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Relationship between intracellular proton buffering capacity and intracellular pH

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Relationship between intracellular proton buffering capacity and intracellular pH. In a recent publication the widely held view that the intracellular proton buffering power [defined as the amount of acid or base that has to be added to the cytosol to change the intracellular pH (pH_i) by one pH unit] increases as the intracellular pH decreases, has been challenged, with the opposite relationship being proposed. In that publication, buffering was defined not in terms of pH change, but in terms of the change in proton concentration. The reason for this re-definition was the fear that the conventional analysis, using as it does a logarithmic function (pH_i), could bias the outcome in favor of an increasing buffering power with decreasing pH_i. The new system uses a "buffering co-efficient," defined as the number of protons necessary to be added to the cytosol to change the intracellular proton concentration by 1 mM. We report the use of both of these methods to analyze the relationship of pH_i and buffering power, using human peripheral leucocytes loaded with the pH-sensitive fluorophore BCECF examined over a very wide range of pH_i values (pHi 6.0 to 7.5). The most common method for pH_i perturbation for the measurement of buffering is used, the rapid diffusion of ammonia across the cell membrane. In this study, analysis for both a bicarbonate-containing "open" system and for a Hepes-buffered "closed" system was performed. Unlike the previous publication, the intracellular and extracellular conditions were such that the change in pH_i induced by the extracellular addition of an ammonia-containing compound (NH_4Cl) was the same (0.15 to 0.25 pH unit) across the pH_i range of 6.0 to 7.5. The pH_i-buffering relationship varied depending on the analysis used, but taking into account the known properties of Na^+/H^+ exchange and the desirability of defence of the cell against intracellular acidosis, the traditional relationship seems conceptually to be the more satisfactory.

The concept of buffering has been of interest for more than 90 years [1], but until the work of Henderson [2] in 1908, and later Koppel and Spire [3], Van Slyke [4], and Michaelis [5], understanding was limited. Buffering against pH change is important both inside and outside cells; in either case too extreme a pH perturbation is likely to prejudice physiological processes severely. For cells the maintenance of a steady pH_i range is of paramount importance for function and reproduction [6–8]; the mechanisms employed by cells to maintain their pH within that range include pH regulatory mechanisms such as Na^+/H^+ exchange and Cl^-/HCO_3^- exchange, and powerful cellular buffers [9–11]. The current and long-established definition of buffering power (or capacity) is the result of dividing the amount of acid or base that must be added to a solution to cause a certain change in pH (conventionally a change in pH of one unit) by the observed change in pH. Buffers are traditionally and sensibly divided into those that are in a "closed" or one compartment system and those that are "open," that is, in a two or more compartment system [12]. The most physiologically important buffering system is in the "open" category (extra- and intracellularly): the CO_2/HCO_3^- pairing.

Buffering of the cytosol against pH change within the cell can take place by a variety of mechanisms that comprise physicochemical and biochemical reactions, and transport of acid or base equivalents across the membranes of intracellular organelles [11–13]. These properties have been the subject of considerable research. One interesting property of cellular buffering power is its variation with the ambient pH_i. Many studies have concluded that the buffering capacity of a variety of different cells increases as the pH_i falls [14–19]; if one considers that much of the "intrinsic", that is, "closed" cellular buffering comes about by reaction with monoprotonic moeities (such as imidazole groups on proteins) the maximal reactivity will occur when pH is equal to the pKa of the buffer. The demonstration of rising cellular buffering with falling pH_i has now been challenged by a recent paper [20], in which the authors, worried that the traditional definition of buffering power may itself produce a "spurious" pH-dependence of this sort, re-define buffering not in terms of a pH change, but in terms of the notional change in intracellular proton concentration that the pH change measured represents. This they call a buffering co-efficient, and they proceed to show that the cellular buffering co-efficient has the opposite relationship with ambient pH_i, namely a fall in power as pH_i becomes more acidic.

