Control and Modulation of Intracellular pH in Mammalian Central Neurones

by

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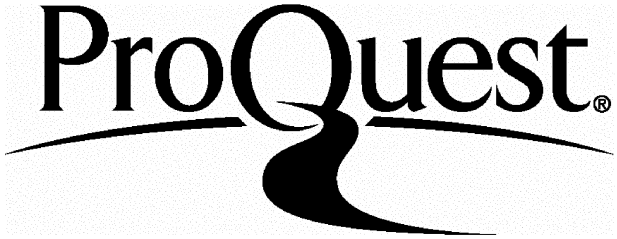

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ABSTRACT

This thesis describes single cell microfluorimetry studies of intracellular pH (pH_i) in rat central neurones maintained in culture. The work aimed to determine the proton buffering power of these neurones as well as define the contribution of CO_2 to this buffering. Furthermore, the relationship between buffering power and pH was to be investigated as well as the nature of pH_i-regulating transport mechanisms present in these cells. In addition to investigating these pH_i homeostatic mechanisms, the modulation of neuronal pH_i by neurotransmitter action was investigated.

In order to estimate proton buffering power, cells were challenged with a variety of weak acids and bases (NH_4Cl, trimethylamine, butyric acid, CO_2). Experiments performed in the absence of CO_2/HCO_3^- yielded estimates of intrinsic buffering power (10mmoles/l at pH 7.25). In the presence of CO_2 buffer, estimates of total buffering power were obtained. The contribution of CO_2 to proton buffering appeared to be minimal. Furthermore, the presence of a carbonic anhydrase inhibitor had no effect on estimates of total buffering power. The values of buffering power appeared to be independent of the acid/base used to obtain them, with the exception of those estimated using butyric acid. In this latter case some monocarboxylate ion transport apparently occured causing an overestimation of buffering power. Both total and intrinsic proton buffering were found to increase with decreasing pH over the range measured (6.7-7.7). The method of measuring buffering power was such that pH_i-regulating mechanisms had a negligible effect on buffering power estimates.

The effect of glutamatergic agonists on neuronal pH_i were investigated. Challenges of 50μM L-glutamate, or the metabotropic glutamate agonist tACPD, elicited a sustained intracellular acidification. In HEPES buffer these acidifications took the form of a step change in pH_i whereas in CO_2 buffer a continuous decline in pH_i was observed. One mechanism by which the metabotropic glutamate receptor agonist appeared to work was via suppression of a bicarbonate-dependent pH_i-regulating process. This effect was possibly G-protein mediated.

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Abbreviations:

ß	Buffering power
$ß_T$	Total buffering power
$ß_i$	Intrinsic buffering power
$ß_{CO2}$	Buffering power due to CO_2
K_a	Acid dissociation constant
α	Solubility coefficient of CO_2
A23187	4-bromo-A23187
BCECF	2'7'-bis(carboxyethyl)-5(6)-carboxyfluorescein
CA	Carbonic anhydrase
CCTV	Closed circuit television
CHC	α-Cyano-4-hydroxy-cinnamate
DIDS	4,4'-dinitrostilbene-2,2'-disulphonic acid
DMEM	Dulbecco's modified Eagle medium
DRG	Dorsal root ganglion
EGTA	Ethylene glycol-bis-N,N,N',N'-tetra acetic acid
EIPA	Ethylisopropyl amiloride
EPSC	Excitatory postsynaptic current
GABA	γ-amino-butyric-acid
$[HCO_3]_i$	Intracellular bicarbonate
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]
HVA	High voltage activating current
LAP3	L(+)-2-Amino-3-phosphonopropionic acid
LTD	Long term depression
MCPG	(±)-α-methyl-4-carboxyphenylglycine
MEM	Minimum essential medium
NaL	Locke's solution
NMDA	*N*-methyl-D-aspartate
NMDG	*N*-methyl-D-glucamine
NMR	Nuclear magnetic resonance
PCO_2	Partial pressure of CO_2
$PCO_{2\ inside}$	Intracellular partial pressure of CO_2
$PCO_{2\ outside}$	Extracellular partial pressure of CO_2
pH_i	Intracellular pH
SID	Strong ion difference
tACPD	(±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid
TMA	Trimethylamine

CHAPTER 1: INTRODUCTION

The need to regulate pH in biological systems exists due to the high reactivity of the H^+ ion. When free in solution (probably existing as $[H_3O^+]_n$) it can affect many processes by changing the degree of ionisation of various molecular species. Any chemical group that has the propeties of a weak acid or weak base will change its degree of ionisation as pH changes and this may have a profound influence on macromolecular function. Its effect on proteins, for example, is demonstrated by the pH sensitivity of enzyme activity and ion channel activity. This is strikingly illustrated by the pH sensitivity of the glycolytic enzyme phosphofructokinase which can increase its activity twentyfold when pH is increased from 7.1-7.2. Consequently there is a limit to the range of the pH values of main body fluids that are compatible with life. (Individual membrane bound compartments, however, may have very acidic pH values eg lysozomes - pH≈5.5). Cells continually produce H^+ ions through metabolic processes such as ATP hydrolysis. As the H^+ ion is so reactive, cells need to resist large swings in intracellular pH (pH_i). This is achieved by immediate buffering of pH changes and subsequent longer term pH_i-regulating transport processes. In addition to this, the sensitivity of many biochemical processes and molecules to pH changes may permit a role for H^+ ions as intracellular messengers.

Eukaryotic animal cells have a number of methods at their disposal to resist changes of pH_i. For clarity these have been divided by time course into immediate proton buffering and pH_i-regulating transport of acid/base ionic species (Roos & Boron, 1981). This Thesis is principally a study of the former process in rat central neurones but an outline investigation of pH_i-regulatory transport mechanisms is included. Some preliminary work on the role of H^+ ions in intracellular signalling is also included.

Proton buffering

All excess protons must ultimately be extruded across the plasma membrane but this takes a finite time. In the very short term, shifts in pH_i are moderated by buffering. The study of buffering H^+ ions has been of interest since the turn of the century (Henderson,

1908, Koppel & Spiro, 1914; see Roos & Boron 1980). Immediate buffering of H^+, familiar in the sense of buffer solutions in chemistry, is performed by weak acids or bases. This may be through free acid for example, or basic groups on a protein. A weak acid, HA for example, will partially dissociate into protons (H^+) and the acid anion A^-. The balance of the three different species is determined by a constant, the K_a, describing the dissociation equilibrium. In buffering excess acid, A^- will combine with H^+ to form HA, thus removing free H^+ from solution. Conversely, to buffer excess base HA will dissociate into A^- and H^+. Metabolic processes may also remove protons from solution (such as the consumption of H^+ in oxidative phosphorylation) as might sequestration into organelles (into mitochondria for example). These processes may also therefore contribute to intracellular proton buffering.

Intracellular candidates for proton buffering in neurones include the principal titratable groups: α-aminos, carboxyl groups and phosphate groups. The intracellular concentrations of these groups are generally fixed, designating them as 'closed' buffers. Van Slyke (1922) and Koppel & Spiro (1914); (see Roos & Boron 1980) showed that in a closed system (i.e. no exchange of buffer species with any other compartment) buffers will work best at pH values close to the buffer pK value if buffering is measured in terms of pH changes (as opposed to $[H^+]$ changes). We would expect intracellular buffering to be most effective when pH_i values are close to the pK_a of the most dominant buffering groups in a cell.

Koppel & Spiro (1914)(see Roos & Boron, 1980) also showed that the maximal buffer value of any weak acid or base of equal activity is the same and numerically equal to $0.58[T_A]$, where $T_A=([A^-]+[HA])$. At at a pH value equal to the pK_a of the buffer the system will buffer at a strength of $0.58[T_A]$/pH unit for each L of solution. However, the special case of a buffer system where one component can permeate the cell membrane needs to be considered. This is particularly important in biology due to the presence of CO_2 and its dissolved species and was first referred to by Henderson (1908). CO_2 hydrates readily (though slowly, time constant=30s) at body temperature to form carbonic acid. Carbonic acid (pK_a 3.12) in turn is highly dissociated into H^+ and HCO_3^- ions. The overall reaction is:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The system buffers acid as excess protons combine with HCO_3^- and can thus be removed from solution as CO_2. With excess base, entry and hydration of CO_2 occurs and H_2CO_3 is formed which subsequently dissociates into protons and HCO_3^- ions. The maintenance of a fixed PCO_2 (and hence $[H_2CO_3]$) means that this system is much more powerful in buffering than a closed system. This point is illustrated by consideration of the Henderson-Hasselbalch equation (see appendix 1 for derivation):

$pH=pK+\log[A^-]/[HA]$ Equation (1).

pH= pH of buffered solution
pK= pK_a of weak acid (pK'_a -apparent pK_a is used in calculations)
$[A^-]$= concentration of acid anion in solution
[HA]= concentration of undissociated acid in solution

The pK_a term is a constant, so pH will only change if either or both $[A^-]$ and [HA] change. Consider the situation where initial pH is equal to pK ie $[A^-]$=[HA], so $\log[A^-]/[HA]$= 0. If the concentration of $[A^-]$ under these conditions is 1mM, and we add 0.1mM hydrogen ions to solution then $[A^-]$ drops by 0.1mM and [HA] increases by 0.1mM (in reality only a proportion of the added protons combine with A^-). Hence the term $\log[A^-]/[HA]$ is now -0.087, ie pH drops away from the pK value by 0.087 units. This is the case for a closed buffer system. Now consider the case where [HA] is constant (as in the case of H_2CO_3 when PCO_2 is fixed). In this open buffer system addition of 0.1mM H^+ will cause $[A^-]$ to drop by 0.1mM but [HA] remains constant so $\log[A^-]/[HA]$ changes by -0.045. ie the pH drop is about half that seen in a closed buffer system. In fact, whereas maximal buffer value of a closed system is $0.58[T_A]$ (where $[T_A]$=concentration of acid) that of an open system is $2.3.[A^-]$ (or $2.3[BH^+]_i$ for a base; derivation in appendix 2). In the case of the CO_2-HCO_3^- system this translates into $2.3[HCO_3^-]_i$. So, taking the example of a weak acid, in a closed system maximal buffering occurs at pH value equal to the buffer pK_a and the maximum buffer value is equal to $0.58[T_A]$ whereas in an open system maximum buffer value is equal to $2.3[A^-]$ and thus maximal buffering occurs at greatest $[A^-]$ ie at high (alkaline) pH values. The effectiveness of open buffers has been shown in snail neurones using CO_2 (Thomas, 1976) and weak bases (Szatkowski, 1989).

From these considerations a neurone sitting at neutral pH [1] would apparently be dependent on closed system buffers (phosphate pK_a 6.5, carboxyl pK_a 4.5) in acid transits and increasingly dependent on an open system buffer (the CO_2-HCO_3^- system) for resisting alkaline excursions.

That buffers work best at pH values equal to pK_a depends on the consideration of pH-buffering. The rationale behind choosing changes in pH rather than $[H^+]$ is that we are generally interested in the relative change in $[H^+]$ rather than the absolute change in $[H^+]$. In terms of 'pH-buffering', buffers in a closed system work best at pH values near their pK value, as first shown by Koppel & Spiro in 1914 (see Roos & Boron, 1980). In terms of 'H^+-buffering', buffers will work best at alkaline pH values. This is due to the fact that at very alkaline pH values the buffer is nearly all in the A^- form and so will react with most of the free protons added to remove them from solution, thus making the change in free $[H^+]$ small. At acidic pH values, where most of the buffer is in the HA form, many of the added protons will stay in solution unable to react with A^- and thus the change in H^+ will be large i.e. for a given amount of added protons the change in free proton concentration will be greater at acidic pH values than alkaline ones.

The special nature of the CO_2-HCO_3^- buffering system has led to the customary division of cellular buffering power into two components, intrinsic ($ß_i$) and that provided by open buffers (Roos & Boron, 1981). Intrinsic buffering ($ß_i$) is that contributed by endogenous buffers such as phosphate groups. That contributed by open buffers is designated $ß_{CO2}$ - mainly being that portion contributed by the CO_2-HCO_3^- system. The total buffering power of a cell, $ß_T$, is the sum of $ß_i$ and $ß_{CO2}$, ie $ß_T = ß_i + ß_{CO2}$.

The value of $ß_i$ will obviously vary between cell types with high values being reported for muscle cells (due to the presence of much protein material) and lower values being reported for neurones (e.g. 40mM for striated muscle, Curtin, 1986, 8mM for cerebellar

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[1]This term needs qualification. It is commonly assumed that neutral pH=7.0. In fact, a more helpful definition of neutral pH is the case when $[H^+]=[OH^-]$. The product of $[H^+]$ and $[OH^-]$ is K_w, the ionic product of water. This value is temperature dependent; K_w is $=1x10^{-14}$ $mol.l^{-1}$ in pure water at 25°C only. At 37°C, $K_w = 1x10^{-13.6}$, ie neutral pH is 6.8.

granule neurones, Pocock & Richards, 1992). As PCO_2 is maintained at a fixed value the contribution of $ß_{CO2}$ to $ß_T$ should only be dependent on the pH prevailing inside the cell as this will determine the $[HCO_3^-]_i$. Thus cells of identical pH_i in a fixed PCO_2 should show equal $ß_{CO2}$ values. In many papers published $ß_i$ is measured but $ß_{CO2}$ is assumed (e.g. Gaillard & Dupont, 1990). Subsequently calculated $ß_T$ values may then be used in proton flux calculations.

Although some investigators have found a definite increase in buffering power in the presence of a constant PCO_2 (Thomas, 1976, Aickin & Thomas, 1977, Szatkowski, 1989), as would be expected for an open system, not all have (e.g. Aickin, 1988, Baro *et al*, 1989, Aickin, 1994). Pocock & Richards, 1992, noted that the presence of CO_2 did not seem to increase the proton buffering power of cerebellar granule neurones. This observation was the motive for a more thorough investigation into the role of CO_2 in proton buffering power in CNS neurones. For an open buffer system the difference in $ß_i$ and $ß_T$ should be equal to $2.3[HCO_3^-]_i$ and the increase in $ß_T$ over $ß_i$ should be most noticeable at alkaline pH values as here $[HCO_3^-]_i$ is greatest. The data presented in this Thesis show that CO_2 does not increase buffering power as expected for an open system. This suggests that it would be expedient to measure $ß_{CO2}$ rather than assume its value.

Estimation of buffering power

The buffering power of neurones is of particular importance in view of the apparent sensitivity of central neurones to acidotic damage (Siesjö, 1989). Differences in susceptibilty to ischaemic acidosis may reflect differences in both neuronal buffering power and pH_i regulation. There have been no previous studies comprehensively studying the buffering power of vertebrate central neurones or the contribution of CO_2 to this buffering. Moreover, estimates of buffering power are necessary for any calculation of the fluxes that underlie changes in pH_i.

The modern definition of buffering power, as first defined by Van Slyke in 1922, is described by equation (2).

$$\beta = \frac{[Acid]}{\Delta pH} \qquad \text{equation (2).}$$

Where:
[Acid] = the quantity of acid (or base) added to cause a pH change of ΔpH units.

As pH has no units, buffering power has conventionally been expressed in mmoles/l of solution (cytoplasm in the case of intracellular buffering).

Koppel and Spiro (1914) had previously defined buffering power in a term which took into account the self buffering of water, but as this is important only at pH extremes the derivation suggested by Van Slyke is more commonly used.

Historically, estimation of buffering power has been estimated either by direct injection of acid or base into the cytoplasm (Thomas, 1976; Szatkowski, 1989) or by extracellular application of weak acids or bases (e.g. Vaughan-Jones & Wu, 1990; Pocock & Richards, 1992). The magnitude of the resultant change in pH_i is used to estimate intracellular proton buffering power. Injection of acid or base requires impaling the cell with microelectrodes which is undesirable and impractical in cells as small as mammalian central neurones. Extracellular application of weak acids or bases is less invasive but buffering power estimates made from this method rely on a number of assumptions. I have used the weak acid/base method. This method does not allow us to distinguish the relative roles of metabolic, organellar and physicochemical buffering.

Jacobs (1940) was the first to show the higher permeability of the plasma membrane to uncharged as opposed to charged forms of any given species. This phenomenon has been used in acidifying and alkalinising cells by simply bathing the cells in solutions containing weak acids or weak bases which permits uncharged molecules of acid or base to enter the cell and dissociate in the cytosol into ionic species. Thus, in the case of

butyric acid, C_3H_7COOH will permeate the membrane and enter the cytosol. Here some of the molecules will dissociate into H^+ and $C_3H_7COO^-$, acidifying the cell interior. The concentration of uncharged form is assumed to be in equilibrium across the membrane. Knowing the internal pH and assuming that the K_a (the acid dissociation constant) is the same inside the cell as it is outside we can calculate the concentration of $C_3H_7COO^-$ from the Law of Mass Action. As for each dissociated butyric acid anion there are an equal number of H^+ ions released, the acid load on the cell can be estimated. The extent of the pH_i change that this acid load causes will depend on the proton buffering power of the cell.

Sharp and Thomas (1981) derived an expression from the Henderson-Hasselbalch equation to estimate intracellular buffering power from the addition of weak acids (see Methods). The assumptions that have to be made in using this weak acid/base method to estimate buffering power are:

1) The concentrations of uncharged weak acid or base rapidly attain equilibrium across the membrane.
2) The apparent dissociation constant describing the balance of ionic and non-ionic weak acid/base species is the same in both extracellular and intracellular environments. This dissociation is very rapid.
3) Only the uncharged form of an acid/base species is membrane permeant.
4) No active transport of the acid anion or basic cation occurs across the plasma membrane.
5) All acid/base that enters the cell subsequently leaves it during wash off.
6) The native concentrations of weak acid/base species are negligible.

Besides these assumptions , it is important to recognise that the estimates of buffering power are dependent on accurate pH_i measurements. Dye methods of pH measurement such as those employed here probably have an error of about 0.1 units. Moreover, the value of pK_a depends on the microenvironment. Further problems arise from the pH dependency of buffering power. As estimating buffering power involves changing pH, then estimates must necessarily be made at an average pH value. Previous investigators

have also drawn attention to the action of pH_i-regulating transport mechanisms which may lead to an overestimation of buffering power (e.g. Vaughan-Jones & Wu, 1990). This arises from the fact that any pH_i-regulation occuring during the acid/base challenge would mask the true extent of the pH_i change and thus make the buffering appear more effective than it actually is. Consequently, caution must be taken in assessing buffering power estimates.

Steps can be taken to minimise errors from pH_i-regulation including back-extrapolation (e.g. Thomas, 1976, Aickin & Thomas, 1977) to the time of base/acid addition. Pharmacological poisoning of pH_i-regulating transport mechanisms has also been employed (e.g. Vaughan-Jones & Wu, 1990). The system used to obtain the data presented here, however, had the advantage of very fast intracellular concentration changes which in itself minimised interference from pH_i-regulation.

In the context of this knowledge this study aims to answer certain questions. Specifically, what is the value of intrinsic buffering and does CO_2/HCO_3^- buffer behave as expected for an open system? Furthermore, no attempts have been made to model this buffering in terms of the known titratable groups in brain tissue but the relationship between ß and pH should give clues as to the groups involved. Previously, the values of $ß_i$ and $ß_T$ have been investigated only to a limited extent, especially in mammalian central neurones.

pH_i-regulating transport processes

That H^+ ions are not distributed across cell membranes according to a Donnan equilibrium was recognised as early as the 1930's. Later microelectrode studies (from the 1950's onwards eg Caldwell, 1954) confirmed that pH_i was higher than would be explained by passive distribution of protons at the resting membrane potential. These studies provided the basis for hunting down the ion transporters that regulated pH_i.

