO_3^-]_i$ (e.7)

Similar to the calculation procedures above for NH_4^+ and, the $[HCO_3^-]_i$
can then be calculated as

$$[HCO_3^-]_i = [C/(10^{(pK-pHo)} +1)] \times 10^{(pHi-pHo)} \quad (e.8)$$

Solutions

Standard HEPES-buffered Tyrode solution (air equilibrated) contained (mM): NaCl, 140; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$ 2.5; glucose, 11; HEPES, 20; pH adjusted to 7.4 with 4N NaOH. Unless otherwise stated, pH adjustments of all HEPES-buffered solutions (including those where ionic-substitutions are made; see below) are performed at 37oC. Standard bicarbonate-buffered Tyrode solution (equilibrated with 5%CO_2/22 mM HCO_3^-) is the same as above, except that the NaCl concentration is reduced to 117 mM, and 22 mM $NaHCO_3$ is added instead of HEPES (pH 7.40 at 37°C).

Ion-substituted solutions: In Na^+-free, HEPES-buffered Tyrode solution, NaCl is replaced with 140 mM N-methyl-D-glucamine (NMDG), and the pH adjusted to 7.4 with HCl. Cl^--free, CO_2/HCO_3^--buffered Tyrode solution contained (mM): sodium gluconate, 117; potassium gluconate, 4.5; calcium gluconate, 12; $NaHCO_3$, 22; $MgSO_4$, 1; glucose, 11. When 10 mM ammonium chloride was used, it was added directly as a solid to the solution without osmotic compensation.

Nigericin calibration solutions contained: KCl, 140 mM ; $MgCl_2$, 1 mM; 10μM nigericin; buffered with one of the following organic buffers: 20 mM 2-(N-morpholino) ethanesulphonic acid (MES, pH 5.5), 20 mM HEPES (pH 7.5) or 20 mM 3-(cyclohexylamino)-2-hydroxy-1-propane-sulphonic acid (CAPSO, pH 9.5), and is adjusted (37°C) to the correct pH with 4N NaOH. All the drugs mentioned above were obtained from Sigma-Aldrich (United States).

Statistics

Data are reported as mean±standard error of the mean (SEM). Levels of significance is assessed using either the paired or unpaired Student's *t*-test, and $P < 0.05$ is considered significant. Reported *n* values represent number of experiments, even from a single batch of cells.

RESULTS

Determination of βtot by ammonium removal

The addition of NH_4Cl induces a rise in pH_i due to the entry of the membrane-permeant form of the weak base NH_3, which then associates with H^+ thus raising pH_i. Removal of NH_4Cl induces a fall of pH_i as $[NH_4^+]_i$ rapidly dissociated into H^+ and NH_3, the latter diffusing out of the cell. It is desirable to inhibit pH_i regulation when quantifying intracellular buffering power. Such regula-

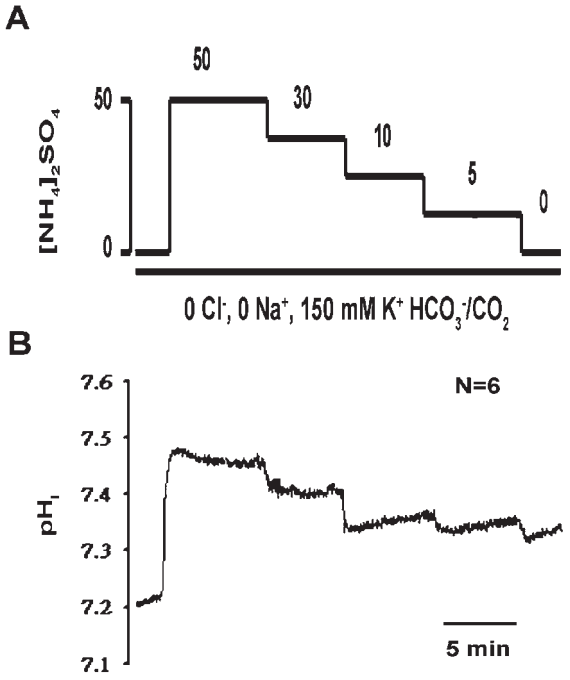

Fig. 1 Experiment to determine pH_i dependence of β_{tot} using NH_4Cl removal in 0 Cl^-, 0 Na^+, 150 mM K^+, bicarbonate-buffered Tyrode solution. A: External NH_4Cl was added and then removed stepwise as indicated. B: The trace shows the changes of intracellular pH (pH_i).