This study examined these issues in a similar fashion and with broadly similar techniques. We were careful to ensure that the pH transients used in the derivation of the buffering were small and, as far as possible, constant across the pH range studied. The buffering capacity rose sharply with falling pH_i using the conventional definition, which is in broad agreement with other studies, including one from the same group that published the paper to which we are responding. Using their approach, the buffering co-efficients across the same pH range were also calculated, and these *decreased* sharply with decreasing pH_i. In addition to the analysis of the buffering properties, it was possible to calculate and analyze initial rates of pH change in the recovery from intracellular acidosis, and thereby total proton efflux rates. Comparison of the outcomes reveals that

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although the steep pH-dependence of activation of Na^+/H^+ exchange is common to both analyses, the more traditional approach is more appealing conceptually with regard to cellular buffering and the defense of the cell against pH disturbances.

Methods

Materials

The buffers used were Earle's, containing physiological amounts of sodium, potassium, bicarbonate, calcium, magnesium, phosphate and glucose, which when gassed with 5% CO_2 at 37°C had a pH of 7.4 and a pH of 7.2 when gassed with 10% CO_2, and a Hepes buffer (composition as above except Hepes 10 mmol/liter provided the buffering rather than bicarbonate). All solutions used contained chloride ions. The amiloride analogue 5 (N,N hexa-methylene) amiloride was purchased from Dr. E.J. Cragoe, Jr., 2211, Worthington Lane, Ohio. The acetoxymethyl ester of bis-carboxyethyl carboxyfluorescein (BCECF-AM), Nigericin, and non-esterified fatty acid-free bovine serum albumin (NEFA-BSA) were obtained from Sigma Chemicals (St. Louis, Missouri, USA). All other chemicals were from Sigma or BDH Chemicals (Poole, Dorset, UK).

Laboratory methods

Cell isolation, measurement of intracellular pH and calibration. Leucocytes were isolated by sedimentation with 3% Dextran and residual erythrocytes removed by hypo-osmotic shock. The procedure for isolating the cells and then loading them with BCECF-AM have been described [19, 21], but in brief: the concentration of BCECF-AM used was 6 μmol/liter and incubation was for 30 minutes. All experiments and measurements took place at 37°C. Calibrations were performed for each study using a high potassium/nigericin medium [22] involving sequential titrations with 0.1 M potassium hydroxide while recording fluorescence output and extracellular pH (Whatman pH meter with micro-probe). The calibration relationship was linearised, and the gradient and least squares linear regression computed (Epson PC AX-2). All regressions had an r value greater than 0.995. After the loading period, and after incubation for 20 minutes in either bicarbonate or Hepes buffer, the pH_i of a cell suspension of 1 to 3 million cells/ml was recorded over a period of 60 seconds.

Initial rate of change of pH (recovery from intracellular acidosis). pH recovery from intracellular acidosis induced either by clamping of pH_i using the ionophore nigericin or by ammonium chloride prepulse has been shown to be mediated by Na^+/H^+ exchange [16, 21, 23]. This study demonstrated that in the absence of extracellular sodium or in the presence of 10 μmol/liter 5-(N,N hexamethylene) amiloride, there was no measurable recovery in pH over a 40 second time period. At the concentration used the amiloride analogue is highly unlikely to have had significant protonophoric capacity, particularly given the short exposure time. Thus the pH changes measured in the presence of extracellular sodium represent Na^+/H^+ exchange activity, and non-Na^+/H^+ mediated recovery from intracellular acidosis is negligible. Using the nigericin technique cells' pH_i could be clamped to various levels between 5.9 and 7.2. As in other studies [16, 18, 19] we were able to demonstrate that at a pH_i of 6.0 the initial rate of intracellular re-alkalinization (proton efflux rate) is very close to its maximal value for normal subjects (V_{max}) (Fig. 4).

For the study of dynamic fluorescence data aliquots of cells were suspended in the high potassium buffers at varying pHs (5.9 to 7.2) in the presence of 2 μmol/liter nigericin for five minutes, to clamp the pH_i at the desired level. The cells with their pH_i clamped were then washed and resuspended in 100 μl of a sodium-free (choline isosmotically substituted for sodium) Hepes buffer at pH 7.0 containing 5 g/liter NEFA-BSA in a fluorescence cuvette at 37°C. To these cuvettes in turn was added 2.9 ml of Earle's medium (final concentration of sodium 135 mmol/liter, potassium 6.5 mmol/liter, pH 7.4). The fluorescence (495/440 nm) of the cell suspension was recorded from the moment of addition of the 2.9 ml aliquot for 40 seconds. The initial rates of recovery over the first 20 seconds were analyzed by computer and treated as being linear; least squares linear regression co-efficients were deemed acceptable if greater than or equal to 0.95. For each experiment this was performed in duplicate, with the final result being the mean of the two values. If there was more than a 25% difference between the two readings, the reading with the lower linear regression value was discarded. The intra-assay coefficient of variation for this protocol was 21%, with the inter-assay coefficient of variation 23%. From the calibration relationship it was possible to convert the initial 20 seconds of alkalinization into the initial rate of change of pH (in pH U/min).