The regulation of pH_i has generally been investigated by displacing pH_i and watching its return to normal. The mechanisms by which the cell does this are generally assumed to be the same as those that provide normal pH_i-regulation (Thomas, 1984). Whether or

not the primary role of these exchangers is to regulate H^+ loads resulting from metabolism or to modulate localised pH_i excursions in excitable cells (resulting from channel mediated ion fluxes) is open to discussion (Kaila *et al*, 1990).

Displacement of pH_i by acid injection or membrane permeation of weak acids has allowed the identification of three main types of exchanger involved in pH regulation; the Cl^-/HCO_3^- antiporter, the Na^+/HCO_3^- symporter and the Na^+/H^+ antiporter. The Na^+-dependent or Na^+-independent Cl^-/HCO_3^- antiporter has been identified in snail neurones (Thomas, 1976), rat central neurones (Gaillard & Dupont, 1990, Pocock & Richards, 1992 and Schwiening and Boron, 1994) and sheep cardiac muscle (Vaughan-Jones, 1982) as well as other preparations. A Na^+/HCO_3^- symporter has been identified in renal proximal tubule (Frelin *et al* , 1988) and in central neurones (Pocock & Richards, 1992). The Na^+/H^+ exchanger has been demonstrated in salamander kidney (Boron & Boulpaep, 1983), human fibroblasts (Moolenaar *et al*, 1983), rat sympathetic ganglion cells (Tolkovsky & Richards, 1987), rat cerebellar purkinje cells (Gaillard & Dupont 1990), rat hippocampal cells (Raley-Susman *et al*, 1991), and rat cerebellar granule cells (Pocock & Richards, 1992). These exchangers regulate pH_i by the transport of H^+ (or OH^-) or via the pH consequences of HCO_3^- transport. All of these exchangers have been identified in rat central neurones. The Cl^-/HCO_3^- antiporter may only be active in adult neurones however (Raley-Susman *et al*, 1993). Besides these exchangers proton pumps and H^+-channels have been implicated in pH_i-regulation (Frelin *et al*, 1988).

In order to make accurate ß estimates the effects of any pH_i-regulation must be considered. In turn this requires knowledge of the pH_i-regulating mechanisms present. As cultured neocortical and hippocampal neurones present such a heterogenous population of cells it is inexpedient to characterise the pH_i-regulating mechanisms present in a single cell. A more pragmatic approach is to identify the variety of mechanisms present and examine their individual effects on ß estimates. Therefore, this study will identify the diversity of pH_i-regulating mechanisms present and evaluate their effects on pH_i levels.

H^+ ions and intracellular signalling

Although various mechanisms obviously exist to keep extracellular and intracellular pH within narrow limits the sensitivity of some proteins to pH changes within physiological range suggests a role for H^+ ions in cellular signalling (such as ion channels and enzymes; see Chesler (1990) or Busa & Nuccitelli (1984) for a review).

In neurones the action of neurotransmitters on intracellular pH (pH_i) has been the subject of only very limited investigation, excepting the action of GABA which is well documented (Kaila, 1994, for a review). There is limited data referring to activity or depolarisation induced changes in pH_i (Brown & Meech, 1979, Chesler, 1990). However, direct demonstration of neurotransmitter modulation of neuronal pH_i has been confined to recent publications (Kaila *et al*, 1993, Dixon *et al*, 1993, Irwin *et al*, 1994). So, excepting the work of Endres *et al* (1982) in frog motorneurones. So, although ion-sensitive fluorescent dyes and microelectrodes have been widely used to measure changes of $[Ca^{++}]_i$, the pH_i consequences of activating neurotransmitter receptors in the CNS have largely been neglected. This may arise partially from the belief that pH_i is not a variable under normal physiological conditions. Morever, complications are introduced due to the difficulty of measuring pH_i in slices.

In contrast, activity induced shifts in *extracellular* pH have been reported for a number of brain preparations (for a review see Chesler, 1990) demonstrating the pliant nature of $[H^+]$ in physiological solution. Microelectrode studies have shown activity-induced glutamate dependent interstitial pH shifts in turtle cerebellum (Chesler & Rice, 1991) and rat hippocampal slices (+0.07 units from a 5s 20Hz stimulation; Chen & Chesler, 1992). The mechanism by which glutamate elicits these pH shifts is unidentified, though both NMDA and non-NMDA receptors can be involved. These pH shifts are not caused by cell firing *per se.*

Reports of central neurotransmitters changing pH_i have mainly been limited to the action of GABA. Through activation of a channel that is permeable to Cl^- and HCO_3^- anions GABA can cause an intracellular acidification in central neurones due to an electrochemical gradient favouring HCO_3^- efflux from cells (Kaila *et al*, 1993). In HEPES

buffered conditions, where PCO_2 is negligible, GABA application does not elicit a pH_i change. Recent publications have demonstrated that glutamate can affect intraneuronal pH (Dixon *et al*, 1993), in one case through NMDA receptor activation (Irwin *et al*, 1994). Both of these investigations were performed in HEPES buffer.

Due to the high sensitivity of some proteins to pH_i changes within the physiological range a second messenger role for intracellular H^+ ions has been postulated (Busa & Nuccitelli, 1984). Recently GABA-induced changes of pH have been shown to modulate synaptic transmission (Taira et al 1993). Also Dixon *et al* (1993) have demonstrated glutamate modulation of the HVA calcium current in catfish bipolar horizontal cells mediated by changes in intracellular pH. At extracellular concentrations as low as 1μM L-glutamate can supress the peak HVA calcium current by 15%.

The heterogeneity of resting pH_i values seen in neurones in culture and the possibility of H^+ acting in a second messenger role spawned the idea of other neurotransmitters affecting pH_i. This Thesis specifically studies the action of glutamate on neuronal pH_i. I have found that L-glutamate applied to mammalian central neurones by pressure ejection or bath application elicits an intracellular acidification. This acidification is sustained unlike the transient changes seen in other studies (Dixon *et al*, 1993) and is qualitatively different in HEPES or CO_2-HCO_3^- buffer. Furthermore these effects can be mimicked by the metabotropic glutamate receptor agonist tACPD.

The pH_i-regulating transporters seem a likely target for any signalling mechanism involving pH_i changes. Demonstration of neurotransmitters and hormones acting on these transporters has mainly been limited to the Na^+/H^+ antiporter. The Na^+/H^+ antiporter is responsive to vasoconstrictors, growth factors, cell shrinking and intracellular acidification (Poch *et al*, 1994). Neurotransmitters shown to activate the antiporter include noradrenaline (Ho *et al*, 1989) in rat pinealocytes . The Cl^-/HCO_3^- has been shown to be modulated by arginine vasopressin in mesangial cells (Ganz *et al*, 1989).

Metabotropic glutamate receptors

Besides the report that NMDA receptor activation can elicit an intracellular acidification (Irwin *et al*, 1994) glutamate receptor activation has not been directly shown to affect pH_i. Indirect evidence, however, comes from glutamate changing pH_i in catfish horizontal bipolar cells (Dixon *et al*, 1993) and rat central neurones (Hartley & Dubinsky, 1993) when applied at neurotoxic levels. The family of metabotropic glutamate receptors have not yet been implicated in pH_i modulation. Various roles have been assigned to mGluR activation including involvement in LTP (Bashir *et al*, 1993), suppression of EPSC's (Gereau & Conn, 1994) and modulation of ion channel activity (Rothe *et al*, 1994). Current knowledge encompasses seven different mGluR types. Primary sequence studies show that mGluRs are of the order 900 amino acids long with a large extracellular carboxyl terminus. They are directly linked to second messenger systems and have short cytoplasmic NH_2 termini in comparison to other G-protein linked receptors. As yet they have been generally found to either downregulate cAMP levels or to activate phospholipase C thus increasing IP_3 levels in cells.

pH Measurements

Accurate buffering power estimates and investigation of pH_i-regulation are critically dependent on accurate pH measurements. Measuring biological pH values has been of interest since the turn of the century. Early studies used pH sensitive electrodes to measure the pH of cell lysate/homogenates. Weak acid distribution was also used as a technique to measure pH_i. This technique relied on estimates of intracellular and extracellular waterspace and was very limited in time resolution. Contemporary methodology uses pH sensitive dyes, pH sensitive microelectrodes or nuclear magnetic resonance (NMR) spectroscopy (eg Pocock & Richards, 1992, Szatkowski & Thomas, 1989 and Pirttilä & Kauppinen, 1993, respectively).

With whole slices of brain, NMR spectroscopy can be used (eg Pirttilä & Kauppinen, 1993) but the signal from individual neurones is too small for this technique. Consequently an average signal is obtained for all elements in the slice, limiting the

spatial resolution. This problem, combined with poor temporal resolution, precludes NMR techniques from effective assessment of buffering power in individual neurones. Whilst double barreled pH-sensing microelectrodes have been used successfully in snail neurones (Thomas 1976) they are unsuitable for use in the small cells of mammalian CNS. Consequently pH sensitive dyes offer the only practical method for measuring the pH_i of these neurones.

The use of pH sensitive dyes is dependent on the phenomenon of fluorescence. Certain molecules emit light when excited at the appropriate wavelengths. This results from the excitation of electrons in the molecule which then drop back down to the ground state. The resultant energy emitted is fluorescence (this differs from phosphoresence in having a faster decay time). The sensitivity of this fluorescence to quenching by specific ions in some molecules has allowed the development of dyes whose fluorescence alters with ion concentration.

In the last 15 years the development of ratiometric dyes has reduced errors and artefacts common with single wavelength dyes. Ratiometric dyes change their excitation or emission spectra with changes in the concentration of specific ions. This allows collection or excitation of fluorescent signals at two different wavelengths. The dual excitation or emission allows collection of two signals which are subsequently expressed as a ratio. As such, the final ratio value is independent of dye leakage, photobleaching, variations in cell thickness and other factors that could give rise to artefacts if light at a single waveband was collected.

The pH sensitive dye used in these investigations, BCECF, is a dual excitation dye. Cells loaded with the dye are excited at 440nm and separately at 490nm. The signal emitted (collected at 510-550nm) is pH sensitive with 490nm excitation but fairly insensitive to pH at 440nm. The two signals are collected and ratioed. As pH decreases the 490/440nm signal ratio decreases, and an increase in ratio is seen as pH rises. As the signal is a ratio it is independent of dye concentration and is therefore independent of changes in cell volume. Also dye leakage from a cell (which could be mistaken for a pH change with a single wavelength dye) will affect each wavelength proportionately and

thus the ratio remains unchanged. Furthermore, spurious fluorescence effects of exogenously applied substances can often be detected by changes of the pH insensitive 440nm signal. BCECF and other pH sensitive dyes have been successfully used to measure pH in a number of preparations (eg Baro *et al*, 1989, Gaillard & Dupont, 1990, Pocock & Richards, 1992, Goldsmith & Hilton, 1992, Dixon *et al*, 1993, Irwin *et al*, 1994). The ratiometric dye technique is used in this study. Although the dye will load non-specifically into various intracellular compartments when cells are permeabilised almost all the dye signal is lost rapidly (⟨1 min.), implying that the BCECF signal largely reflects cytosolic pH changes.

In summary, this Thesis set out to answer the following questions:

1) What is the value of intrinsic buffering power in mammalian CNS neurones?
2) How does buffering poer vary with pH_i?
3) Does the CO_2/HCO_3^- buffer behave as an open system in CNS neurones?
4) What role might agonist induced pH_i changes play in neuronal signalling?

These investigations of buffering and pH regulation may prove fruitful in helping to explain the varying susceptibility of mammalian central neurones to acidotic damage. Also, the modulation of pH_i by neurotransmitters may play a role in the control of neuronal excitability.

CHAPTER 2: METHODS

Cell culture

Cells used in investigations were obtained from young rats and maintained in culture for short periods before use. Neonatal rats (3-4 days old) were anaesthetised with isoflurane and then decapitated. Whole hippocampi and sections of neocortex were aseptically dissected and placed in HEPES buffered minimum essential medium (MEM). The separate tissue samples were roughly chopped with iridectomy scissors and then triturated through progressively finer bore pasteur pipettes to mechanically dissociate individual cells. In between triturations the cell suspensions were kept on ice. The cells were then spun at 500 r.p.m. for 5 min to separate intact cells and debris. The supernatant was removed and the pellet resuspended in HEPES buffered MEM. The cells were then plated at a density of $0.5x10^{-6}$cells ml^{-1} onto poly-L-lysine (0.1mg ml^{-1}, Sigma) coated coverslips in 60mm culture dishes (Nunclon). The dishes contained CO_2-buffered DMEM (16 mM bicarbonate, pH 7.3) supplemented with 10% foetal calf serum (Gibco) and glutamine (1mM)(Sigma). The cells were incubated at 37°C for 6-12 days in a water saturated atmosphere containing 5% CO_2 before use. The medium bathing the cells was changed every 5 days.

At x400 magnification a number of cell types were obvious present in the cultures. Some were very distinctive such as type-II astrocytes. However, for experimental purposes cells were broadly divided into neurones and glia. Neurones were distinguished from glia by their morphology, size and visual density under phase contrast microscopy. Central neurones in culture are phase bright and have neurites with few recurrent branches which are not symmetrically distributed, unlike those of type-II astrocytes. In order to avoid potential errors in identification, classification of cells as neuronal was limited to those individuals with one, two or three neurites present only.

Solutions

During experiments cells were bathed in either HEPES or CO_2-HCO_3^- buffered sodium Locke's solution. The HEPES buffered (HEPES NaL) solution was 140 mM NaCl, 3 mM KCl, 1.8 mM $MgCl_2$, 1.25 mM $CaCl_2$, 5 mM glucose and 16 mM HEPES, pH adjusted to 7.3 using 1M NaOH. The composition of the CO_2-HCO_3^- buffered solution (CO_2-HCO_3^- NaL) was the same except HEPES was replaced by 16mM $NaHCO_3$. This solution was gassed with 95% O_2-5% CO_2 which gave pH 7.3 at 37°C. Sodium-free solutions contained equimolar n-methyl, d-glucamine and chloride free solutions contained methanesulphonate in place of Cl^-.

For buffering power experiments, weak acids or bases were directly added to, or removed from, the solution covering the cells by completely changing the bathing medium. The bases used were NH_4Cl (pK_a 9.02, 1-20mM) and trimethylamine (TMA, pK_a 9.8, 1-15mM). Butyric acid (pK_a 4.82) and CO_2 (overall pK_a 6.1) were used to acidify the cells. When measured, the pH of the bathing solution was not found to change on addition of the weak acid/bases.

For the experiments designed to explore the effect of neurotransmitters on pH_i, L-glutamate was dissolved in NaL and applied to individual neurones by pressure ejection from a glass pipette. Application consisted of two 250ms pulses separated by a 1 second gap. Solutions of (±)-1-aminocyclopentane-trans-1,3-dicarboxcylic acid (tACPD) were applied via the bath (for 30s) or pressure ejection (2x 250ms pulses). All other chemicals used in investigations (NH_4Cl, L-amino phosphonopropionic acid and α-methyl carboxyphenylglycine) were bath applied. In most experiments involving glutamate or tACPD the cells were perfused at a rate that completely changed the bath volume in about 30s.

pH measurements

Buffering power estimates were made from the changes in pH_i caused by addition and removal of weak acids and bases (for a review of this method see Roos and Boron, 1981). Extracellular pH was measured directly using a standard glass electrode and pH-meter. pH_i was estimated using the pH sensitive fluophore BCECF which was chosen for these studies as the experimental system was already adapted specifically for this dye. An alternative dye would be the more recently available SNARF. However, this dye has a pH-sensitive emission maxima at wavelengths which are specifically used for direct visual monitoring of the cells using a CCTV. In addition, photomultipliers are less sensitive at the long wavelength emissions of SNARF. Finally, the pK_a of BCECF is about 7 (close to the resting pH of these cells) whereas the pK_a of SNARF can be much more alkaline (up to 7.8, see Haugland 1992). Thus BCECF is the prefered indicator when both acid and alkaline excursions from pH 7.0 are to be investigated.

Fluorescence measurements were made on a photomultiplier based system centred on an inverted microscope (Diaphot, Nikon). A drawing of the apparatus is shown in Figure 1. Individual coverslips on which the cells were grown formed the base of a thermoregulated chamber placed on the microscope stage. Cells were bathed in either HEPES buffered or CO_2-HCO_3^- buffered Locke solution and were loaded with BCECF using the AM ester loading technique (10μM BCECF-AM for 15 min at 37°C, Rink *et al*, 1982). After loading, extracellular dye was removed by washing. Any solution changes were made by draining the bath completely (400μl volume) and refilling immediately. This method allowed rapid changes in the extracellular concentration of weak acids/bases. All the experiments were performed at 37°C. In some experiments (where glutamate was applied) the bath was perfused at a rate of $100\mu l.min^{-1}$.

The cells were excited alternately at 440nM and 490nM by means of a filter wheel and emissions at 520-580nM were captured by a photomultplier. The ratio of these emissions was taken as a direct measure of pH_i. Neutral density filters were used to keep irradiation levels low and a diaphragm placed in between the cells and the collecting photomultplier allowed isolation and study of single neurones. The cell under study was observed throughout the experiment via a video camera. Most recordings were stored and analysed

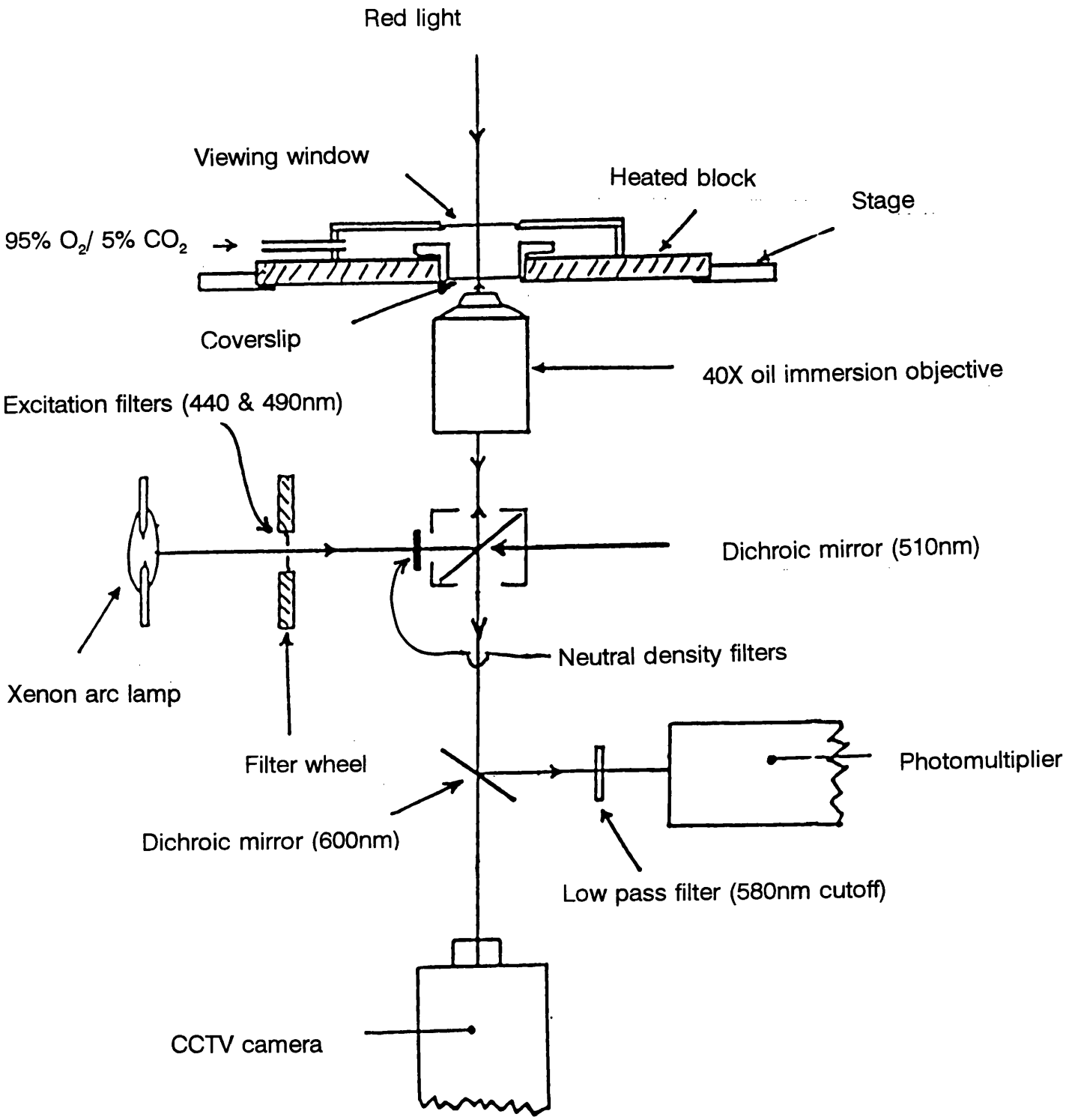


FIGURE 1: Diagram of the experimental set-up.
Excitation light was provided by a 75W xenon-light source and attenuated with neutral density filters (ND2-ND8 plus a 1% transmission filter). A filter wheel was placed in the light path, limiting excitation to 440 and 490nm (Ealing filters, 10nm bandwidth transmission). The field of view was illuminated with red light which allowed constant observation of the cells with CCTV. Interference of this light source with the fluoresence signal was minimised by using a 600nm dichroic mirror to reflect the signal to a photomultiplier tube. Also a 580nm low pass filter placed in front of the collecting photomultiplier. The experiments were performed in a dark room to minimise background light levels. With a 400ms collection time for each wavelength of excitation and 300ms for filter wheel rotation the collection time was about 1.1s. (Modified from Pocock & Richards, 1992).

on computer using software supplied by Newcastle Photometric Systems. Figure 1 shows the experimental setup schematically. The cells were illuminated throughout experiments by a red filtered microscope source light. Most of this light was prevented from reaching the photomultiplier tube by a 600nm dichroic mirror that passed light to a CCTV. A barrier filter (cutoff 580nM) in front of the photomultiplier tube prevented any other source light interfering with the dye emission signal.