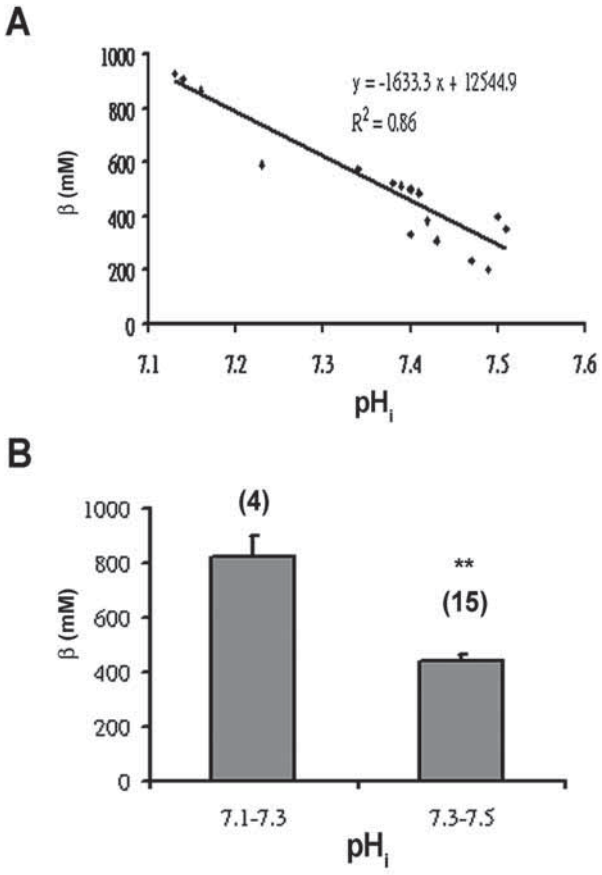

Fig. 2 β_{tot} decreases as pHi becomes more alkaline. A: Each value of β_{tot} is plotted versus pH_i. (pH_i was taken as the mid-point of each stepwise acid load followed by NH_4Cl reduction; see Figure 1). Line is fitted by linear regression (correlation coefficient, 0.86) and drawn according to the equation: β_{tot}= -1633.3$[pH]_i$ +12544.9. B: Histograms show the total intracellular buffering power (β_{tot}) averaged over two groups of pH_i (7.1~7.3; 7.3~7.5). **: p< 0.01 vs. pH_i 7.3~7.5.

tion may blunt the rapid pHi-change associated with weak acid/base addition and removal.[12] In the present study, acid extrusion is inhibited by superfusing a Na^+-free solution (Na^+ replaced isosmotically with N-methyl-D-glucamine) and high potassium solution (150 mM K^+). This inhibits pH_i recovery following an internal acid-load.[13] Figure 1A shows the protocol of small stepwise reductions of external NH_4Cl (from 50 to 0 mM). The stepwise reductions of pH_i caused by small stepwise reductions of external NH_4Cl in CO_2/HCO_3^--buffered are shown in Fig. 1B. Figure 2A shows the calculated values of β_{tot} plotted versus pH_i (*See Material and methods for detail of the calculation*). The relationship is a good linear and can be described empirically by the equation:

β_{tot}= -1633.3 [pH_i]+12544.9(correlation coefficient R=0.86).

It means that the values of β_{tot} increase as pH_i rises. Figure 2B shows the comparisons of β_{tot} in two different pH_i ranges of 7.3~7.5 and 7.1~7.3. The former pH_i range represents the normal physiological resting condition while the latter pH_i range represents the slightly acid condition. Fig. 2B shows that averaged β_{tot} of 415.2±25.6 mM has nearly doubled to 817.5±32.9 mM when pH_i shifts to a slightly acid condition (-0.1~-0.3 pH unit).