The Hill (or stochastic) equation was used to model the Na^+/H^+ exchanger kinetics:

$$V = V_{max} \times [H^+]_i^{nH}/K_H + [H^+]_i^{nH} \quad (1)$$

(where V = velocity of reaction, V_{max} = maximum velocity of reaction, $[H^+]_i$ = Internal proton concentration, K = pK_a (point of half-maximal activity), H = Hill coefficient, n = stoichiometry of binding).

Buffering capacity (BC). This was estimated using the immediate partitioning across the cell membrane of ammonia (by adding a small aliquot of ammonium chloride, final concentration 1 to 5 mmol/liter). At pH values above 7 this addition produced a change in pH_i of between 0.15 and 0.25 pH units. To measure the buffering capacity at pH levels lower than this (5.9 to 7.2), to calculate the H^+ efflux rates, ammonium chloride at final concentration 1 to 2.5 mmol/liter was added to cells that had been pH-clamped (with the ionophores scavenged with NEFA-BSA) while suspended in sodium-free (choline) medium at pH 7.4. In the absence of sodium there was no measurable pH recovery, while the addition of the ammonium chloride produced a pH_i change of approximately 0.15 to 0.2 units. Using the equations:

$$[NH_4^+]_o = [NH_3]_o \times 10^{pK_a - pH_o} \quad (2)$$

$$[NH_4^+]_i = [NH_3]_i \times 10^{pK_a - pH_i} \quad (3)$$

$$BC = d[NH_4]_i/d(pH_i) \quad (4)$$

which can be shown to be:

$$BC = [NH_4]_o/d(pH_i) \times 10^{pH_o}/10^{d(pH_i)} \times 1/10^{pH_i(start)} \quad (5)$$

Equation (5) here is the same as equation (5) in [20]. $[NH_4]_o$ and $[NH_4]_i$, $[NH_3]_o$ and $[NH_3]_i$ are the extracellular and intracellular concentrations, respectively, of ammonium ions and free

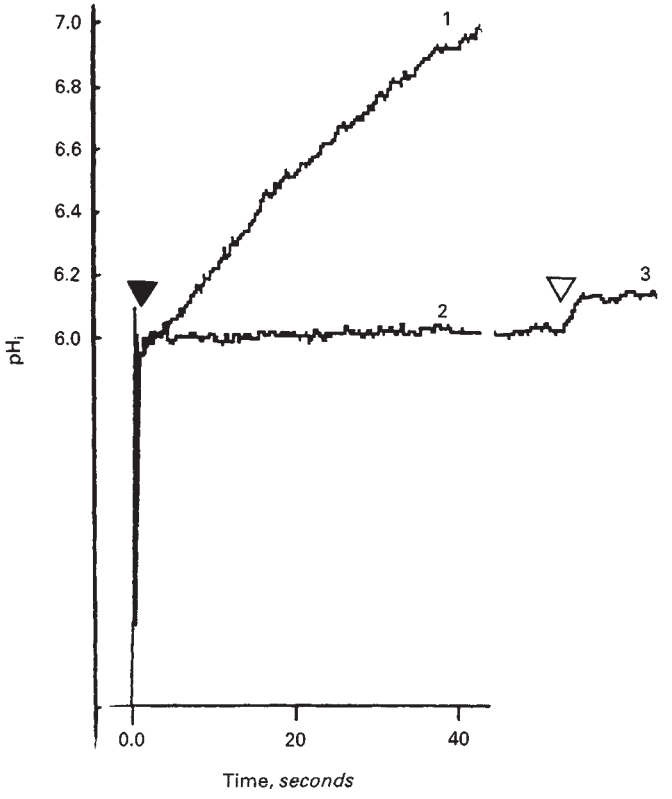

Fig. 1. *Trace of pH_i of a suspension of leucocytes clamped to pH 6.0 and then allowed to recover towards resting pH [by removal of ionophore nigericin and addition of intracellular sodium (at filled arrow)].* With and without the amiloride analogue HMA shows that recovery is mediated by Na^+/H^+ exchange. The second trace is the effect on pH_i of an addition of an aliquot of ammonium chloride to a suspension of leucocytes (at open arrow) at pH_i 6.0, in the absence of extracellular sodium.