When signal to noise ratios were visibly poor they were improved by increasing the illuminating intensity. As noise increases only to the square root of signal intensity increase then when cells showed poor loading (and poor signal to noise) a reduction in irradiation neutral density filtering from Nikon ND2 to Nikon ND8 (corresponding to 50% and 12.5% transmission respectively) usually improved the signal substantially. (A 1% transmission filter was kept in place at all times). Autofluorescence and background signals were subtracted prior to ratio estimation, but were always less than 1% of total signal.

Calibration of BCECF signals

Accuracy in estimating buffering power depends on the accuracy of the pH_i measurement. For dye methods this means confidence in the calibration performed. Records were calibrated using nigericin (Thomas *et al*, 1979). This is a K^+-H^+ ionophore which will equilibrate pH across the plasma membrane when $[K^+]_{out}=[K^+]_{in}$. The calibrating solutions consisted of 140mM KCl, 5mM NaCl, 1.8mM $MgCl_2$, 1.25mM $CaCl_2$ and 15mM HEPES for solutions set at pH values 7.0 to 8.0. For solutions of pH less than 7.0 the HEPES was replaced by 15mM PIPES. All calibrating solutions contained 3μM nigericin, drawn from a 1mM stock dissolved in ethanol. Equipment in contact with bathing solutions was soaked in between experiments in Micro detergent and washed thoroughly in distilled water to reduce nigericin contamination (Richmond & Vaughan-Jones, 1993).

Following a successful experiment the medium bathing the cell was changed for one containing nigericin at a known pH. When a steady state value had been attained the

solution was changed for one at a different pH until a full calibration of ratio against pH_i had been obtained. The ratios for particular pHi values varied somewhat from cell to cell (see Chapter 3) so this necessitated calibration of individual experimental traces. Why this variation occurs is not clear. Some of this variation may be explained by the two different types of glass slips that were used for cell culture. It is possible that the slips varied in thickness and transmission qualities. Consequently, the shorter wavelength irradiation may not be transmitted as well through the thicker slips, for example, giving mean higher average ratios for a given pH. Alternatively (or in addition) the calibration procedure may be at fault. The nigericin technique depends on setting $[H^+]_o=[H^+]_i$ when $[K^+]_o=[K^+]_i$. The same concentration of K^+ (140mmole) was used in all nigericin calibrating solutions. However, in an heterogenous population of cells where $[K^+]_i$ might vary as much as 40mM a lower value of $[K^+]_i$ would lead to an overestimation of $[H^+]_i$. This would shift the pH/ratio relationship for that cell relative to another where the $[K^+]_i$ had been near 140mmole. This in turn would affect ß estimates. Furthermore, if the cells had consistently different $[K^+]_i$ in CO_2-HCO_3 NaL as compared to HEPES NaL an error in estimating $ß_T$ with respect to $ß_i$ would occur. The pK_a value for BCECF will depend on the microenvironment within the cell and in an heterogenous population of cells it might vary. This would shift the pH/ratio relationship for some cells relative to others. In contrast to the variation in the calibration curves the estimated resting pH_i values were remarkably consistent. Moreover, the nigericin technique has been shown to correspond well to the null point method for steady state values in central neurones (see Eisner *et al*, 1989, Pocock & Richards, 1992).

The accurate assessment of pH_i, and therefore ß, is limited by the errors inherent in the technique of measurement and calibration procedure. The dye will be subject to non-specific loading- it may load into any membrane bound organelles. This means the pH_i signal estimated will be an average intracellular pH value rather than purely cytosolic pH. However, treatment of these cells with 5µM digitonin (selectively permeabilising the plasma membrane, Ishijima *et al*, 1991) gives rise to a rapidly disappearing fluorescence signal (⟨1 min.). This indicates that the signal is mainly cytosolic. The dye itself will contribute to buffering, this has been discussed in the previous section.

Ca^{++} measurements

Intracellular calcium was measured using the fluorescent dye Indo-1 excited at 340nm with emissions collected at 400 and 480nm. Cells were loaded with 15μM Indo-1-AM for 10 min at 37°C. Signals were calibrated with the calcium ionophore 4-bromo-A23187 (5μM) and the calcium chelator EGTA (20mM). The value of $[Ca^{++}]_i$ was then estimated using the equation derived by Grynkiewicz *et al* (1985).

Buffering power estimates

Buffering power is most simply measured by displacing intracellular pH using a weak acid or base applied extracellularly.(see Introduction). The resultant shift in intracellular pH for an estimated amount of base added is a measure of buffering power. For calculating intracellular buffering power Sharp and Thomas (1981) derived a single equation that estimates ß by insertion of pK′, pH_o, pH_i, extracellular concentration of added acid and the change in pH_i. Similarly they derived one for weak base addition using the same principles. Buffering power estimates in this thesis have been made using these two equations (equations (3) & (4)).

For weak acid addition $$ß = \frac{C.10^{(pHi-pK')}}{\Delta pH_i.(1+10^{(pHo-pK')})}$$ equation (3).

For weak base addition $$ß = \frac{C.10^{(pK'-pHi)}}{\Delta pH_i.(1+10^{(pK'-pHo)})}$$ equation (4).

In the case of equation 3:

C = total extracellular weak acid concentration (mM)

pH_i = intracellular pH at peak acidification

pH_o = pH of bathing medium

pK′ = weak acid dissociation constant

ß = proton buffering power, mmoles/l

For weak base addition C is the extracellular concentration of base and pK′ is the weak

base dissociation constant. These equations provide ß estimates in line with those values obtained by calculating buffering power from first principles. As described in the Introduction certain assumptions have to be made in using this method. These are:

1) The concentrations of uncharged weak acid or base rapidly attain equilibrium across the membrane.
2) The apparent dissociation constant describing the balance of ionic and non-ionic weak acid/base species is the same in both extracellular and intracellular environments. This dissociation is very rapid.
3) Only the uncharged form of an acid/base species is membrane permeant.
4) No active transport of the acid anion or basic cation occurs across the plasma membrane.
5) All acid/base that enters the cell subsequently leaves it during wash off.
6) The native concentrations of weak acid/base species are negligible.

A complication is introduced by the fact that buffering power is pH dependent. Consequently we require that buffering power values be stated at a particular pH. As estimating ß necessarily involves a change in pH_i, ß values given are stated for a mean pH_i value. Obviously it is preferable that the pH change used to measure buffering power is as small as practicable. An example of ß estimation is shown in figure 2. This is the addition of a weak base so ß can be estimated from equation (4) in the Introduction. Extrapolation is used to minimize errors due to pH regulation (Vaughan-Jones & Wu, 1990) where possible, though the time course of changes in pH_i following addition or removal of extracellular [base] or [acid] is very quick (usually less than 1s) so back-extrapolation is not essential.

The contribution of BCECF to buffering must be considered as it is a weak acid of approximate pK_a 7.0. As this will be a closed buffering system the maximal buffer value of BCECF is equal to $0.58[T_A]$. So, even at an internal concentration as high as 5mM the cellular buffering would only be enhanced by about 2.9mM at pH 7.0. In context of the errors in estimating ß it can be assumed that BCECF does not have a significant effect on increasing ß values.

Statistics

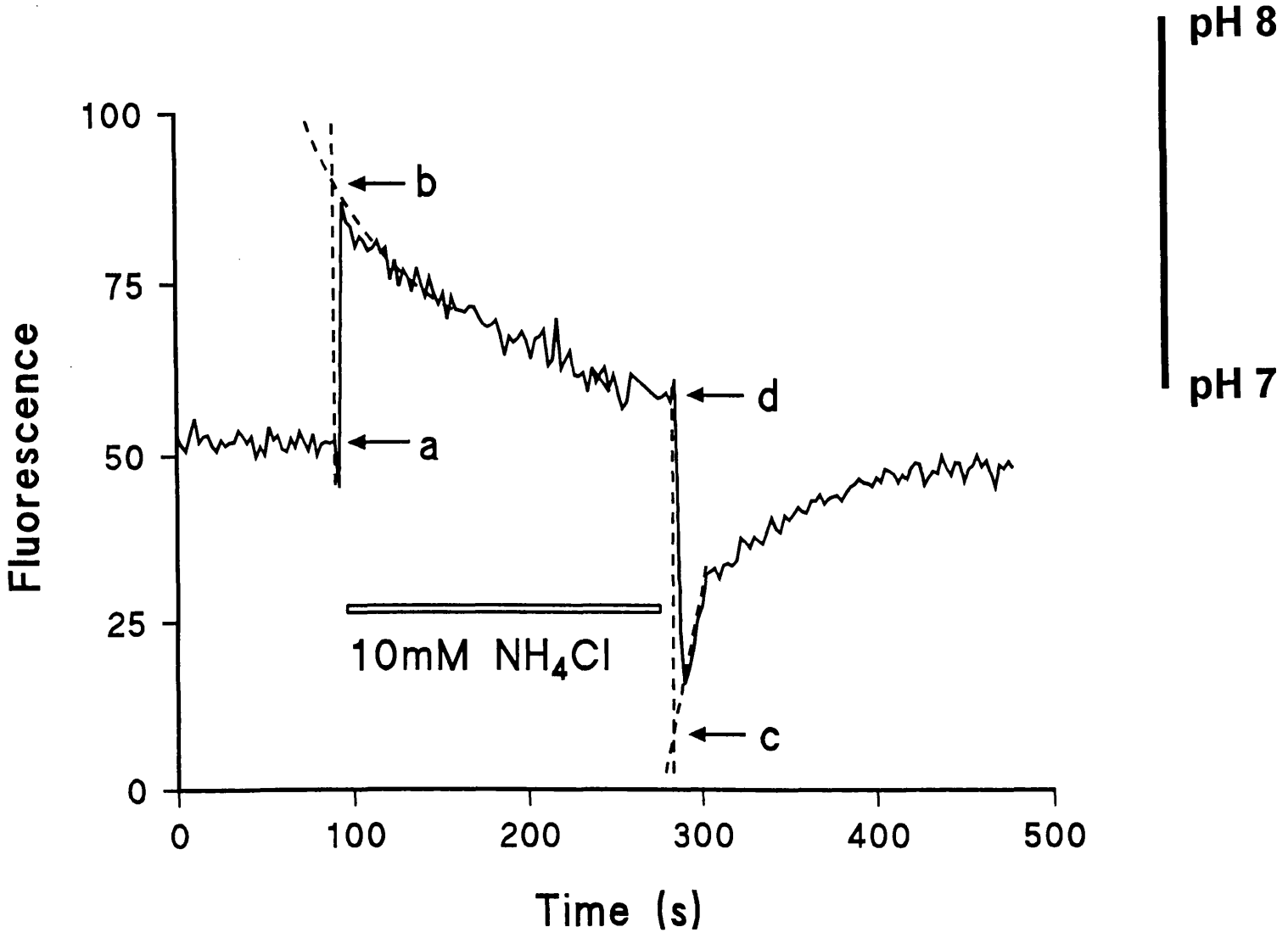
All statistics given in this thesis are means (± standard error of the mean) unless otherwise stated. Comparisons data have been done by paired or unpaired Student's t-tests unless otherwise stated, and the p values given are two-tailed.

Figures

Experimental records show fluorescence ratio (a direct measure of pH once calibrated) against time. Increases in ratio represent alkalinisation of the cytoplasm and decreases mean an increase in $[H^+]_i$. With early records, where data files could not be converted to ASCII format, an arbitrary fluorescence value is plotted. All data were obtained as ratios however. All records were individually calibrated.

FIGURE 2. This trace shows the pH_i of a cell bathed in HEPES NaL and estimation of its intrinsic buffering power ($ß_i$) using a NH_4Cl challenge. An increase in fluorescence represents an alkalinisation, a decrease is acidification. The first 100s of record show that the cell has a stable resting pH_i. Once calibrated the fluorescence signal of resting pH_i corresponds to a value of 6.95. On addition of 10mM NH_4Cl to the bathing medium an intracellular alkalinisation occurs due to NH_3 entry. It is possible that this pH change evokes some pH regulation by the cell. As the extent of the pH_i change caused by the addition of NH_4Cl is critical to estimation of the buffering power (see equation (2)) the pH_i trace must be corrected for any pH-regulation. The extrapolation technique (eg Thomas 1976) is used to estimate the pH_i change at the actual moment of NH_4Cl application. This helps to reduce errors in estimating buffering power caused by pH regulation. In practice the extrapolation is minimal as the actual ΔpH_i is very rapid.

The pH change *ab* is estimated to be 0.7 units, the pK_a of NH_4Cl is taken to be 9.02 (Aickin & Thomas, 1976) and the extracellular pH is 7.25. From equation (4) buffering power from NH_4Cl application is 5.5mM at an average pH_i 7.3. This is an estimate of intrinsic buffering power as the cell was bathed in HEPES NaL which is nominally free of CO_2. Although this is a good example for illustrative purposes, the large size of the pH_i change makes it unsatisfactory as an accurate estimate of buffering power. The buffering power estimated form the change *cd* is 14.5mM at average pH_i 6.6.


pH 8
pH 7
100
75
50
25
0
Fluorescence
b
a
d
c
10mM NH_4Cl
0
100
200
300
400
500
Time (s)

CHAPTER 3

Results I: Buffering power of CNS neurones

Resting pH_i and $[Ca^{++}]_i$.

Steady state pH_i ("resting" pH_i) was measured in individual neurones maintained in culture for 6-12 days. In a single series of experiments in CO_2-HCO_3^- NaL (pH 7.3) cells showing a stable baseline pH had a resting pH_i of 7.07±0.05 (n=22). Cells bathed in HEPES NaL (pH 7.3-7.35) had a resting pH_i of 7.03±0.04 (n=22). These two values are not significantly different (p=0.53). Approximately half of all cells examined showed a declining pH_i in HEPES buffered media. Replacement of HEPES NaL with CO_2-HCO_3^- NaL usually led to a reversal of this decline and establishment of a stable resting pH_i. Cells with unstable pH_i were excluded from the sample.

Cells initially bathed in HEPES NaL and subsequently bathed in CO_2-HCO_3^- NaL invariably showed an acid pH_i transient on CO_2 addition. This was usually followed by pH_i recovery to a new stable level. The acidification was caused by CO_2 entering the cell and subsequently being hydrated to H_2CO_3 which in turn ionises into H^+ and HCO_3^-. The H^+ released in this reaction acidify the cell. A CO_2-induced acidification is shown in figure 3. Conversely, changing from CO_2-HCO_3^- NaL to HEPES NaL led to an intracellular alkaline transient due to CO_2 efflux from the cell. This efflux pushes the equilibrium of the reaction $CO_2+H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ to the left. Consequently H^+ tends to associate with HCO_3^- to form H_2CO_3 which then dehydrates to CO_2. The removal of H^+ from solution causes the intracellular alkalinisation. The rate of pH change was variable with the range of values of 1.8×10^{-3} units.s^{-1} to 4×10^{-3} units.s^{-1}, as shown in figure 4. In an 11 cell sample, 4 cells showed an immediate acidification on increasing PCO_2 whereas the rest demonstrated a slowly changing pH_i that was complete within 32s ±4.6. The deionisation reaction is very fast but dehydration of H_2CO_3 takes longer. It may be speeded *in situ* by the enzyme carbonic anhydrase. The variation in rates of pH_i change seen in the two panels of figure 4 may reflect a variation in carbonic anhydrase activity between the two cells. However, it should be noted that carbonic anhydrase is not generally thought to reside in rat central neuronal tissue (Trachtenberg & Sapirstein,

1980) though recent evidence shows its activity in the rat hippocampus (Pasternack *et al*, 1993). As the $\tau_{½}$ for CO_2 hydration is 38°C is 5s we would expect the uncatalysed reaction to be more or less complete at 30s. The figures for pH_i changes on increasing PCO_2 suggest that carbonic anhydrase is present in some cells but not others.

As a further parameter of cell health, intracellular calcium levels were measured using Indo-1. Resting $[Ca^{++}]_i$ in HEPES NaL was estimated at 145±17nM (n=8) and in CO_2-HCO_3^- NaL it was 147±20nM (n=7).