Determination of β_i by ammonium removal

Figure 3A shows the protocol of the experiment for the determination of β_i. Human atrial myocardium is bathed in a HEPES-buffered solution. Estimates of β_i is derived from the pH_i-fall and followed by stepwise of NH_4Cl reduction from 50 mM to zero in the superfusate of HEPES. Each fall of pH_i can be used to estimate β_i. Figure 3B shows the original pH_i records of the experiment.

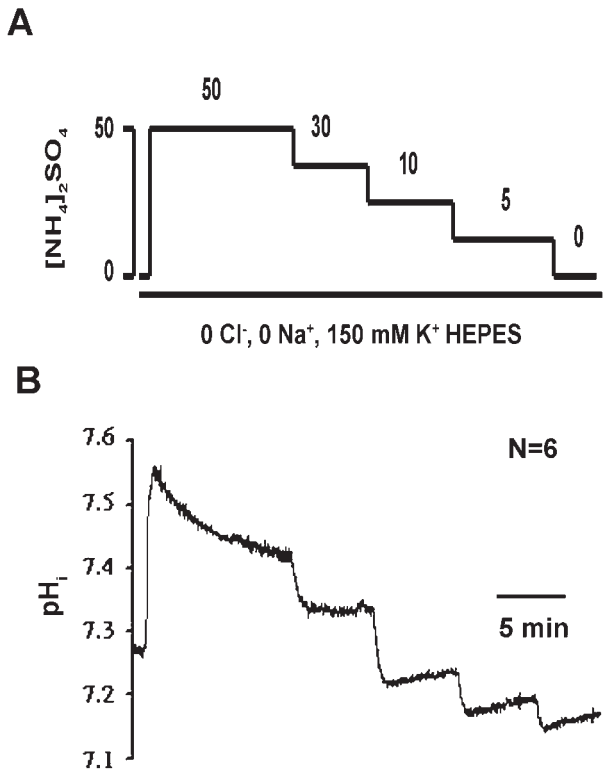

Fig. 3 Experiment to determine pH_i dependence of β_i using NH_4Cl removal in 0 Cl^-, 0 Na^+, 150 mM K^+, HEPES-buffered Tyrode solution. A: External NH_4Cl was added and then removed as indicated. B: The trace shows the changes of intracellular pH (pHi).

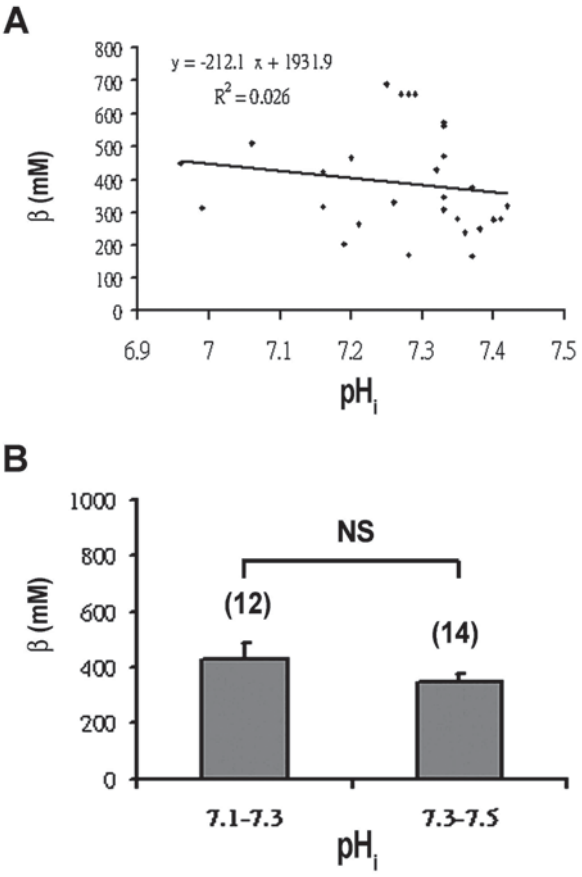

Fig. 4 β_i has no significant correlation with pH_i. A: Each value of β_i is plotted versus pH_i. pH_i was taken as the mid-point of each stepwise acid load following NH_4Cl reduction Line is not a good linear regression (correlation coefficient, 0.026) and drawn according to the equation: $\beta_i = -212.1[pH]_i + 1931.9$. B: Histograms show the intrinsic buffering power (β_i) averaged over two groups of pH_i (7.1~7.3; 7.3~7.5). NS: $p > 0.05$ vs. pH_i 7.1~7.3.