ammonia; pH_i = intracellular pH; pH_o = extracellular pH; pK_a = half-maximal buffering and BC = buffering capacity.

Knowing the variables pH_i (start and finish), pH_o (start and finish), and extracellular ammonium chloride concentration, it was possible to calculate the change in internal ammonium ion concentration, using a value of 8.89 for the pK_a of the dissociation of ammonia in solution at 37°C, and assuming that this figure applies to the intracellular environment and that the free ammonia is at equilibrium across the cell membrane. Using the derived change in intracellular ammonium ion concentration and the measured change in pH_i, the buffering capacity was calculated. To calculate the buffering coefficient the same equation as in the previous study (eq 7 in [20]) was used, namely

$$BC = [NH_4]_o \times 10^{pH_o}/10^{d(pH_i)} - 1 \qquad (6)$$

Buffering capacity due to bicarbonate/CO_2 was derived by subtracting intrinsic buffering capacity from the total buffering capacity, measured using cells at a range of pH_i values in an Earle's buffer (sodium chloride replaced by equimolar amount of choline chloride).

Statistics

From the data in this study and from previous work we have found that in normal subjects pH_i and the Na^+/H^+exchange values have a normal distribution. Therefore Student's unpaired *t*-test (Oxstat statistical program) was used for the between groups for comparisons. All *P* values are two-tailed. Results are expressed as either mean ± SEM or median values with the range in parentheses.

Results

Figure 1 shows data from the recordings of leucocytes clamped to pH 6.0 and exposed to a 140 mmol/liter sodium solution, allowing a rapid recovery towards resting pH. The lack of recovery in the presence of the amiloride analogue HMA (or in the absence of extracellular sodium) is also shown. The separate trace shows the effect on leucocyte pH_i of the addition of an aliquot of ammonium chloride to a suspension of non-recovering (sodium-deprived) acidified cells.

Figure 2A shows the buffering capacity (traditional method) increasing steeply as the pH_i falls. Figure 2B uses the same data, but re-calculated as buffering co-efficients. This shows a marked rise as pH_i rises.

Figure 3A shows the initial rate of pH change in the recovery from intracellular acidosis, across a wide pH range. Figure 3B shows the initial change in intracellular proton concentration (derived from Figure 3A) for cells under the same conditions.

Figure 4A shows the proton efflux rate (the product of cellular buffering and initial rate of pH change) across the pH range; this reached a maximum at around pH 6.0. Figure 4B shows a similar relationship (this time derived from the product of the cellular buffering co-efficient and the initial rate of change of proton concentration). There is not numerical identity with Figure 4A because of the various approximations involved in the calculations.

Figure 5A is a representation from all the experiments of the total cellular buffering power across the pH range. The contributions from intrinsic and from bicarbonate/CO_2 buffering are shown. Figure 5B shows the same data, presented as buffering co-efficients.

Discussion

The most common technique to measure cellular buffering is the use of a weak acid or base to penetrate the cell membrane and thereby to cause a measurable change in pH_i. There are many exacting experimental requirements that have to be fulfilled for the derived results to be both valid and meaningful, and this technique rests on a number of assumptions [13]. Many agents have been used and each has specific advantages and drawbacks depending on the experimental circumstances; the NH_4/NH_3 pairing is a significant physiological buffer and is often used experimentally to measure intracellular buffering power. A small aliquot of ammonium chloride is added to the extracellular environment, which results in the presence of some free ammonia by dissociation. This free ammonia, whose precise concentration depends both on the concentration of ammonium ions and on the extracellular pH (reflecting the $pH:pK_a$ relationship), rapidly diffuses across the cell membrane into the cell, where initially its concentration must equal that outside the cell (law of mass action). Inside the cell the free ammonia combines with intracellular protons to cause an intracellular alkalinization. The extent of the reduction in intracellular proton concentration should exactly equal the formation of ammonium ions within the cell. The extent of this intracellular