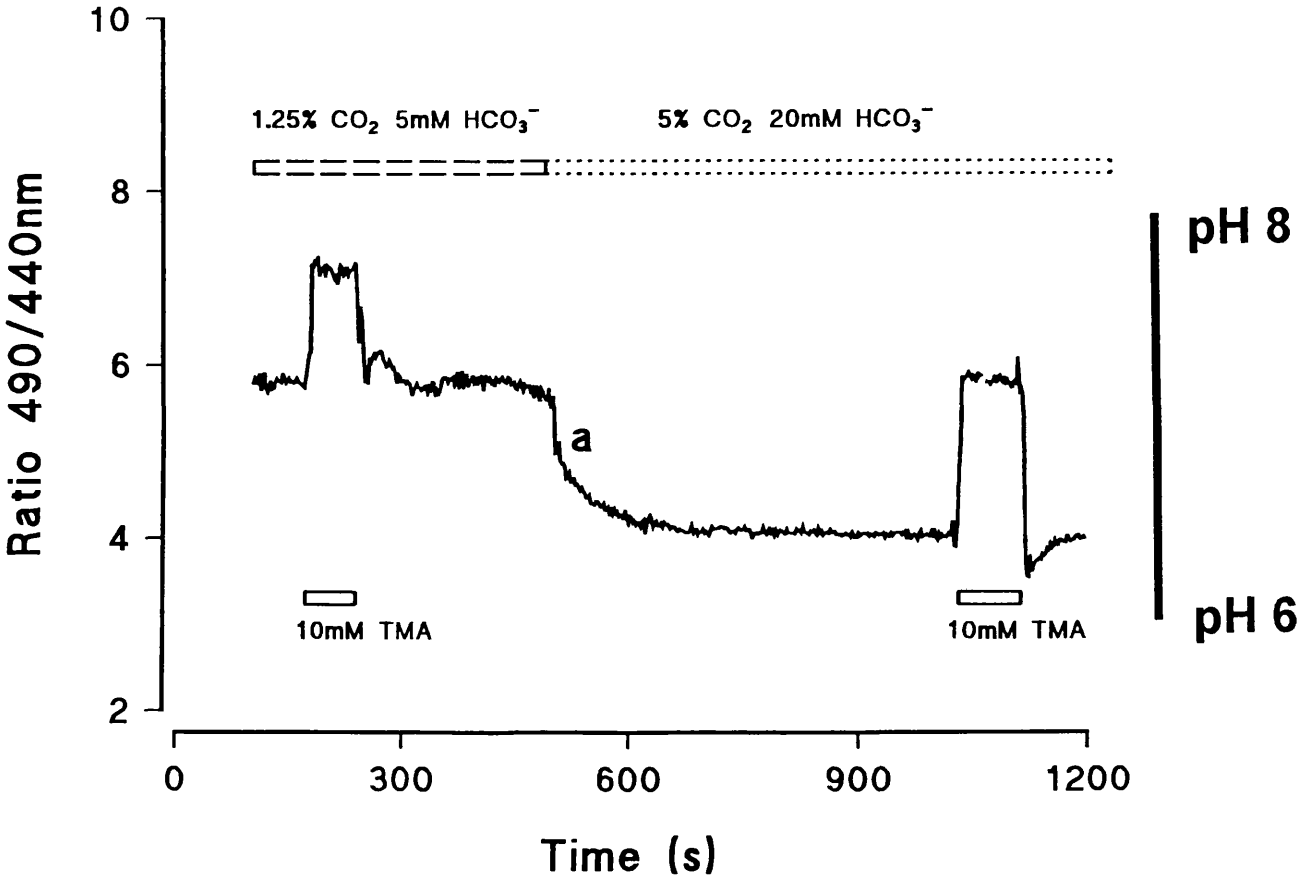
Estimates of buffering power using weak bases.

Estimates of ß using NH_4Cl.

Cells bathed in HEPES NaL were challenged with NH_4Cl (1-15mM). Figure 2 shows the typical pH_i challenges evoked by an NH_4Cl challenge. On bath application of NH_4Cl all neurones showed an immediate intracellular alkalinisation caused by NH_3 influx which then removes protons from solution by forming ammonium ions, NH_4^+ (AB). There then followed a slow acidification probably caused by slow permeation and dissociation of NH_4^+ ions (BD). On removal of extracellular NH_4Cl, cells showed an intracellular acidification (CD) due to the dissociation of NH_4^+ ions into H^+ ions and NH_3 (which subsequently exits the cell). Buffering power was estimated as described in the methods section (see figures 2 and 5). The cell shown in figure 5a had a resting pH_i of 6.95. The buffering power is estimated using NH_4Cl as described in Methods and illustrated in figure 2. The estimates of buffering power obtained (5mM for addition and 13mM for removal) are estimates of *intrinsic* buffering power as PCO_2 is negligible. This record also shows the calibration technique. Solutions of different pH, containing nigericin (3µM), are applied to the cell. Intracellular pH is presumed to equilibrate with extracellular pH under these conditions, and the resultant ratio values allow construction of a pH vs ratio plot for each cell. Estimation of total buffering power ($ß_T$) is shown in figure 5b. The buffering power is calculated in exactly the same way, but as the cell is

FIGURE 3. The rates of intracellular acidification seen on increasing the PCO_2 of the bathing medium varied widely. Due to uncertainties in changing from effectively 0mmHg CO_2 to 38mmHg CO_2, records are shown where CO_2 was already present at about 9.5mmHg and was subsequently increased to 38mmHg.

The acidification seen on increasing PCO_2 was caused by CO_2 entry (see text). The top panel shows a slow rate of intracellular acidification (at *a*) from a high resting pH_i of 7.5 in CO_2-HCO_3^- NaL to a new stable pH_i of 6.75 in HEPES NaL. The rate of acidification is 6.25×10^{-3} 'pH units'.s^{-1}. The new stable resting pH_i value attained may indicate a necessity for HCO_3^- dependent pH_i-regulating mechanisms in this cell. A much faster rate is seen in the bottom panel where the pH_i changes from 6.95 to 6.65 (at *b*) within 3s (rate= 0.1 units.s^{-1}). (The trimethylamine (TMA) challenges are incidental and were used to estimate buffering power under the different conditions).


10
8
6
4
2
Ratio 490/440nm
1.25% CO_2 5mM HCO_3^-
5% CO_2 20mM HCO_3^-
a
10mM TMA
10mM TMA
pH 8
pH 6
0
300
600
900
1200
Time (s)

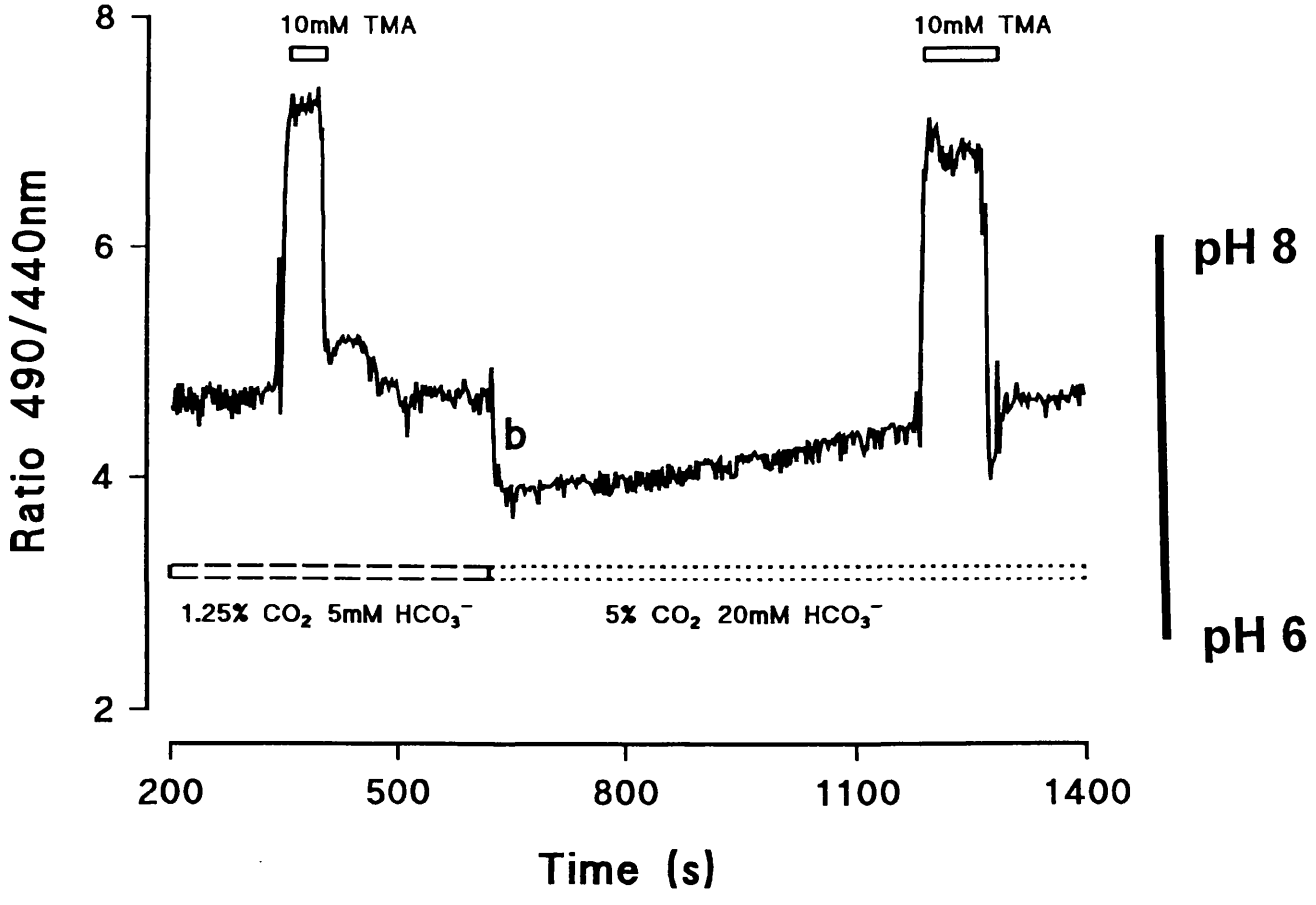
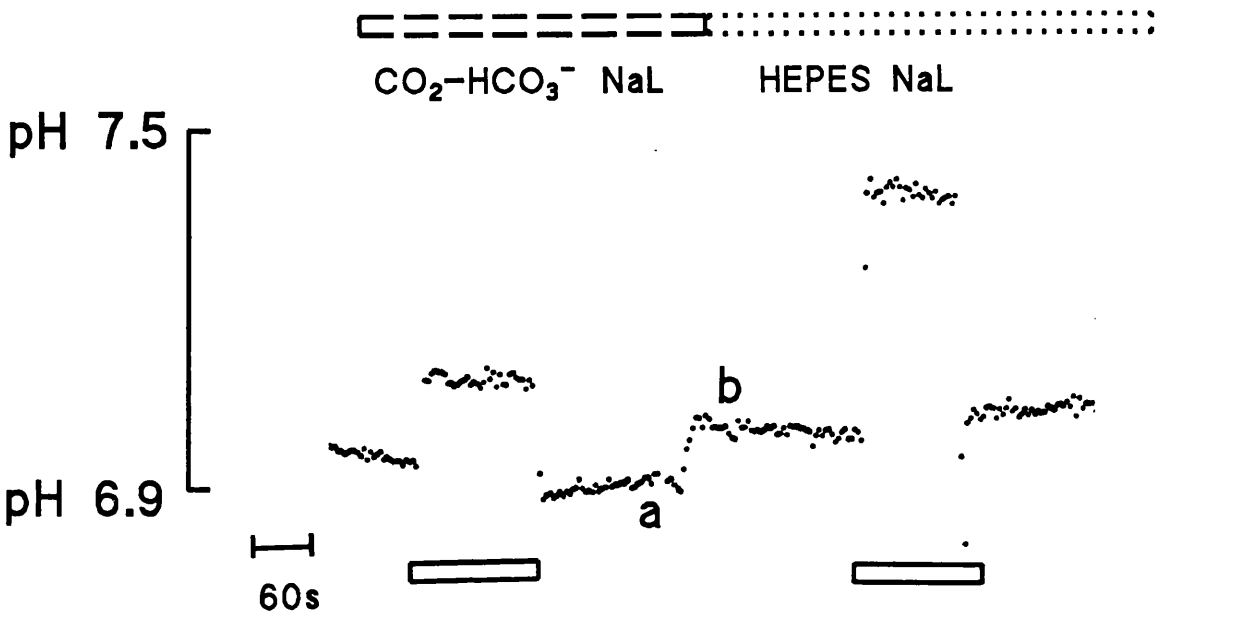
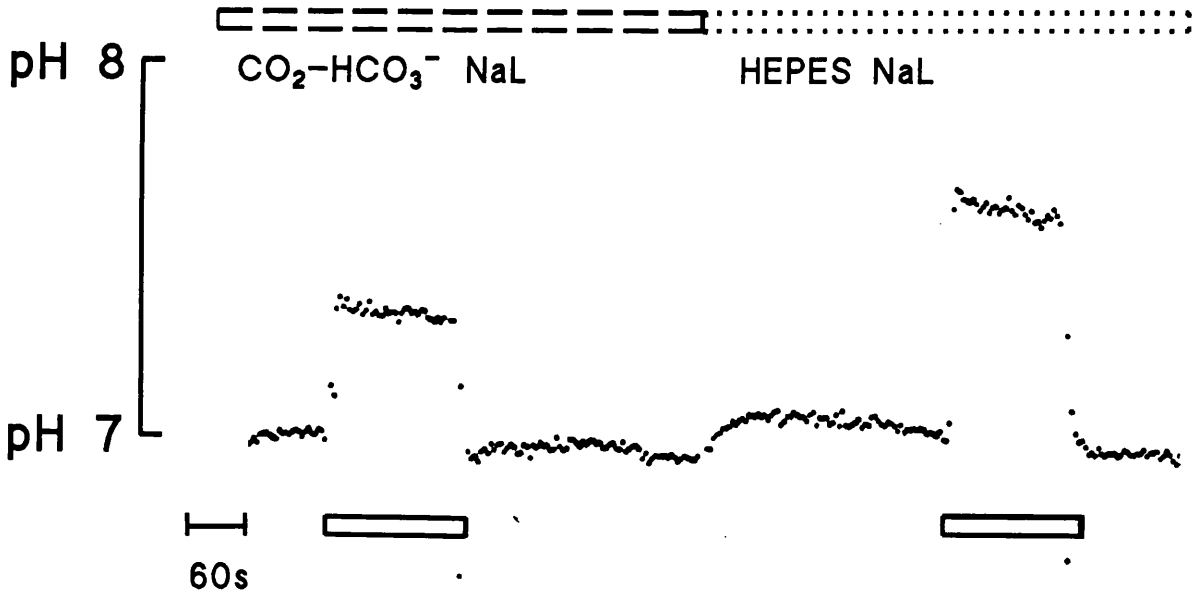

8
6
4
2
Ratio 490/440nm
10mM TMA
10mM TMA
b
1.25% CO_2 5mM HCO_3^-
5% CO_2 20mM HCO_3^-
pH 8
pH 6
200
500
800
1100
1400
Time (s)

FIGURE 4. These two traces show the variability in the rate of pH_i change on removing CO_2 from the bathing medium. The top panel shows a cell resting at a pH_i of 6.9 (point *a*) when bathed in CO_2-HCO_3^- NaL (pH 7.3). On changing the bathing medium to HEPES NaL (pH 7.3) the pH_i changed to 7.0 within 25s (point *b*) so the rate of intracellular alkalinisation was $4x10^{-3}$ "pH units".s^{-1}. The lower record shows a much slower rate of alkalinisation ($1.8x10^{-3}$units.s^{-1}) from a similar resting pH_i in CO_2-HCO_3^- NaL of 6.95. The bars underneath the traces represent TMA applications, used to estimate buffering power.


CO_2–HCO_3^- NaL
HEPES NaL
pH 7.5
pH 6.9
b
a
60s


pH 8
CO_2–HCO_3^- NaL
HEPES NaL
pH 7
60s

bathed in CO_2-HCO_3^- NaL the estimates obtained include $ß_{CO2}$ and are therefore of *total* buffering power.

The inverse relationship between proton buffering power and pH_i over the physiologically relevant pH range is well documented (Szatkowski & Thomas, 1989, Wenzl & Machen, 1989, Vaughan-Jones & Wu, 1990, Goldsmith & Hilton, 1992, Corbett *et al*, 1992). Therefore it is necessary give the specific pH_i values at which the buffering power was measured. As buffering power estimation necessarily involves a change in pH_i, any ß estimates are given for an *average* pH value: that is the mean of pH_i in the absence of weak acid/base and the pH_i at peak acidification/alkalinisation. (This latter value may be an extrapolated value to reduce errors from pH_i regulation). The pH change evoked is preferably small enough to limit the necessary averaging of buffering power over a pH range but large enough to ensure accurate measurement above signal noise levels.

Table 1. *Buffering power values estimated using NH_4Cl challenges*

NH_4Cl challenge	CO_2-HCO_3^- NaL; $ß_T$	Mean pH_i	HEPES NaL; $ß_i$	Mean pH_i
Addition	13.1±1.5	7.25±0.05	9.0±1.6	7.27±0.05
Removal	12.4±0.85	6.88±0.04	14.2±3.6	6.77±0.04

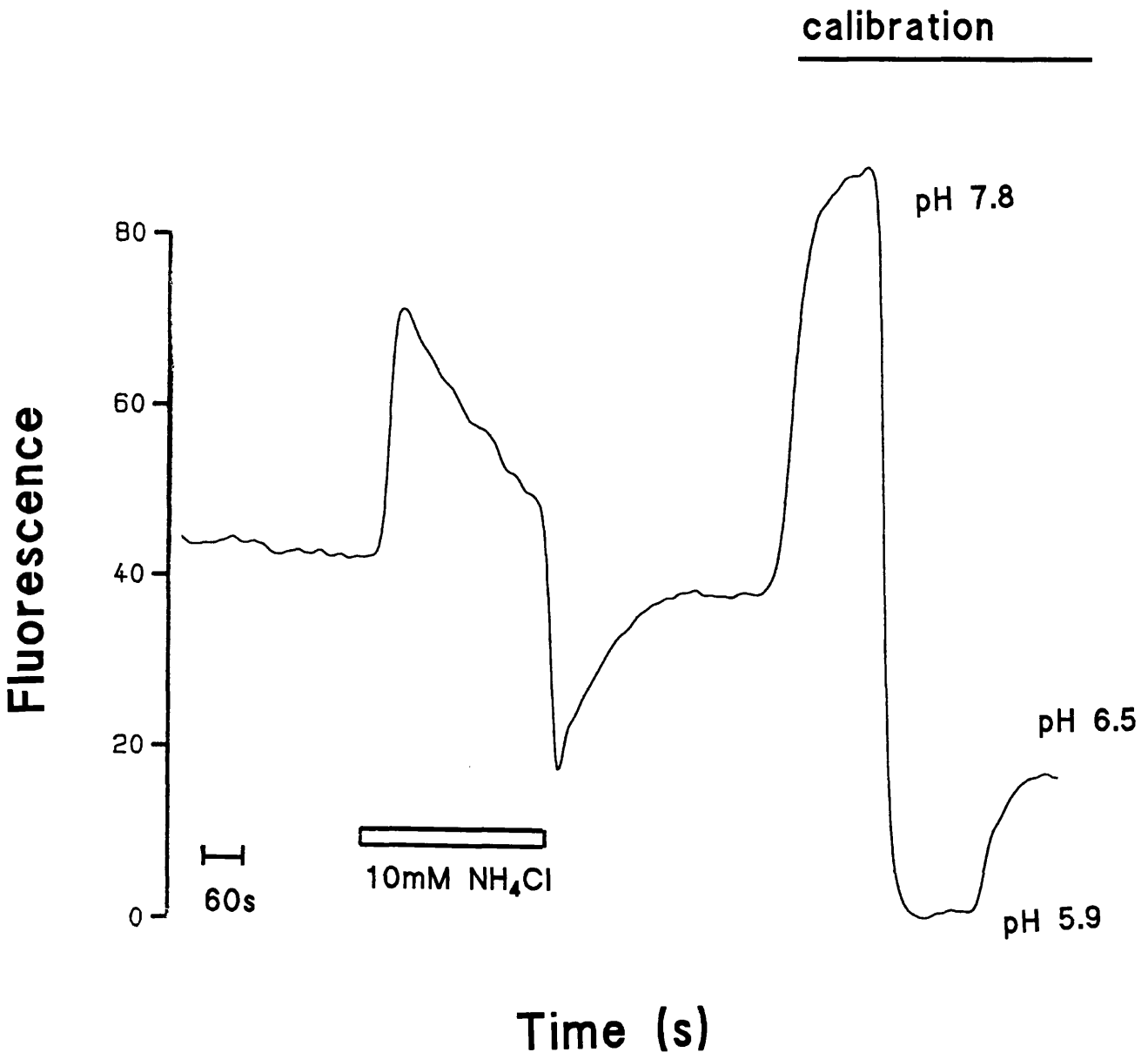
$ß_i$ and $ß_T$ are intrinsic and total buffering power respectively (mmoles/l of cytoplasm). Each column reports data as means±s.e.m. for 13-27 estimates.