Figure 4A shows the calculated values of β_i is plotted versus pH_i. The relationship is not obvious and can be described roughly by the equation: $\beta_i = -212.1\ [pH_i] + 1931.9$ (linear regression, correlation coefficient 0.026). Figure 4B shows comparisons of two groups of β_i in pH_i ranges of 7.1~7.3, 7.3~7.5, respectively. The averaged values of β_i are 412.2 ± 35.2, 395.3 ± 26.8. There is not a significant difference between these two groups ($p > 0.05$). This result represents that values of β_i is not a good relationship with pH_i changes.

Characterization of βCO_2

Note that the pHi changes in each step associated with the addition and removal of NH_4Cl are considerably larger in HEPES-buffered conditions than in CO_2/HCO_3^--buffered conditions. These results indicate that the intracellular buffering capacity is increased considerably in the presence of a CO_2/HCO_3^- buffer system. The β_{CO2} is derived as (β_{tot}-β_i). Figure 5A pools results of β_{CO2} *versus* pH_i. The relationship is a good linear and can be described empirically by the equation:

$\beta_{CO2} = 3670.7[pH_i]^2 - 54082.7\ [pH_i] + 199255.4$ (linear regression, correlation coefficient 0.95). In order to compare of the values of β_{tot} and β_i, two kinds of β are grouped into pH_i ranges of 7.1~7.3 and 7.3~7.5 as shown in Figure 5B. There is a significant difference in pH_i ranges of 7.1~7.3 ($p < 0.01$). But, in pH_i ranges of 7.3~7.5, there is no significant difference ($p > 0.05$). The means that in the acidic condition, β_{CO2} becomes more important on intracellular buffering power.

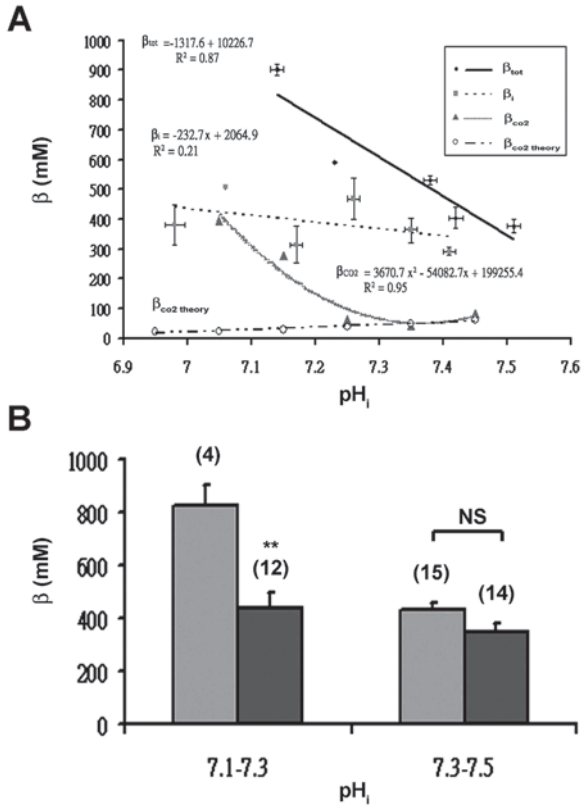

Fig. 5 β_{CO2} increases as pH_i becoming more acidic. A: Each value of β_{C02} is the result of the mean β_{tot}- mean β_i over four ranges of pH_i. Line is fitted by linear regression (correlation coefficient, 0.98) and drawn according to the equation: $\beta_{CO2} = 3670.7[pH]_i^2 - 54082.7[pH]_i + 199255.4$. B: Histograms show the significant difference between β_i and β_{tot} in the ranges of pH_i (7.1~7.3). **: $p < 0.01$.