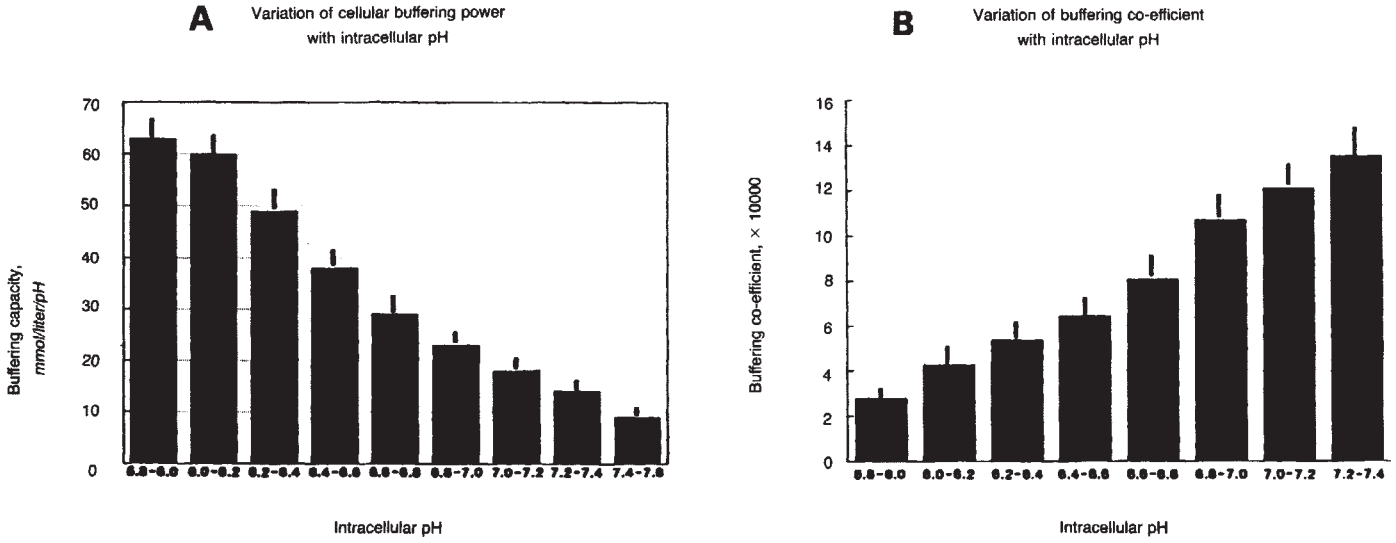

Fig. 2. *The relationship between cellular buffering capacity and pHi (A) and the cellular buffering co-efficient and pH_i (B).* Data are means ± SEM.