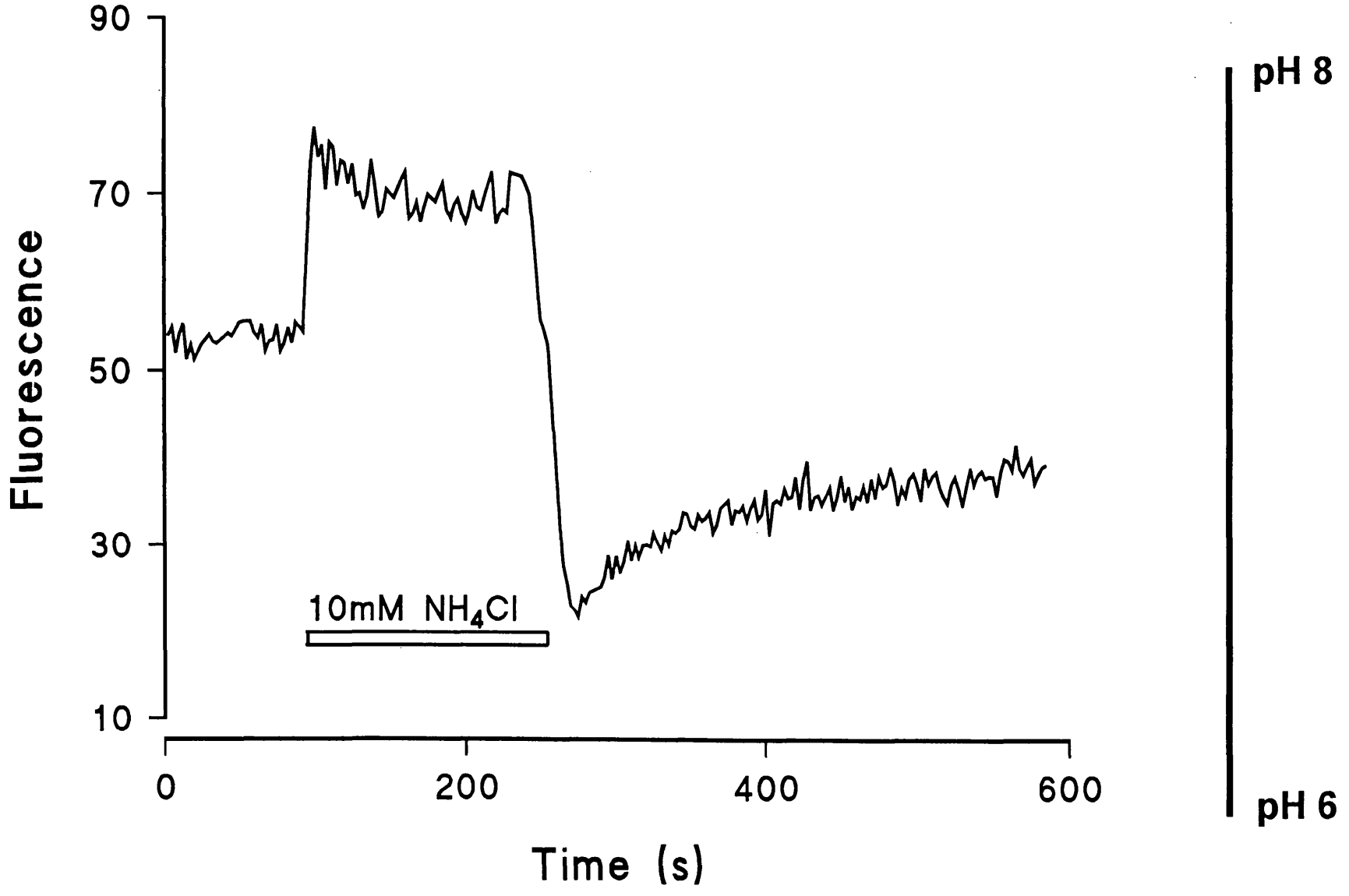
The acidification caused by washing off NH_4Cl means that ß estimates from removal are at a lower average pH_i value than those made from NH_4Cl addition. The data are summarised in table 1. The average values of $ß_i$ are in good agreement with other studies of central neurones stating 8mM pH $unit^{-1}$ in cerebellar granule cells (Pocock & Richards, 1992) and 11mM pH $unit^{-1}$ in cerebellar purkinje cells (Gaillard & Dupont, 1990). However, the expected increase in buffering power due to the presence of CO_2 is not

FIGURE 5. Figure 5a shows the filtered (0.25Hz) pH_i record of a cell bathed in HEPES NaL. The cell had a resting pH_i of 6.95. The buffering power is estimated using NH_4Cl as described in Methods and illustrated in figure 2. The estimates of buffering power obtained (5mM for addition and 13mM for removal) are estimates of *intrinsic* buffering power as PCO_2 is negligible. The cell showed a rapid rate of pH_i recovery following NH_4Cl removal, $8x10^{-3}$ units.s^{-1}. This record also shows the calibration technique. Solutions of different pH, containing nigericin (3μM), are applied to the cell. Intracellular pH is presumed to equilibrate with extracellular pH under these conditions, and the resultant ratio values allow construction of a pH vs ratio plot for each cell.

Estimation of $ß_T$ is shown in figure 5b. The buffering power is calculated in exactly the same way, but as the cell is bathed in CO_2-HCO_3^- NaL the estimates obtained are of *total* buffering power. $ß_T$ is low in this cell, 4mM for both addition and removal of NH_4Cl at average pH values 7.6 and 7.25. The rate of pH_i recovery in this cell is $7x10^{-3}$ units.s^{-1}.

N.B. Figures 5a and 5b are separate cells.


calibration
pH 7.8
pH 6.5
pH 5.9
80
60
40
20
0
Fluorescence
60s
10mM NH_4Cl
Time (s)


90
70
50
30
10
Fluorescence
10mM NH4Cl
0
200
400
600
Time (s)
pH 8
pH 6

apparent. In fact at similar mean pH_i values $ß_i$ and $ß_T$ are not significantly different (p=0.33 for NH_4Cl addition and 0.61 for removal, Student's t-test).

The natural variation in ß between different neurones necessitated measurement of $ß_T$ and $ß_i$ in the same individual cell. Cells were serially bathed in CO_2-HCO_3^- NaL and HEPES NaL and buffering power was measured under the two conditions thus providing estimates of $ß_T$ and $ß_i$ in the same cell (see figure 6). The pooled results from thisprotocol are displayed in table 2.

Table 2. *Paired measurements of $ß_T$ and $ß_i$ using NH_4Cl*

NH_4CL Challenge	$ß_i$	Mean pH_i	$ß_T$	Mean pH_i
Addition	11.4±3	7.07±0.03	14.1±2	7.11±0.04
Removal	14.8±3	6.71±0.04	13.3±2	6.84±0.03

$ß_i$ and $ß_T$ are intrinsic and total buffering power respectively (mmoles/l of cytoplasm). Data (mean±s.e.m.) from 4 cells serially subjected to HEPES NaL and CO_2-HCO_3^- NaL i.e. 16 separate buffering estimates.

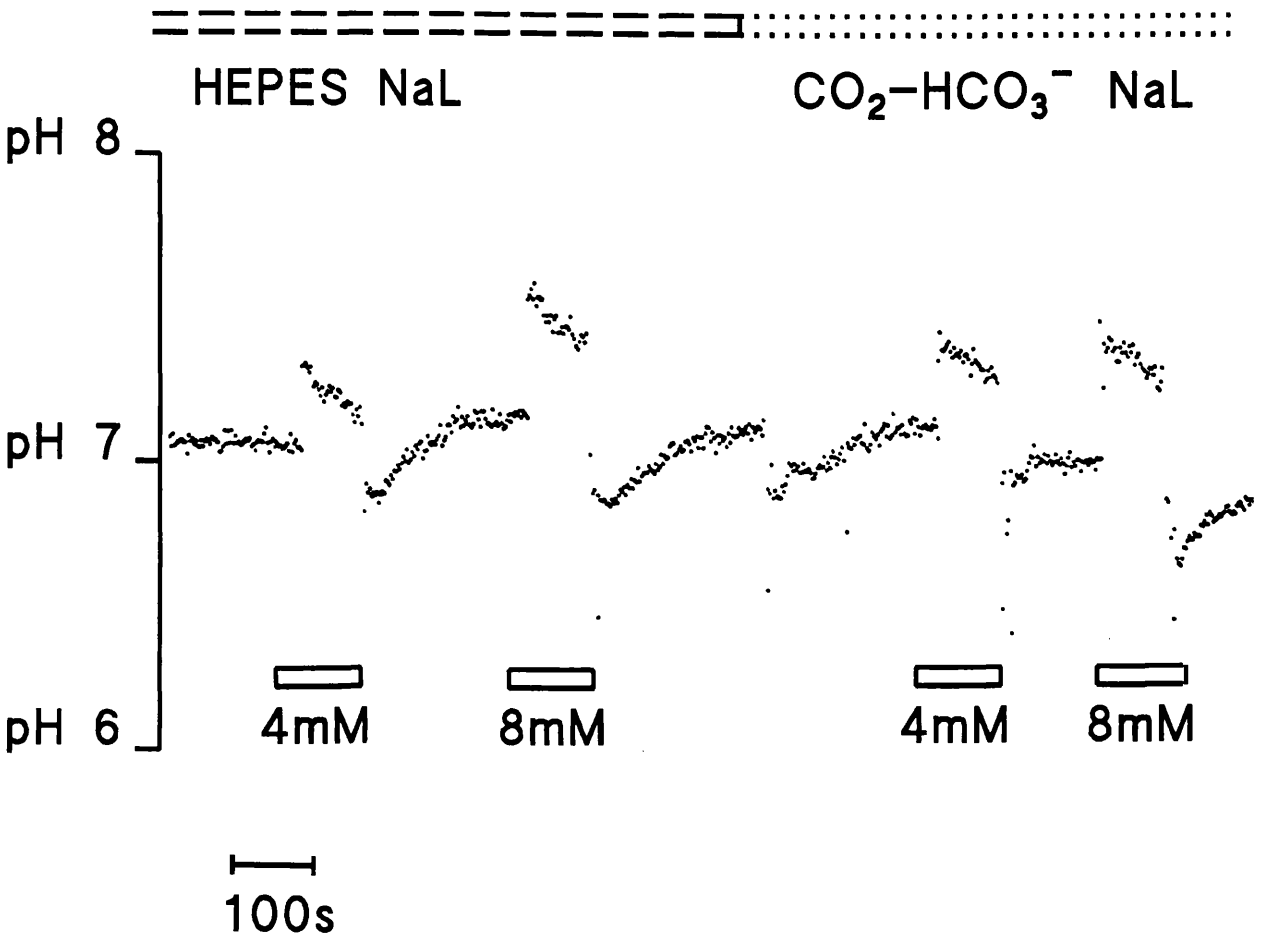
The paired two-tailed p-value comparing $ß_i$ and $ß_T$ estimated from NH_4Cl addition is 0.53 and that from removal is 0.57. This method of estimating $ß_i$ and $ß_T$ in the same cell is limited by the change in resting pH_i when swapping between CO_2-HCO_3^- NaL and HEPES NaL. Consequently the buffering power estimates are made at slightly different mean pH_i values.

FIGURE 6. Estimation of $ß_i$ and $ß_T$ in the same cell using sequential NH_4Cl challenges. This trace shows a cell initially bathed in HEPES NaL and subjected to separate 4mM and 8mM NH_4Cl challenges in order to estimate $ß_i$. The challenges were then repeated in CO_2-HCO_3^- NaL to yield $ß_T$ estimates. On changing from HEPES NaL to CO_2-HCO_3^- NaL the cell undergoes a reversible acidification.

$ß_i$ estimated from NH_4Cl addition was 15.7mM at pH 7.23 and 11mM at pH 7.35. Removal of NH_4Cl provided $ß_i$ estimates of 18.5mM at pH 7.0 and 19.3mM at pH 7.13.

$ß_T$ estimated from NH_4Cl addition was 9.3mM at pH 7.3 and 10.4mM at pH 7.27. Removal of NH_4Cl provided $ß_T$ estimates of 8.7mM at pH 7.08 and 12mM at pH 6.98.

That intrinsic buffering is apparently greater than total buffering is indicative of the errors in pH (and hence buffering power) estimation. This makes the acquisition of a large number of estimates essential. Also it is not obvious why the cell shows an apparently muted acid recovery from the second 4mM NH_4CL challenge. The cell demonstrates a healthy pumping of acid from the subsequent 8mM challenge. This variability in pH_i is not uncommon during prolonged procedures that alter pH_i.


HEPES NaL
CO_2–HCO_3^- NaL
pH 8
pH 7
pH 6
4mM
8mM
4mM
8mM
100s

Estimates of ß using TMA

In order to avoid any error specific to estimating buffering power with NH_4Cl the weak base trimethylamine (TMA) was also used. As with NH_4Cl, TMA was bath-applied in concentrations of the range 1.25-20mM. Intrinsic and total buffering power were estimated by performing experiments in HEPES NaL and CO_2-HCO_3^- NaL respectively.

The ionic form of TMA does not appear to permeate the cell membrane to any great extent. Consequently, TMA application evoked a 'flat top' alkaline response unlike alkalinisation and subsequent acidification seen with NH_4Cl (compare figure 2 with lower panel of figure 7). Thus buffering power values are generally at the same average pH_i whether estimated from TMA addition or removal. The top panel of figure 7 shows the pH_i of a cell bathed in CO_2-HCO_3^- NaL. On addition of 8mM TMA to the bathing medium the intracellular pH changed from 7 to 7.65. Assuming the pK_a of TMA to be 9.8 and the extracellular pH to be 7.3 then equation (4) can be used to estimate total buffering power. $ß_T$ estimated from TMA challenge is 5.5mM at pH 7.33 (the intracellular concentration of $TMAH^+$ at peak alkalinisation is estimated to be 3.56mM and the ΔpH_i it produces is 0.65 units). The results of buffering power estimates made from TMA applications are displayed in Table 3.

Table 3. *Buffering power estimates made from TMA challenges.*

$ß_T$	Mean pH_i	$ß_i$	Mean pH_i
10±0.78	7.27±0.05	9±1.12	7.34±0.04

$ß_i$ and $ß_T$ are intrinsic and total buffering power respectively (mmoles/l of cytoplasm). Each column reports data as mean±s.e.m., n=46 for $ß_i$ estimates and n=30 for $ß_T$.

Estimates of ß measured with TMA, as with those measured using NH_4Cl, show no increase in $ß_T$ over $ß_i$ (p=0.54 Student's t-test). This suggests that the lack of CO_2-HCO_3^- contribution to $ß_T$ is not specific to measurement with ammonium. Also the ß levels are similar whether measured by NH_4Cl or TMA challenges.

Estimates of $ß_T$ and $ß_i$ in the same cell were made by challenging cells bathed serially in CO_2-HCO_3^- NaL and HEPES NaL with TMA. A limitation of this approach is the inevitable change in pH_i on addition/removal of CO_2, which means that ß values will be measured for slightly different average pH_i values. Figure 8 shows one such experiment. Estimates of both $ß_i$ and $ß_T$ from individual cells are displayed in table 4.

Table 4. *Paired measurements of $ß_i$ and $ß_T$ using TMA*

Cell	Mean $ß_i$	Mean $ß_T$
(1)	6	8.9
(2)	8.6	7.1
(3)	6.4	6.2
(4)	10.4	14.4

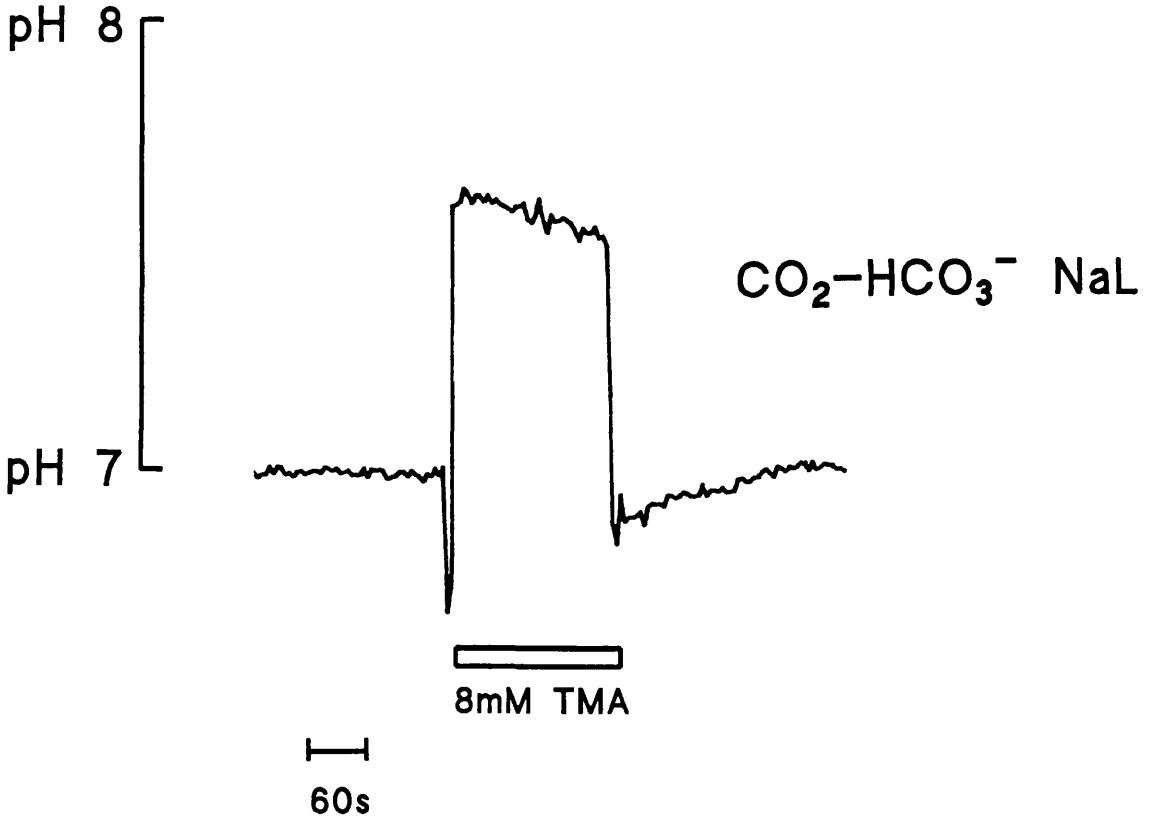
$ß_i$ and $ß_T$ are intrinsic and total buffering power respectively (mmoles/ll of cytoplasm). Means are of 2 estimates in each case, TMA addition and TMA removal.

There was no significant difference between $ß_i$ and $ß_T$ for individual cells subjected to this treatment ($p=0.38$ Student's t-test, $n=4$ pairs). It should be noted that between cells the resting pH_i varied. However, the TMA challenges produced step changes in pH_i and the intrinsic and total buffering power estimates were at the same average pH_i value.

FIGURE 7. Estimation of $ß_T$ and $ß_i$ using TMA. The top panel shows the pH_i of a cell bathed in CO_2-HCO_3^- NaL. On addition of 8mM TMA to the bathing medium the intracellular pH changed from 7 to 7.65 and from 7.55 to 6.75 on removal. Assuming the pK_a of TMA to be 9.8 and the extracellular pH to be 7.3 then equation (4) can be used to estimate total buffering power. $ß_T$ estimated from TMA challenge is 5.5mM at pH 7.33 and 6mM at pH 7.15.