DISCUSSION

CO_2/HCO_3^--dependent buffering dose not consists with an open CO_2-system

One factor influencing the value of β_{tot} is the contribution of CO_2/HCO_3^- to cell-buffering. In an open system, it is assumed that two compartments exist, with the outer compartment acting as a reservoir of uncharged buffer species (CO_2) that freely enters and equilibrates with the inner compartment. If one considers the intracellular & extracellular spaces as the two compartments and assumes that the cell is open to CO_2 (recall that CO_2 is highly membrane-permeant), then the intracellular concentration of CO_2 should remain constant, even during changes in intracellular pH. In such a system, intracellular buffering power is increased even when the values of pH are very different from the apparent pK. In an open system, providing CO_2-permeation and CO_2 hydration/dehydration reaction are sufficiently rapid, $\beta_{CO2} = 2.3 \times [HCO_3^-]_i$.[14] In the sheep cardiac Purkinje fiber[15], a component of β_{tot} due to CO_2/HCO_3^- buffer was measured experimentally and was very similar to that predicted for an open, fully equilibrated system for CO_2. However, estimates of β_{tot} in smooth muscle cells of guinea-pig ureter[16] and rabbit mesenteric artery[17] have suggested that intracellular buffering caused by the presence of CO_2/HCO_3^- is negligible. A similar observation has been made recently in rat cerebellar granular cells.[18] A recent study, however, in isolated human detrusor smooth muscle cells[19] and pulmonary vascular smooth muscle[20,21] suggest that a significant fraction of β_{tot} is caused by the presence of a CO_2/HCO_3^- buffer.

In the previous study, in guinea-pig cardiac myocytes, it is apparent that β_{CO2} can be calculated by using the equation: $\beta_{CO2} = 2.3 \times [HCO_3^-]_i$[3,6] and then calculated theoretical β_{CO2} = 21.3 mM at pH_i 7.04. In the human atrial myocardium, as in the equation we estimated: $\beta_{CO2} = 3670.7[pH_i]^2 - 54082.7 [pH_i] + 199255.4$ (linear regression, correlation coefficient 0.95) and calculated theoretical β_{CO2} = 438.9 mM at pH_i 7.04.

According to the equation: $CO_2+H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$, the equation shows that while in the alkaline condition, it is shifted to the right. It also means that $[HCO_3^-]_i$ will be accumulated and the values of β_{CO2} increased. In human atrial myocardium, the result shows that the model of CO_2-dependent buffering power is not fully consistent with an open CO_2- system. We find that as pHi increased, β_{CO2} will be decreased. It means that the factor influencing $[HCO_3^-]_i$ may not simply come from free CO_2-permeation. The activity of carbonic anhydrase may be involved. This result is different from earlier studies, in the sheep Purkinje fiber[15], human smooth muscle cells11 and rabbit single pulmonary vascular smooth muscle cells.[20]

The clinical implication of pH dependent values of buffering power

Bountra et al suggests that an inhibition of glycolysis will stimulate aerobic respiration, which leads to an excessive generation of carbonic acid.[22] This situation generally is found in hypoxia, an acidic condition. The increased carbonic acid will be hydrated intracellularly and reduce pH_i. Wu and Vaughan-Jones[23] exhibited that the pHi recovery from intracellular acidosis is slowed by cyanide. However, since cyanide has no inhibitory effect

on the rise of Na^+, they conclude that cyanide does not inhibit NHE-1 and that the rise in β which is associated with an increased intracellular inorganic phosphate (Pi) levels that might account for most of the slowing of pH_i recovery. These previous studies agree with our study and suggest the increased β values come from other inorganic acids. Therefore, increased β values induced by inorganic phosphate provide a perfect protective mechanism for the human heart upon the impact of a clinical pathophysiological condition, such as ischemia caused by open-heart surgery or angina pectoris. On the contrary, the phenomenon of lower β values in alkalosis condition implicates the fact that human heart cells are more vulnerable to the impact of intracellular alkalosis than that of intracellular acidosis. However, the experiments on the impact of intracellular acidosis and alkalosis need further study.

In conclusion, our study suggests that, in the human atrial myocardium, (1) CO_2/HCO_3^- makes a clear contribution to intracellular buffering power especially in intracellular acidic conditions, (2) the atrial myocardium behaves as a buffered compartment and does not fully open to CO_2 , and (3) other inorganic acids, such as inorganic phosphate (Pi) mediate the increased β values.

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