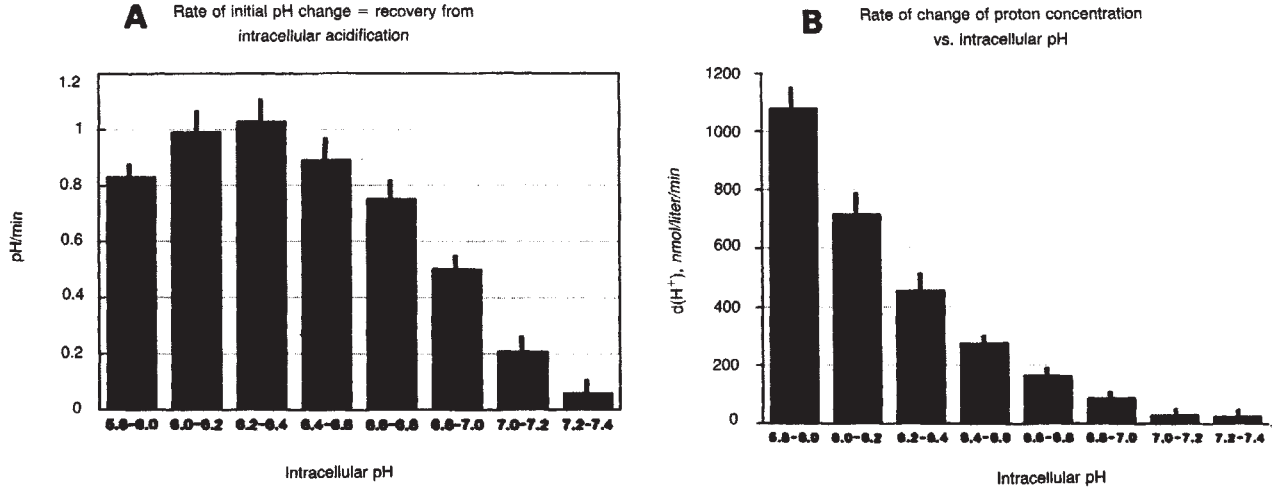

Fig. 3. *The relationship between the initial rate of pH change in the recovery from intracellular acidosis and pHi (A), and the calculated initial rate of change of proton concentration and pH_i (B).* Data are means ± SEM.

alkalinization depends on the concentration of free ammonia and the pH_i. Buffering power is then calculated as the change in intracellular ammonium concentration divided by the change in pH_i. Central to this approach are the assumptions that the cell has a negligible intracellular ammonium ion concentration at the start (in terms of the amount added, true), that the dissociation characteristics within the cell are the same as those outside, that the plasma membrane is freely and immediately permeable to the uncharged species (ammonia), that in the time of the analysis there is no additional entry to or exit from the cell, by means of transport or metabolism, of either the charged or the uncharged form, and that during the analysis no significant pH regulation occurs. This last point is crucial; as the pH being studied deviates further from resting pH, the pH-regulatory ion-transport pathways (Na^+/H^+ exchange and HCO_3^-/Cl^- exchange) will increasingly serve to return the pH_i towards the resting pH level. If the pH recovery is not prevented then there will be a serious overestimation of the true pH change brought about by the ammonia, leading to an underestimation of cellular buffering power.

Of necessity, by altering the pH_i by adding ammonia the very system under examination is altered—the pH_i after ammonia has entered the cell is more alkaline than before (if it were not the buffering capacity would be infinite). Therefore to study buffering at a particular pH value, it must be accepted that this technique is imprecise. If the pH change brought about by the addition of ammonia is too large then the cell is no longer near the pH at which its buffering capacity is being measured. In their study, Saleh, Rombola and Batlle [20] produce a pH change of around 0.5 pH unit, which we consider to be too great (*vide infra*).