The bottom panel shows the addition of 8mM TMA to HEPES NaL bathing the cell caused an intracellular alkalinisation of 0.55 units from a resting pH_i of 7.0. On removal of TMA the pH_i falls from 7.55 to 7.05. The estimates of $ß_i$ from these changes are 8mM at pH 7.28 and 9mM at pH 7.3.

The downward spikes seen on TMA addition and removal are solution change artefacts. These spikes were not always seen.


pH 8
pH 7
CO_2–HCO_3^- NaL
8mM TMA
60s

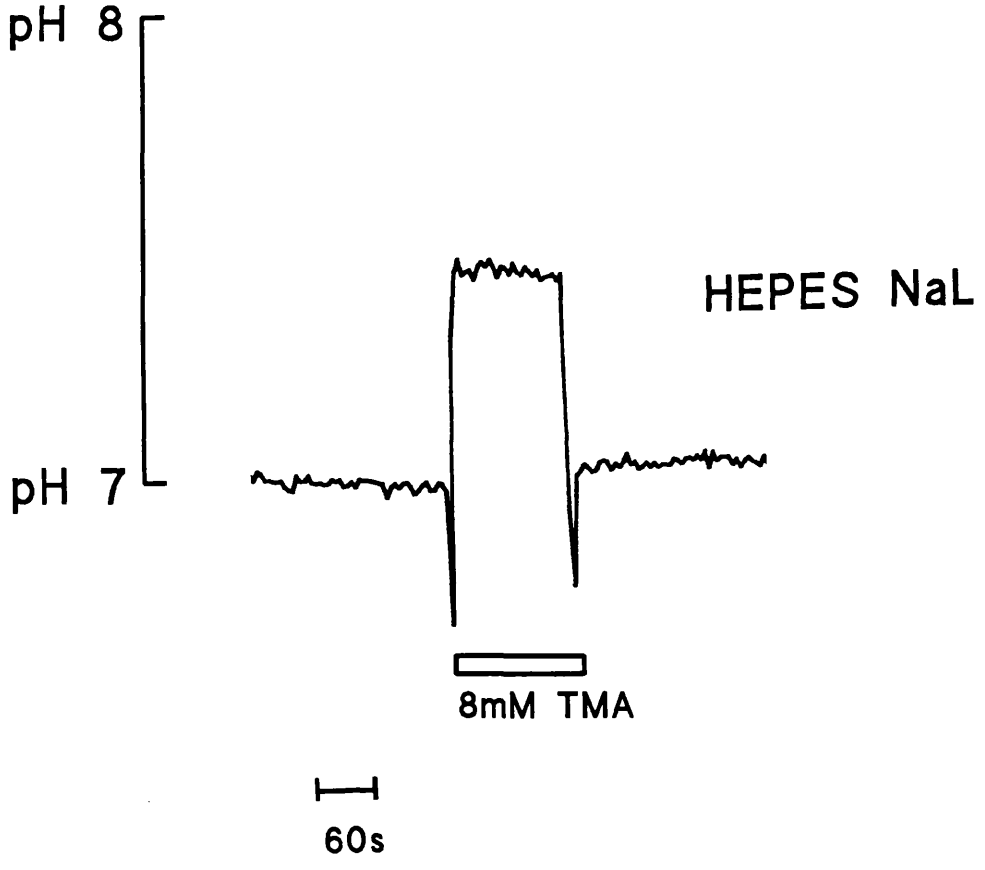
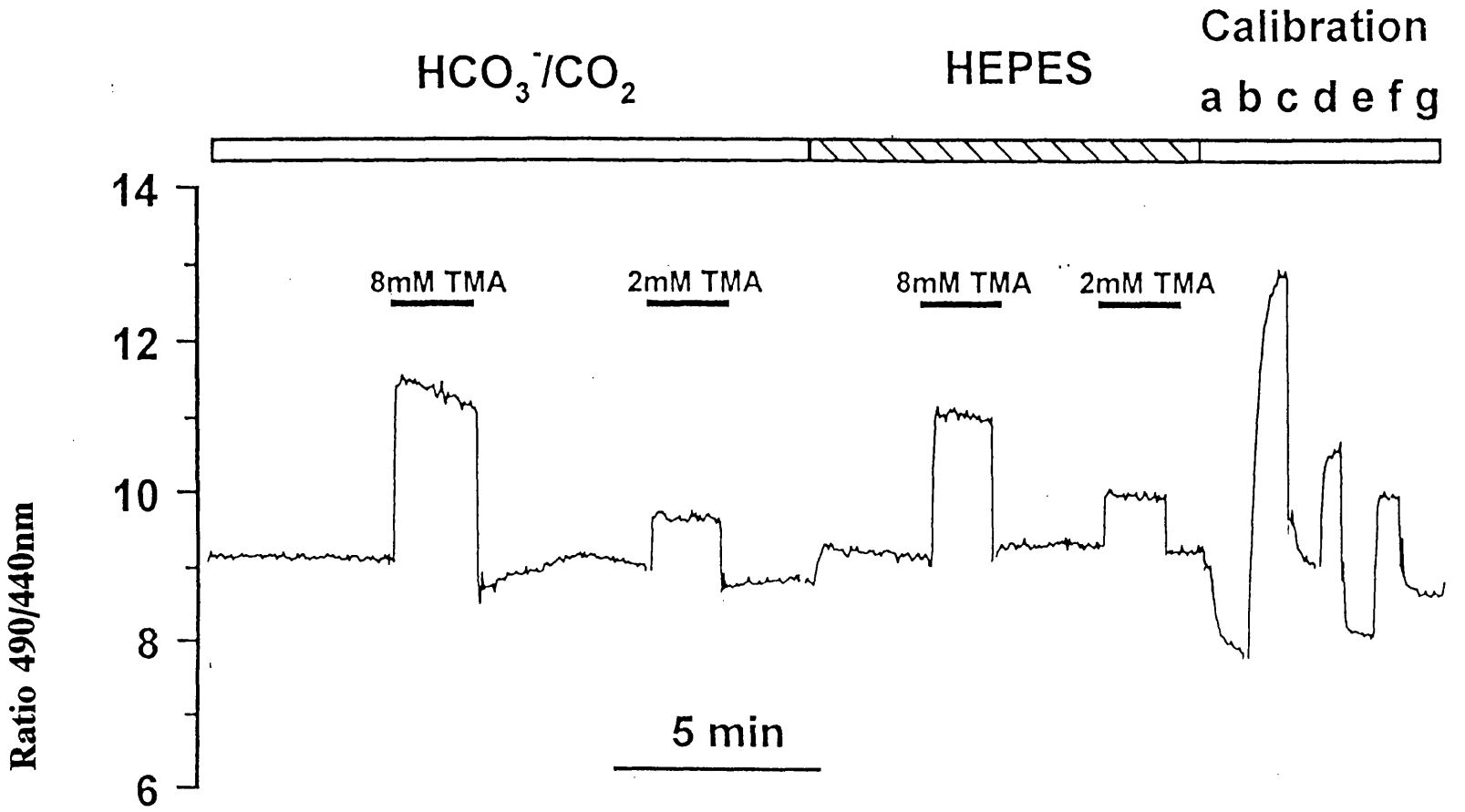

pH 8
pH 7
HEPES NaL
8mM TMA
60s

FIGURE 8. By challenging a cell with TMA in CO_2-HCO_3^- NaL and then HEPES NaL we can estimate $ß_T$ and $ß_i$ respectively. Estimates of $ß_T$ made from the TMA challenges in the first half of the record yielded values of 5.8 and 10mM for addition and 6.5 and 7.7mM for removal. Repeating the TMA challenges in HEPES NaL gave $ß_i$ estimates of 9.2 and 11.2mM for addition and 11.8 and 8.9mM for removal.

On removal of CO_2-HCO_3^- NaL the cell alkalinised due to the efflux of CO_2 from the cell. This record also shows the calibration procedure where high-K^+ solutions of different pH containing 3μM nigericin are applied to the cell. The pH values were a) 6; b) 8; c) 7; d) 7.45; e) 6.5; f) 7.2 and g) 6.8.


HCO_3^-/CO_2
HEPES
Calibration
a b c d e f g
14
12
10
8
6
8mM TMA
2mM TMA
8mM TMA
2mM TMA
5 min
Ratio 490/440nm

Estimates of ß using weak acids.

(i) Butyric acid

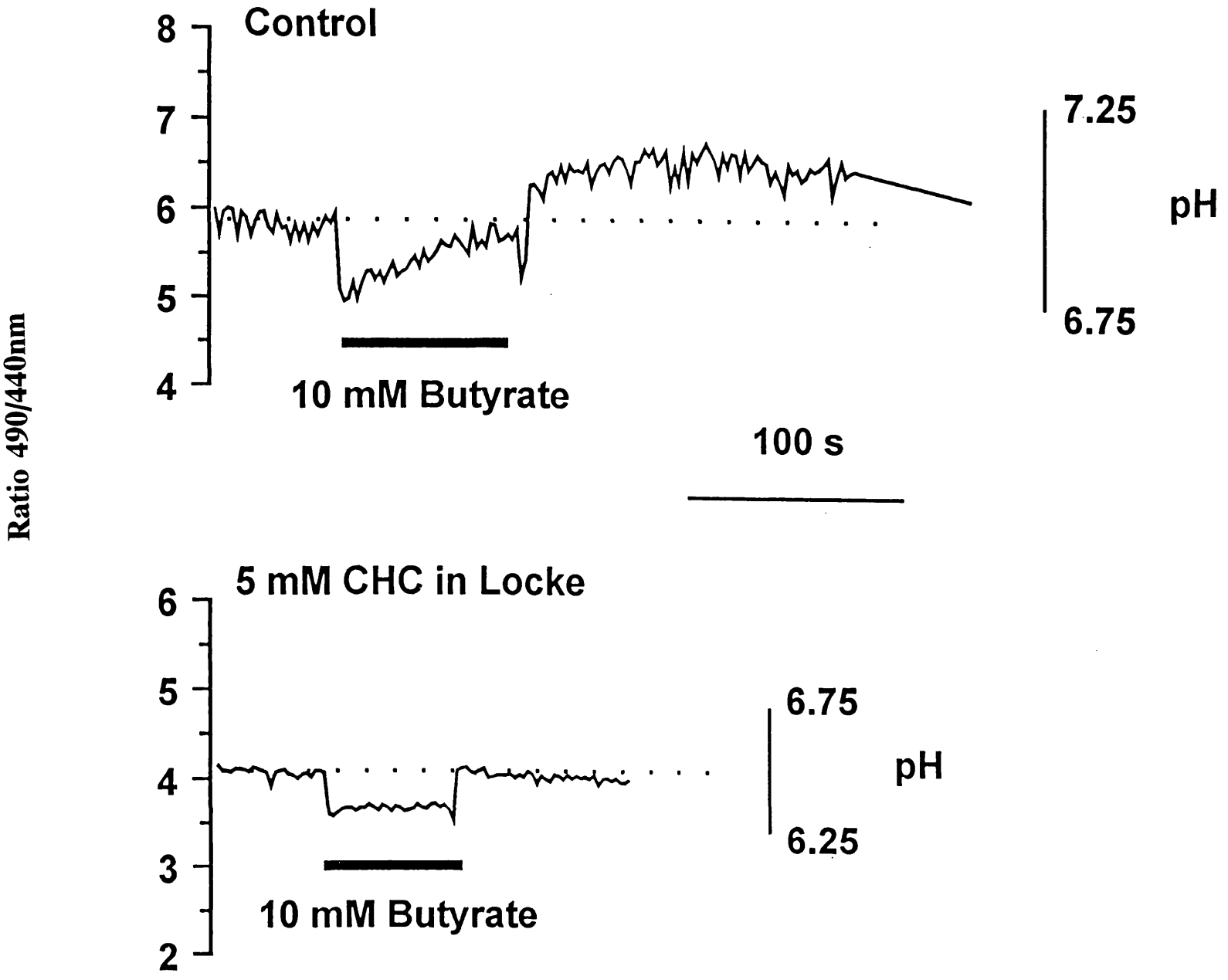
In order to estimate ß values at more acidic pH_i values a weak acid was used, butyric acid bath-applied as sodium butyrate. Immediately upon application of 1-10mM butyric acid an intracellular acidification was seen resulting from dissociation of C_3H_7COOH molecules into butyrate ions and H^+. However, very often a recovery in pH_i towards resting values occured in the continued presence of butyric acid. Both of these observations are shown in figure 9 (top panel). Estimates of total buffering power, calculated using equation 3 (Introduction), were quite high, $ß_T$=24.2±3.2 mM at mean pH_i 6.9, n=8. Individual estimates were as high as 40mM.

Taking into consideration previous studies stating the inverse relationship between ß and pH_i (eg Vaughan-Jones & Wu and Goldsmith & Hilton, 1992) the high values of $ß_T$ may not seem suprising. However, closer scrutiny revealed much larger ß estimates when ß was measured with butyrate than by NH_4Cl *at the same average* pH_i. This implied that one of the assumptions made in the weak acid/base method of measuring ß was being violated. It was possible that some butyrate ion was being transported into the neurones during ß measurement. In this case the apparent acid load on the cell from butyric acid dissociation would be greater than the actual quantity of H^+ released, resulting in an overestimation of ß. The mechanism of any butyrate ion entry is presumably via the monocarboxylate ion transporter, found in many tissues including brain (Assaf *et al*, 1990). The sodium linked form would provide appropriate entry.

In order to block butyrate transport ß was estimated in the presence of the monocarboxylate ion transporter inhibitor α-cyano-hydroxy-cinnamic acid (CHC) (Halestrap, 1976). The effect of CHC on buffering power estimates is illustrated in figure 9 (lower panel). Buffering power estimates made from the addition of 10mM butyrate in the presence of 5mM CHC were much lower than those made in its absence. For eight

FIGURE 9. The effect of blocking monocarboxylate ion transport on ß estimates. The top trace shows the pH_i record of a cell bathed in HEPES NaL subjected to a 10mM challenge of butyric acid. The cell was initially sitting at a pH_i of 7.0 and this shifted to 6.85 on addition of butyrate. The estimate of $ß_i$ from butyric acid addition is 23mM. From removal it is 40mM. The steady increase of pH_i during the butyrate challenge indicates possible monocarboxylate ion transport.

The lower trace shows estimation of $ß_i$ in the presence of the monocarboxylate ion transport inhibitor CHC (see text). Note that no transport of butyrate seemed to be occuring. Estimates of $ß_i$ for both addition and removal of butyric acid are about 14mM.


Control
8
7
6
5
4
7.25
pH
6.75
10 mM Butyrate
100 s
Ratio 490/440nm
5 mM CHC in Locke
6
5
4
3
2
6.75
pH
6.25
10 mM Butyrate

cells $ß_T$ measured with 5mM CHC [2] present was 7.1 ±2.5mM at mean pH_i 6.45. It seems probable that some monocarboxylate ion transport is indeed occuring during buffering estimates and that values of ß more consistent with other estimates can be obtained by inhibiting this transport. However, although CHC effectively blocks monocarboxylate ion transport, being a weak acid it will itself acidify the cell interior to a certain extent. Furthermore, monocarboxylate extrusion was apparently active at rest in some cells as evidenced by a steady decline in pH_i on application of CHC (5mM). Mean rate of decline in cells previously exhibiting a steady resting pH_i was $3x10^{-4}$ units.s^{-1}.

(ii) Estimates of $ß_i$ made from changes in $[CO_2]$ & $[HCO_3^-]$

The method-specific error that arises with butyric acid in these cells prompted us to try other means with which to measure buffering power by acid changes. Changing PCO_2, concomitant with changes in solution $[HCO_3^-]$ in order to maintain constant pH_o, allows rapid challenges to intracellular pH. Although this procedure causes rapid changes in intracellular pH by simply changing the amount of dissolved CO_2 (and hence the $[H^+]_i$), it is difficult to estimate buffering power by the change in $[HCO_3^-]_i$. Problems arise due to the slowness of the pH_i change in many cells (which implies low carbonic anhydrase levels) as well as uncertainty in determining the end point of pH_i change.

Buffering power was estimated from CO_2 addition in 10 cells, as shown in figure 10. Estimates of $ß_i$ were made by rapidly changing the PCO_2 of the bathing medium from 1.25% or 2.5% to 5%. The $[HCO_3^-]$ of the bathing medium was kept at 5, 10 and 20mM respectively thus maintaining the same pH between the solutions. The change in the estimated $[HCO_3^-]_i$ due to increasing the PCO_2 is a direct measure of the acid load on the cell. In the example given in figure 10 the change in pH_i (δ) evoked by doubling the PCO_2 is used to estimate the intrinsic buffering power of the cell. Just prior to the change of CO_2 from 2.5% to 5% the resting pH_i was 7.35, after equilibration it was 7.15. The value of $[HCO_3^-]_i$ can be estimated from the equation $HCO_3^- = \alpha . pCO_2 . 10^{(pHi-pKa)}$, where

[2] The effects of CHC on fluorescent yield was examined by comparing BCECF spectra in the presence and absence of CHC. CHC at the concentrations used externally (1-5mM) was not found to have significant effects on BCECF emission at 520nM from 440nm and 490nm excitation fluorescence spectra.

α=solubility coefficent of CO_2 (0.03mmol.l^{-1} per mmHg) and pK_a="overall" dissociation constant for CO_2. At pH 7.35 (2.5% CO_2) the $[HCO_3^-]_i$ is 9.42mM. At pH 7.15 (5% CO_2) the $[HCO_3^-]_i$ is 12mM. Therefore the increase in $[HCO_3^-]_i$ is 2.58mM and the pH_i change is 0.2 units so $ß_i$ is 12.9mM at pH 7.2. Mean $ß_i$ estimated using this method was 11mM±2.34 (n=10) at average pH_i 7.14.

(iii) Estimates of $ß_T$ at different PCO_2 values.

If $[HCO_3^-]_i$ is a direct measure of $ß_{CO2}$ (Woodbury 1965, Roos & Boron 1981) then a cell should have different $ß_T$ values if $[HCO_3^-]_i$ is changed. At two different PCO_2 levels, if pH is constant, $[HCO_3^-]$ must change. Since $ß_{CO2}=2.3[HCO_3^-]$ for an open system the difference in $[HCO_3^-]_i$ should be a measure of the expected difference in $ß_{CO2}$. Estimates of $ß_T$ in single cells derived from TMA challenges in 2.5% and 5% CO_2 are shown in figure 10 and table 5. In figure 10 TMA challenges (10mM) given in two different CO_2-HCO_3^- states were used to estimate $ß_T$. From the pH_i change *a*, $ß_T$ is estimated to be 6mM at mean pH 7.63. From *b* an estimate of 8mM for $ß_T$ at mean pH 7.5 is calculated. However, the difference in $[HCO_3^-]_i$ at the two mean pH_i values is 8.9mM, so the theoretical difference in $ß_{CO2}$ is about 20mM.