This study investigates the relationship, in both a closed and

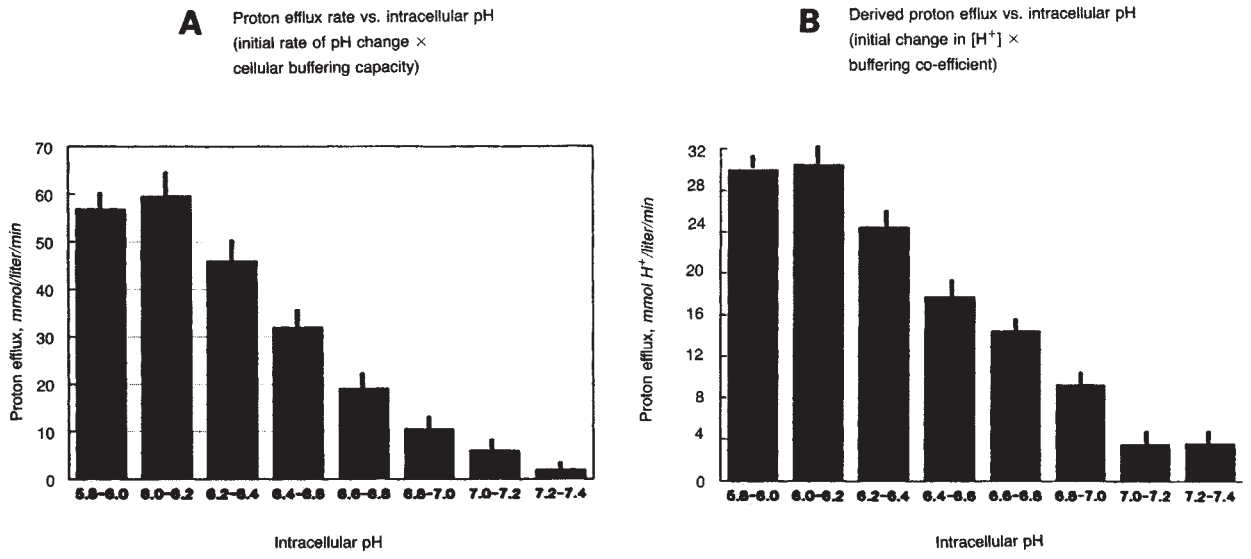

Fig. 4. *The relationship between the total proton efflux rate (product of the initial rate of pH change and the intracellular buffering capacity) and pH_i (A), and the derived proton efflux rate from rate of change of proton concentration and cellular buffering co-efficient (B).* Data are means ± SEM.

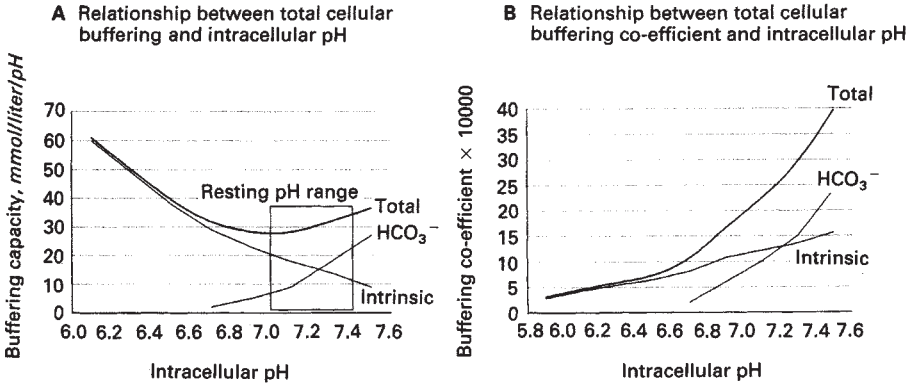

Fig. 5. *Total cellular buffering capacity versus pH_i (A), and total cellular buffering co-efficients versus pH_i (B).*

an open buffering system, between the buffering capacity of human peripheral circulating leucocytes and pH_i. We used a mixed population of cells, mainly neutrophils and lymphocytes; while inter-cell type quantitative differences do exist for buffering capacity, there are no important inter-cell qualitative differences with respect to the regulation of intracellular pH.

To measure pH_i the well-described pH-sensitive fluorophore BCECF, introduced into the cells as the pentaesterified BCECF-AM and cleaved there by native esterases into BCECF [24] was used. BCECF is an excellent fluorophore, whose sensitivity to pH change is greatest at around pH 7 (approximately the pK_a in vitro), but which retains appreciable pH sensitivity even at pH 6.

The fluorescence of suspensions of BCECF-loaded leucocytes was then recorded in a fluorimeter. We were able to acid-load the leucocytes using the nigericin/high potassium method of Grinstein, Cohen and Rothstein [25] and, by removing the ionophore and removing extracellular sodium, clamp the pH_i at a wide range of pH levels, from 5.9 to 7.5. In the absence of extracellular sodium there is negligible recovery from intracellular acidosis even in an alkaline buffer (Fig. 1), as in leucocytes this is dependent on Na^+/H^+ exchange [16, 18, 21]. To cells clamped at the different pH levels, but with the membrane ionophore (nigericin) scavenged by albumin, ammonium chloride was added at concentrations that were predetermined to produce a similar pH_i transient. The limit of accurate measurement precludes a pH transient of less than 0.1 pH unit, while a pH change of greater than 0.25 pH unit would of necessity alter the system being measured to an unacceptable degree; for this reason transients of between 0.15 and 0.25 pH unit were produced. This approach has the corollary that the strength of the ammonium chloride solution was varied across the pH_i range chosen (from 1 to 5 mmol/liter). Using this method, the concept that the authors of the previous study had investigated could be directly examined, namely that the same pH transient does not represent the same proton concentration change (such as pH 6.0 to 6.2 is 370 nmol/liter H^+ while pH 7.0 to 7.2 is 37 nmol H^+/liter), and that because of this fact there is a relative underestimation of the buffering taking place at more alkaline pH values as the concentration of protons is correspondingly much smaller.

Finally, the relative contribution of the bicarbonate and

non-bicarbonate systems to total cellular buffering was examined across the pH range of 6.6 to 7.5. The rates of pH recovery on the re-addition of extracellular sodium to cells clamped to pHs were also measured from 6.0 (at or near V_{max} [16, 18]) to 7.2 (near where the antiporter becomes quiescent [18, 21]); using these and the buffering capacities at the corresponding pH levels total proton efflux rates could be derived [14, 16, 18, 19], and the relationship between proton efflux and pH_i could be investigated.

Using the conventional analysis for the closed buffer system a strong relationship between falling pH_i and rising intracellular proton buffering capacity is shown [Fig. 2a]. This relationship was reversed when the buffering co-efficient was calculated [Fig. 2B]. For the "open", bicarbonate/CO_2 system, this study's results from the conventional analysis of the buffering capacity showed the expected rise in buffering power as pH_i (and hence intracellular bicarbonate concentration) increased [11, 12]: the buffering power from an open system should rise by $2.3 \times [B]$ where [B] is the concentration of the buffer (in this case bicarbonate). This effect is magnified by using the buffering co-efficient approach (Fig. 5 A and B). Intracellular buffering capacity is used in the derivation of Na^+/H^+ kinetics for total cellular proton efflux rates; it might be thought that the steep activation of the Na^+/H^+ exchanger (a co-operative effect of increasing intracellular proton concentration on the efflux of intracellular protons) [26] might not be seen using the newer approach (where the initial rates of change of pH_i seen after at the start of recovery from differing degrees of intracellular acidosis are converted approximately to rates of change of proton concentration). In fact, there is a very similar relationship whatever approach is used (Figs. 3 and 4).

It should be appreciated that although all the discussions have been about intracellular buffering, there is nothing unique in this setting, and for this reason the same arguments can be expressed for all buffering. Buffering with a single closed buffer system will increase as the pH of the solution approaches the pK_a of the buffer in question; the corollary of the use of the buffering co-efficient is that the main non-bicarbonate (intrinsic) buffering systems have a pK_a greater than 7.5, while if the traditional buffering capacity relationship is used instead, the pK_a is probably around 6. It is likely that in vivo there are several important moieties responsible for cytoplasmic buffering (as is the case for plasma). Also the degree of buffering by cytoplasmic organelles will distort the simple pH:pK_a relationship. On this last point, the cytoplasmic organelles in leucocytes are highly acidic—from first principles one might expect their contents to buffer against acid pH changes better than alkaline ones. Leucocytes contain virtually no mitochondria (typically alkaline), which is a point in favor of the traditional interpretation of cellular buffering and pH_i.

Using the conventional analysis, it is clear that the bicarbonate buffering system is important for buffering around and above resting pH_i levels (consistent with the operation of bicarbonate-dependent re-acidifying pH regulatory mechanisms). As the pH_i falls from resting pH, the intrinsic (non-bicarbonate) buffering capacity rises, and with it the activity of Na^+/H^+ exchange (proton efflux) increases. Thus the cell at resting pH is sitting at a "trough" in the overall buffering capacity (Fig. 5A), with both systems poised to defend the cell against pH changes. Most of the other studies in this field, using a variety of techniques (weak acids as well as weak bases [14–19], and even NMR [27]), have all arrived at the conclusion that cellular buffering rises as intracellular pH falls. Only one study that reports the opposite could be found, which was in rat brain synaptosomes [28].

To the same extent that the traditional approach may risk biasing the relationship in favor of an increase in buffering capacity with falling pH_i, so the newer approach will bias the relationship in the opposite direction, as the number of protons conceptually involved in buffering falls dramatically as the pH rises. This figure is the denominator in the new calculations. The use of a logarithmic function, pH, in the derivation of buffering power using a weak acid or base, fits better with the dissociation properties of that weak acid or base (pK_a is, of course, also logarithmic) than the linearization that is a feature of the new approach.

Thus, the new approach to defining the pH-dependency of intracellular buffering capacity, although yielding results that are algebraically correct, does not offer as conceptually satisfactory a picture of the defence of the cell against pH-perturbations, and for this reason has little to recommend it.

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