Table 5. *Estimates of $ß_T$ under different CO_2/HCO_3^- conditions*

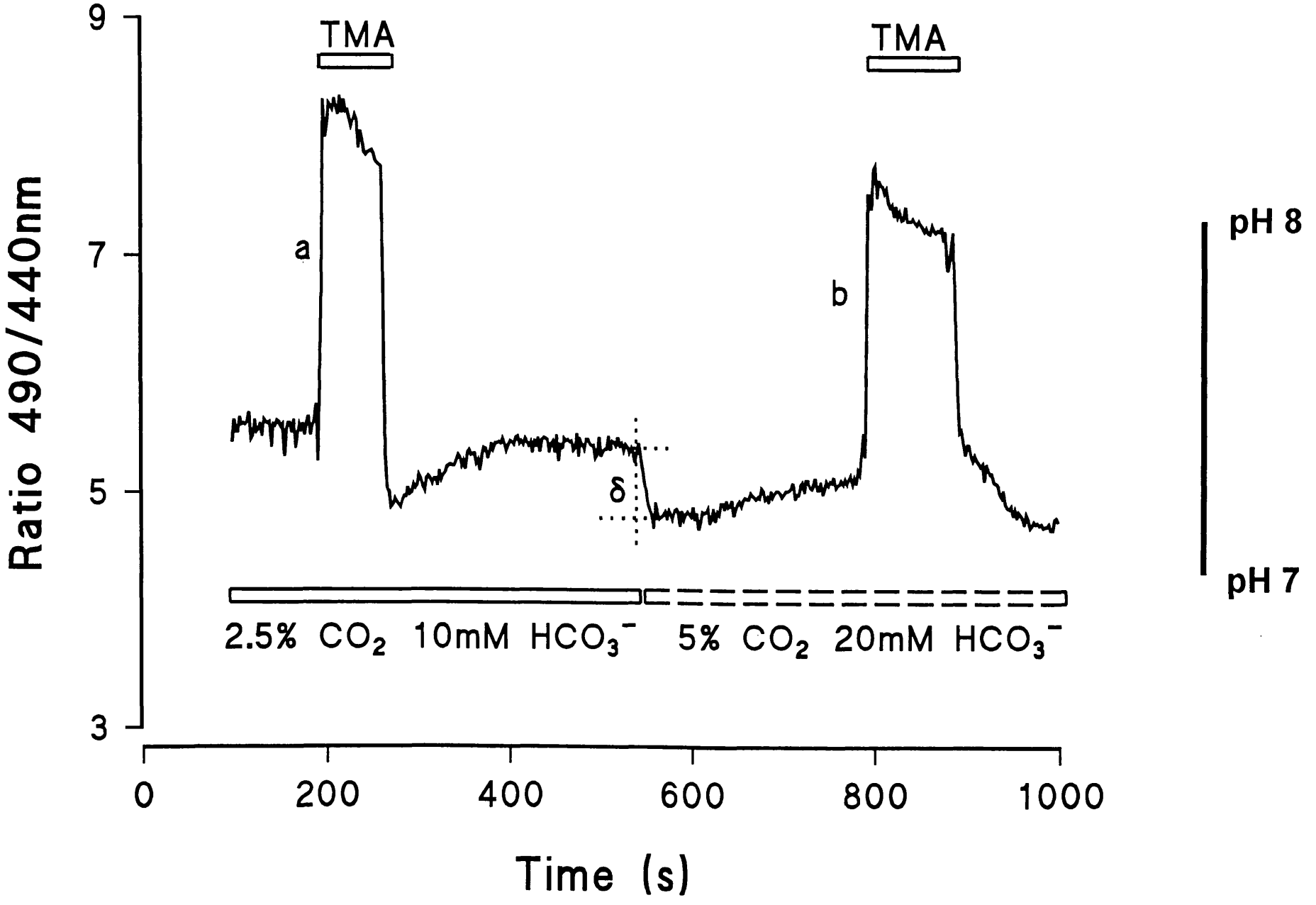
Test condition	Mean pH_i	$[HCO_3^-]_i$	Theoretical $ß_{CO2}$	Measured $ß_T$
2.5% CO_2	7.5±0.04	13.4	31	11.5±1.7
5% CO_2	7.4±0.03	21.3	49	9.99±1.2

$ß_{CO2}$ is carbon dioxide contributed buffering power in an open system, $ß_T$ is total buffering power (mmoles/l cytoplasm). The two mean measured $ß_T$ values are not significantly different, p=0.48. Values given are mean±s.e.m., n=12 for 2.5%CO_2 conditions and n=27 for 5%CO_2 conditions. $[HCO_3^-]_i$ values are estimated concentrations (mmol) for the mean pH_i values given and are calculated as follows: Knowing the pH_i value and assuming an 'overall' pK_a for the dissociation of CO_2 in water into H^+ and HCO_3^- of 6.1 and a carbon dioxide solubility coefficient of 0.03mM/mm Hg the $[HCO_3^-]_i$ can be estimated from the reaction describing the equilibrium dissociation. ie $K_a=[H^+].[HCO_3^-]_i/[CO_2]$. The theoretical value of $ß_{CO2}$ is calculated as $2.3[HCO_3^-]_i$.

The estimated difference in $[HCO_3^-]_i$ between the two conditions is 8mM and therefore the expected difference in $ß_T$ is about 18mM. The CO_2-HCO_3^- system in these cells is clearly not behaving as predicted for an open system. In fact the measured $ß_T$ values fall far short of even the theoretical $ß_{CO2}$ portion of total buffering power.

FIGURE 10. Estimation of $ß_i$ from changing PCO_2. This record shows the intracellular pH of a cell bathed initially in CO_2-HCO_3^- NaL in equilibrium with 2.5% CO_2. The change in pH_i (δ) evoked by doubling the PCO_2 is used to estimate the intrinsic buffering power of the cell. Just prior to the change of CO_2 from 2.5% to 5% the resting pH_i was 7.35, after equilibration it was 7.15. The value of $[HCO_3^-]_i$ can be estimated from the equation $HCO_3^- = \alpha . pCO_2 . 10^{(pHi-pKa)}$, where α=solubility coefficent of CO_2 (0.03mmol.l^{-1} per mmHg) and pK_a="overall" dissociation constant for CO_2. At pH 7.35 (2.5% CO_2) the $[HCO_3^-]_i$ is 9.42mM. At pH 7.15 (5% CO_2) the $[HCO_3^-]_i$ is 12mM. Therefore the increase in HCO_3^- is 2.58mM and the pH_i change is 0.2 units so $ß_i$ is 12.9mM at pH 7.2.

The TMA challenges (10mM) in the two different CO_2-HCO_3^- states were used to estimate $ß_T$. From the pH_i change *a*, $ß_T$ is estimated to be 6mM at mean pH 7.63. From *b* an estimate of 8mM for $ß_T$ at mean pH 7.5 is calculated. However, the difference in $[HCO_3^-]_i$ at the two mean pH_i values is 9mM, so the theoretical difference in $ß_{CO2}$ is about 21mM.


TMA
TMA
a
b
δ
9
7
5
3
Ratio 490/440nm
pH 8
pH 7
2.5% CO2 10mM HCO3-
5% CO2 20mM HCO3-
0
200
400
600
800
1000
Time (s)

pH_i-regulation and ß estimates.

In other studies involving estimation of buffering power (Szatkowski & Thomas, 1989, Vaughan-Jones & Wu, 1990) authors have drawn attention to the need to account for pH regulating mechanisms when measuring the pH_i change necessary for ß estimation. Mechanisms extruding acid for example may cause an artificially evoked acidification to be underestimated and thus ß would be overestimated (as ß=[Acid]/ΔpH, see equation (2), in Introduction). In some of the studies referred to, the pH_i changes caused by weak acid/base addition are slow in their time course compared to our system, taking minutes rather than ≈1 second, as is the case in the present experiments. With these slow changes in extracellular [acid] and [base], cellular pH_i-regulating mechanisms would have a chance to mask the true extent of pH_i change whereas the rapid time course of events in our system largely eliminates this problem. Nevertheless, experiments were conducted to minimize pH regulation as a source of error. Before this could be done, however, it was first necessary to determine the pH_i regulating mechanisms that exist in these cells.

Previously reported mechanisms demonstrated to regulate pH_i in central neurones are the Na^+/H^+ exchanger, the Na^+/HCO_3^- symporter, and the Cl^-/HCO_3^- exchanger. A monocarboxylate ion transporter is also apparently active in some neurones as suggested from experiments where CHC causes a steady decline in resting pH_i.

The Na^+/H^+ exchanger has been reported as amiloride sensitive (Pocock & Richards, 1992 and Gaillard & Dupont, 1990) as well as insensitive (Raley-Susman *et al*, 1991). The Cl^-/HCO_3^- exchanger has been reported as sensitive to DIDS (Schwiening & Boron, 1994; Gaillard & Dupont, 1990) and may only be active in adult neurones (Raley-Susman *et al*, 1993). Also Pocock & Richards reported a Cl^--dependent acidifying mechanism active at rest that was insensitive to DIDS and SITS. Considering the heterogeneity of a preparation such as neocortical neurones maintained in culture it is necessary to perform both pharmacological inhibition and ion replacement protocols in assessing the pH_i regulating mechanisms present. A previous study performed in this laboratory (Pocock & Richards, 1992) has already shown the diversity of mechanisms present in CNS neurones. Consequently it was not felt necessary to perform exhaustive studies of

the pH_i-regulating ion transport mechanisms but merely to assess the distribution of mechanisms present.

Effect of amiloride or replacement of extracellular sodium on pH_i

Application of amiloride (1mM) to cells exhibiting a steady resting pH_i in HEPES buffer caused a steady decline in resting pH_i in 4 of 11 cells. Amiloride (1mM) and a derivative, ethylisopropylamiloride (100μM), completely inhibited recovery from acid loads in HEPES NaL (3/4 cells).

Equimolar replacement of extracellular sodium with NMDG (a non permeant cation) in HEPES NaL where cells showed a stable resting pH_i (and so were presumably not dependent on the Na^+/HCO_3^- symporter or Na^+-dependent Cl^-/HCO_3^- exchanger) caused an acidification in half the cells investigated (3 of 6). Removal of extracellular sodium in CO_2-HCO_3 NaL evoked a decline in pH_i in all cells (n=4).

Effect of DIDS or replacement of external chloride on pH_i

Application of 4,4'-dinitrostilbene-2,2'-disulphonic acid (DIDS, 250μM) to cells demonstrating a stable resting pH_i in CO_2-HCO_3^- NaL had no effect on resting pH_i (6 cells). Equimolar replacement of Cl^- with methanesulphonate in CO_2-HCO_3^- NaL did not affect resting pH_i in 4/5 cells. The cell that did show a dependency for Cl^- demonstrated a dramatic increase in pH_i on Cl^- removal. This was presumably due to the outwardly directed Cl^- gradient causing reversal of Cl^-/HCO_3^- exchange (see figure 11, middle panel).

CO_2-HCO_3^- dependent pH_i-regulating transport mechanisms

Bicarbonate ion dependent mechanisms can be blocked by performing experiments in HEPES buffered solution, with nominally zero CO_2 present. Alternatively, the individual exchangers can be disrupted by replacement of extracellular chloride or sodium ions or pharmacological poisoning (see above). It is possible that the sodium dependent Cl^-/HCO_3^- exchanger or the Na^+/HCO_3^- symporter are active at rest in some cells as

suggested by the declining pH_i in 11 cells of 21 studied seen in HEPES NaL. The general lack of effect of extracellular Cl^- removal would argue against significant Cl^-/HCO_3^- exchange.

These results suggested the presence of:

1) An amiloride sensitive Na^+/H^+ exchange (shown by declining pH_i on Na^+ removal in HEPES NaL and on amiloride application and amiloride inhibition of acid recovery in HEPES).

2) Cl^-/HCO_3^- exchange: limited presence, indicated by change in pH_i on removal of extracellular Cl^- (and possibly not DIDS sensitive).

3) Other HCO_3^--dependent mechanisms: declining pH_i seen in about 50% cells when bathed in normal HEPES NaL -possibly Na^+/HCO_3^- exchange.

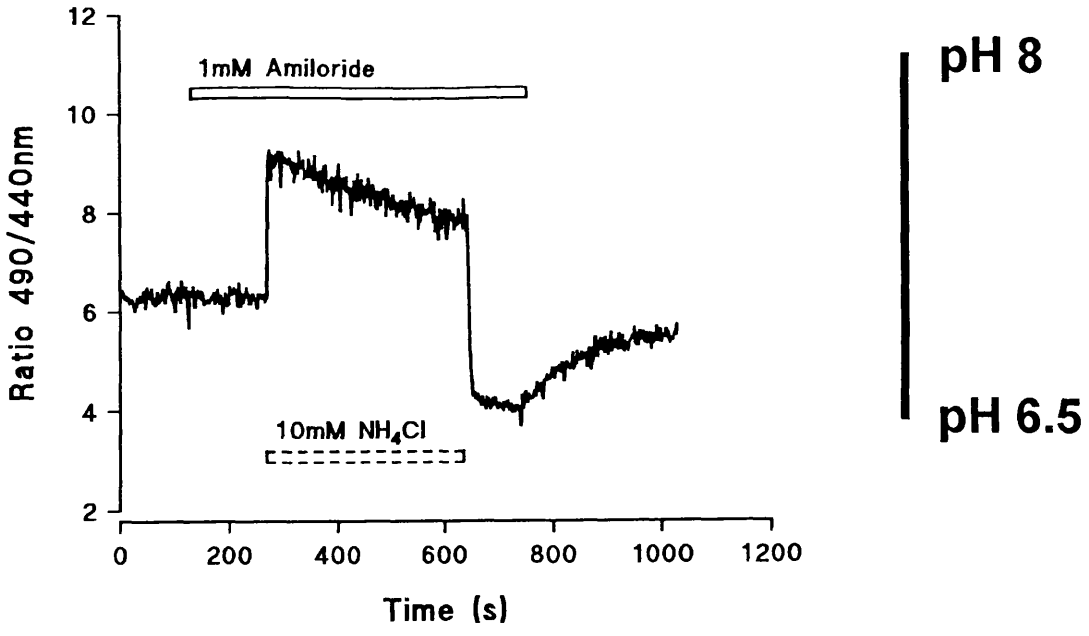
Some of these observations are illustrated in figure 11. In order to reduce contamination of ß estimates by pH_i-regulation, experiments were performed in conditions where these exchangers would be inhibited.

FIGURE 11. Sodium, chloride and bicarbonate dependent pH_i-recovery mechanisms.

The top trace shows a cell challenged with NH_4Cl in the presence of 1mM amiloride (a specific blocker of Na^+/H^+ exchange). On NH_4Cl removal there is an intracellular acidification (shown as a decrease in fluorescence signal ratio). The cell did not recover from the acid load until the amiloride was washed off.

In the middle trace removal of extracellular Cl^- in CO_2-HCO_3^- NaL elicits an intracellular alkalinisation. This is most likely due to the action of the Cl^-/HCO_3^- exchanger, affected by a reversed Cl^- gradient.

The lower trace shows a cell exhibiting HCO_3^--dependent acid recovery. The cell is subjected to an acid load (point 2) in HEPES NaL. No acid load recovery is seen until the HEPES NaL is replaced with CO_2-HCO_3^- NaL (point 3). From energetic considerations this process probably involves Na^+ as well as HCO_3^-. This may represent the action of a Na^+/HCO_3^- co-transporter or the Na^+-dependent Cl^-/HCO_3^- exchanger. This last trace has been smoothed. Notice that the pH_i-regulating mechanisms appear to be restoring pH_i to a value higher than its original resting value. This may suggest that this cell is dependent on CO_2-HCO_3^- for its resting pH_i. However, most cells seem to have similar resting pH_i in the presence or absence of CO_2-HCO_3^-, as detailed at the beginning of the Results section.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Time (s)
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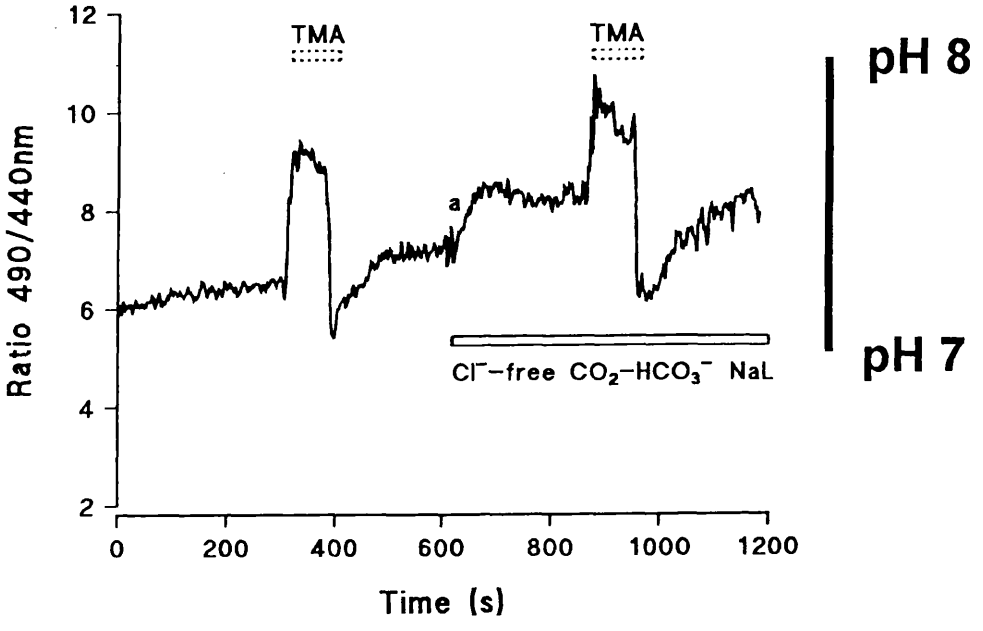
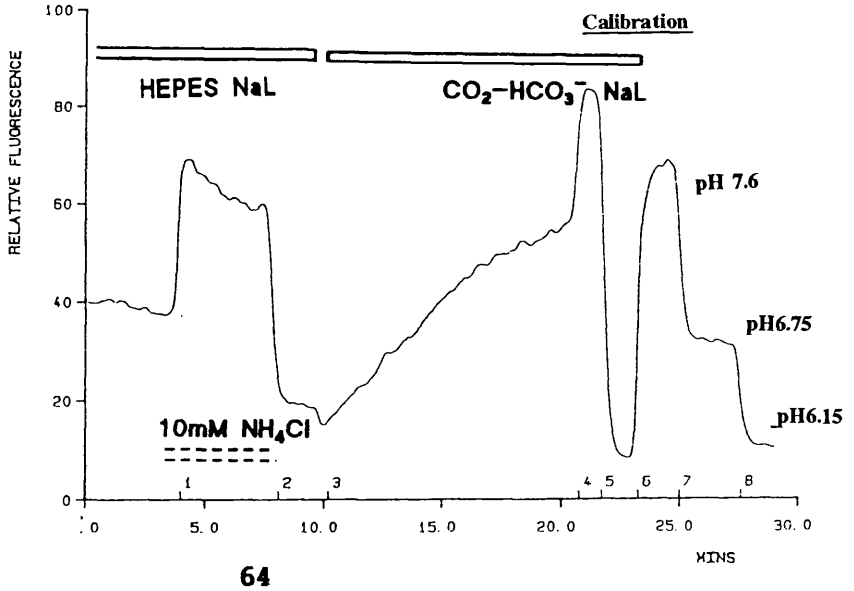
pH 6.5


TMA
TMA
a
Cl^-–free CO_2–HCO_3^- NaL
Ratio 490/440nm
Time (s)
pH 8
pH 7


Calibration
HEPES NaL
CO_2–HCO_3^- NaL
10mM NH_4Cl
RELATIVE FLUORESCENCE
MINS
pH 7.6
pH6.75
pH6.15

Effect of inhibiting pH_i-regulating mechanisms on buffering power estimates

1) Na^+-dependent pH_i-regulating mechanisms.

(a) Estimating $ß_i$ in sodium-free HEPES buffered bathing solutions would block the Na^+/H^+ exchanger. Estimates of $ß_i$ made from NH_4Cl and TMA challenges in HEPES buffer and in Na-free HEPES buffer are shown in table 6 and figure 12. The lack of difference between the two mean $ß_i$ estimates suggests that Na^+/H^+ exchange activity does not cause overestimation of $ß_i$.

Table 6. *Estimates of $ß_i$ with and without external sodium*

Test condition	$ß_i$	Mean pH_i
HEPES NaL	3.6±0.22	7.3±0.04
Sodium free HEPES NaL	3.33±0.29	7.26±0.04

ß values are in mmoles/l cytoplasm. n=6 pairs. The value of $ß_i$ estimated in Na^+-free conditions is no different to that estimated in Na^+-containing solution, p=0.52 for two tailed paired t-test. It should be noted that this test group had buffering power values at the lower end of the range found.

(b) Estimates of $ß_T$ in Na^+-free CO_2-HCO_3^- NaL would show if the action of the Na^+/H^+ exchange or mechanisms dependent on Na^+ & HCO_3^- would give rise to systematic errors in estimates of $ß_T$. Again, Na^+ replacement appeared to have little effect on buffering power estimates.

Table 7. *Estimates of $ß_T$ with and without external sodium*

Test condition	$ß_T$	Mean pH_i
CO_2-HCO_3^- NaL	5.6±1.39	7.3±0.02
Sodium free CO_2-HCO_3^- NaL	3.8±2.3	7.28±0.03

ß Values are in mmoles/l cytoplasm. n=5 pairs. $ß_T$ is no different in Na^+-containing and Na^+-free bathing media, p=0.13 for two tailed paired t-test. However, this significance must be considered in context of the large S.E.M. values.

An example of the effect of Na^+ replacement is shown in the top panel of figure 12. The cell shown was initially sitting at a fairly stable pH_i (6.95) but on removal of Na^+ from the HEPES NaL bathing the cell there was a decline in pH_i of $1.5x10^{-3}$ units.s^{-1}. Estimates of $ß_i$ from 10mM TMA challenge were 4.5mM at pH 7.25 for addition and 6mM at pH 7.08 for removal. The removal of Na^+ was not found to affect $ß_i$ ($p=0.52$ $n=6$ pairs) or $ß_T$ ($p=0.13$ $n=5$ pairs).

(c) Excluding external sodium will prevent the Na^+/H^+ exchanger from working but may have the unwanted consequence of the reversed sodium gradient leading to acid entry. Reversal of Na^+/H^+ exchange can be avoided by inhibiting the exchanger with amiloride or one of its analogues rather than sodium ion replacement. Amiloride and its analogues are commonly used as exchange inhibitors (e.g. Gaillard & Dupont, 1990, Vaughan-Jones & Wu, 1990 and Pocock & Richards, 1992). Application of amiloride (1mM) caused a declining pH_i in some 4 of 11 cells suggesting significant Na^+/H^+ exchange activity at rest. Estimates of $ß_i$ in the presence of amiloride (as demonstrated in figure 12, lower panel) are displayed in table 8. Buffering power was not significantly reduced by the presence of amiloride ($p=0.75$).

Table 8. *Estimates of $ß_i$ in the presence of amiloride*

Conditions	$ß_i$	Mean pH_i
Amiloride (1mM)	5.9±1.29	7.5
Normal	6.49±1.31	7.49

$ß_i$ is in mmoles/l cytoplasm. N=4 in each case.

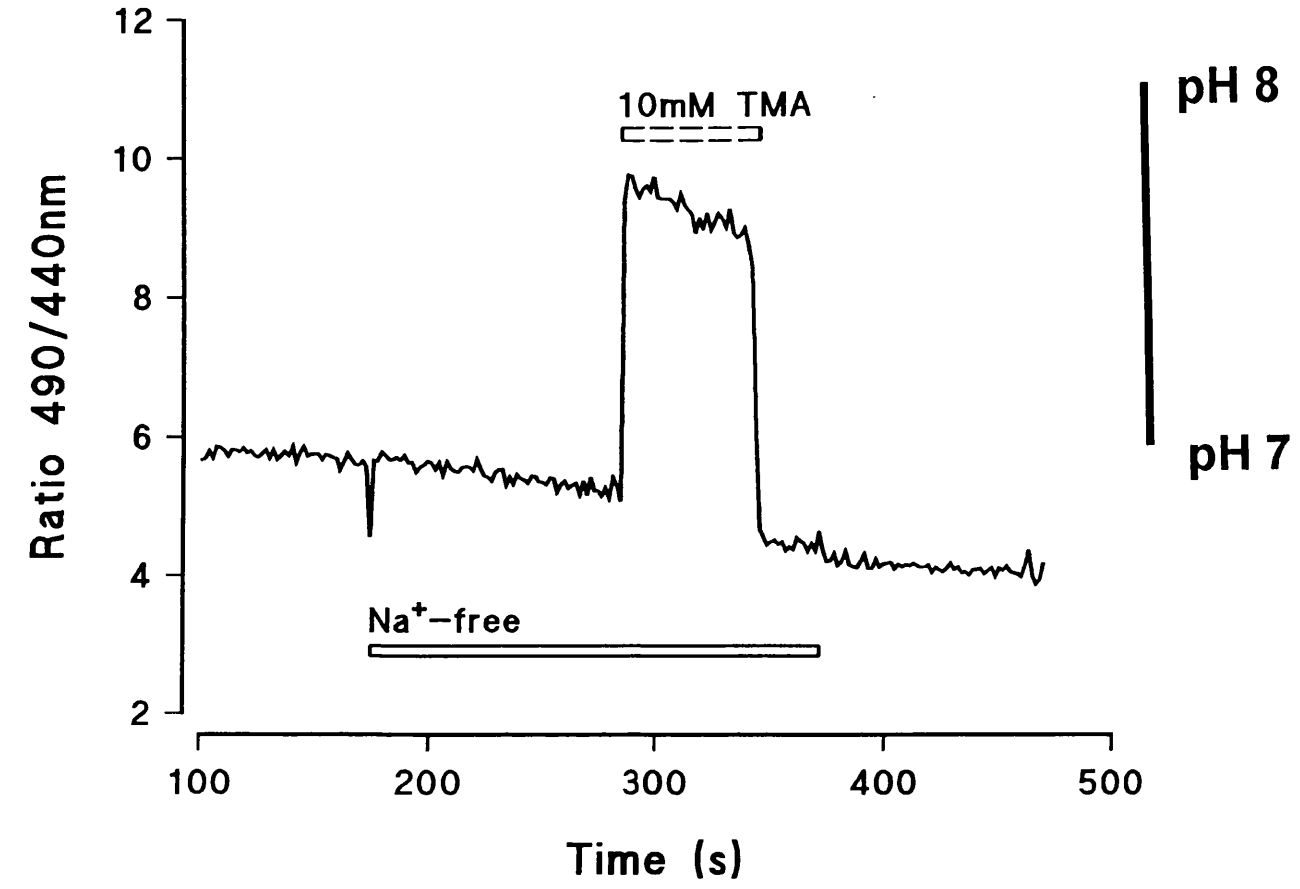
In the example shown in figure 12 (bottom panel) the resting pH_i of the neurone was unaffected by inclusion of 1mM amiloride in the HEPES NaL bathing medium. $ß_i$ was estimated using a 10mM NH_4Cl challenge. For addition the pH_i change was 0.6 units and $ß_i$=8.2mM. On NH_4Cl removal pH_i fell from 7.35 to 6.55 and $ß_i$ was estimated to be 10.9mM. As the Na^+/H^+ exchanger is an acid recovery mechanism we would expect $ß_i$ estimated from removal to be noticeably lower than normal if pH_i-regulation was masking

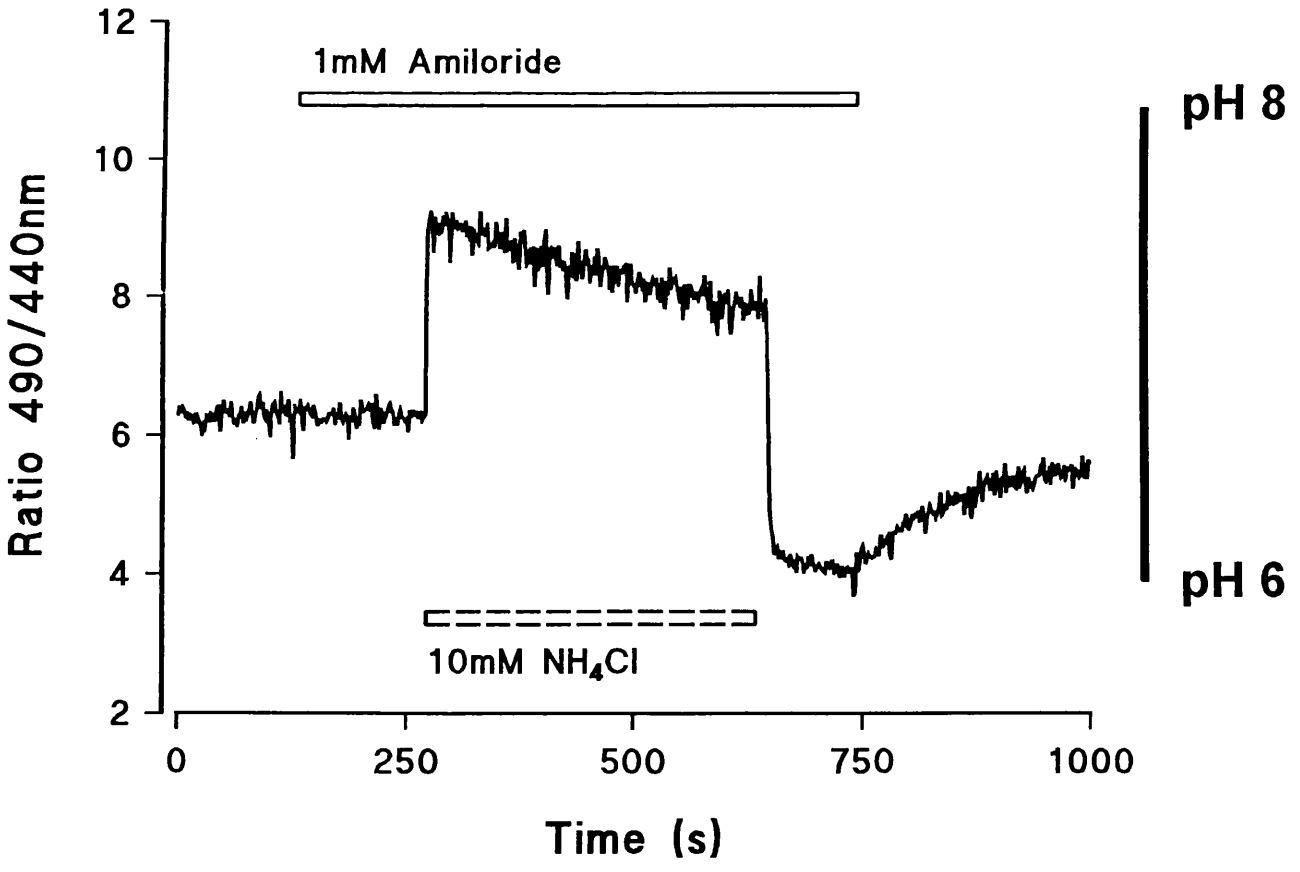
the true pH_i changes. The amiloride was demonstrated to be effective by the pH_i recovery seen on its removal ($2.5x10^{-3}$ units.s^{-1}).

Hippocampal neurones maintained in culture have been shown to possess an amiloride insensitive Na^+/H^+ exchanger by Raley-Susman *et al* (1991). This exchanger seems to be sensitive to harmaline. However, in our investigations harmaline had unwanted effects on single wavelength records not related to pH changes that gave rise to artefactual results. This effect of harmaline has since been noted by Schweining & Boron (1994).

FIGURE 12. Top panel: Estimation of $ß_i$ in the absence of extracellular Na^+. The cell was initially sitting at a fairly stable pH_i of 6.95. On removal of Na^+ from the HEPES NaL bathing the cell there was a decline in pH_i of $1.5x10^{-3}$ units.s^{-1}. Estimates of $ß_i$ from 10mM TMA challenge were 4.5mM at pH 7.25 for addition and 6mM at pH 7.08 for removal. The removal of Na^+ was not found to affect $ß_i$ (p=0.52 n=6 pairs) or $ß_T$ (p=0.13 n=5 pairs).

Bottom panel: Estimating $ß_i$ in the presence of amiloride in order to minimise the effect of Na^+/H^+ exchange. This neocortical neurone had a resting pH_i of 7.0 that was unaffected by subsequent inclusion of 1mM amiloride in the HEPES NaL bathing medium. $ß_i$ was estimated using a 10mM NH_4Cl challenge. For addition the pH_i change was 0.6 units and $ß_i$=8.2mM. On NH_4Cl removal pH_i fell from 7.35 to 6.55 and $ß_i$ was estimated to be 10.9mM. As the Na^+/H^+ exchanger is an acid recovery mechanism we would expect $ß_i$ estimated from removal to be noticeably lower than normal if pH_i-regulation was masking the true pH_i changes. The amiloride was demonstrated to be effective by the pH_i recovery seen on its removal ($2.5x10^{-3}$ units.s^{-1}). Sodium free ß estimates were always estimated *after* measurements in sodium containing bathing media due to the often terminal pH_i decline seen in the latter conditions.


12
10
8
6
4
2
Ratio 490/440nm
10mM TMA
Na+–free
pH 8
pH 7
100
200
300
400
500
Time (s)


12
10
8
6
4
2
Ratio 490/440nm
1mM Amiloride
10mM NH4Cl
pH 8
pH 6
0
250
500
750
1000
Time (s)

2) Cl^--dependent pH_i-regulating mechanisms.

In order to block both Na^+-independent Cl^-/HCO_3^- exchange and $Na^+/Cl^-/HCO_3^-$ exchange activity, Cl^- in the bathing solution was replaced with an impermeant anion (*N*-methyl-D-glucamine). Estimating $ß_T$ in Cl^--free and Cl^--containing CO_2-HCO_3^- NaL is shown in figure 13. The value of $ß_T$ obtained in normal CO_2-HCO_3^- NaL is 5mM at pH 7.35. When $ß_T$ is estimated in the same cell bathed in Cl^--free CO_2-HCO_3^- NaL the same value of $ß_T$ is obtained at mean pH 7.35. Table 9 summarises values of $ß_T$ derived from TMA challenges in chloride-containing and chloride-free solutions for 8 experiments.

Table 9. *Estimates of $ß_T$ with and without external chloride*

Test conditions	$ß_T$	Mean pH_i
CO_2-HCO_3^- NaL	6.0±0.63	7.25±0.03
Chloride free CO_2-HCO_3^- NaL	7.5±0.79	7.25±0.06

ß values are total buffering power in mmoles/l cytoplasm, n=8 for each. The p-value when comparing the two $ß_T$ values is 0.19.

Some HCO_3^--dependent exchange mechanisms that use Cl^- have been reported to be sensitive to the stilbene derivative DIDS (Schwiening & Boron, 1994, Gaillard & Dupont, 1990). I estimated $ß_T$ in the presence of this inhibitor. Such an experiment is shown in figure 14. The results of 7 investigations are shown displayed in table 10.

Table 10. *Estimates of $ß_T$ in the presence of 250µM DIDS*

Challenge	$ß_T$	Mean pH_i
NH_4Cl addition	10.7±1.7	7.4±0.05

ß values are total buffering power in mmoles/l cytoplasm, n=7. Comparing this value of $ß_T$ with that measured at a similar pH using TMA (see table 3) yields a p-value of p=0.7. Ideally paired estimates should be compared.

FIGURE 13. Estimation of β_T in the presence and absence of extracellular chloride. This trace shows the pH_i of a cell initially bathed in standard CO_2-HCO_3^- NaL subjected to a 10mM TMA challenge. The resting pH_i prior to TMA addition was 6.9. Total buffering power estimates from pH_i changes *a* and *b* were 5mM at pH 7.35 and 5mM at pH 7.23. The same cell was then bathed in Cl^--free CO_2-HCO_3^- NaL and the 10mM TMA challenge repeated. The new resting pH_i was 6.9. Estimates of β_T from pH_i changes *c* and *d* were 5mM at pH 7.35 and 6mM at pH 7.15. Removal of extracellular Cl^-, which would prevent any Cl^-/HCO_3^- exchange from reducing the ΔpH_i seen on TMA addition, did not affect β_T estimates.

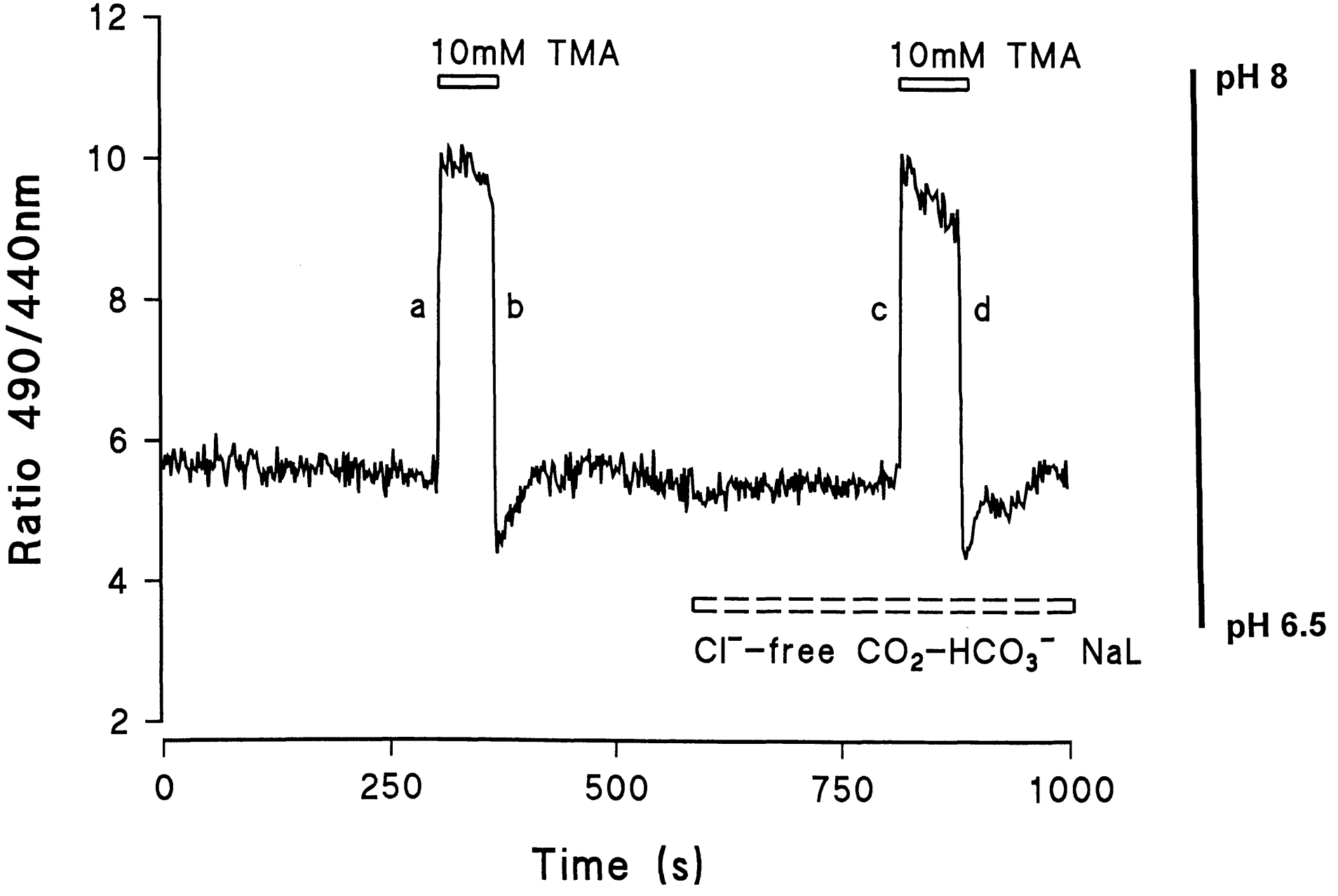
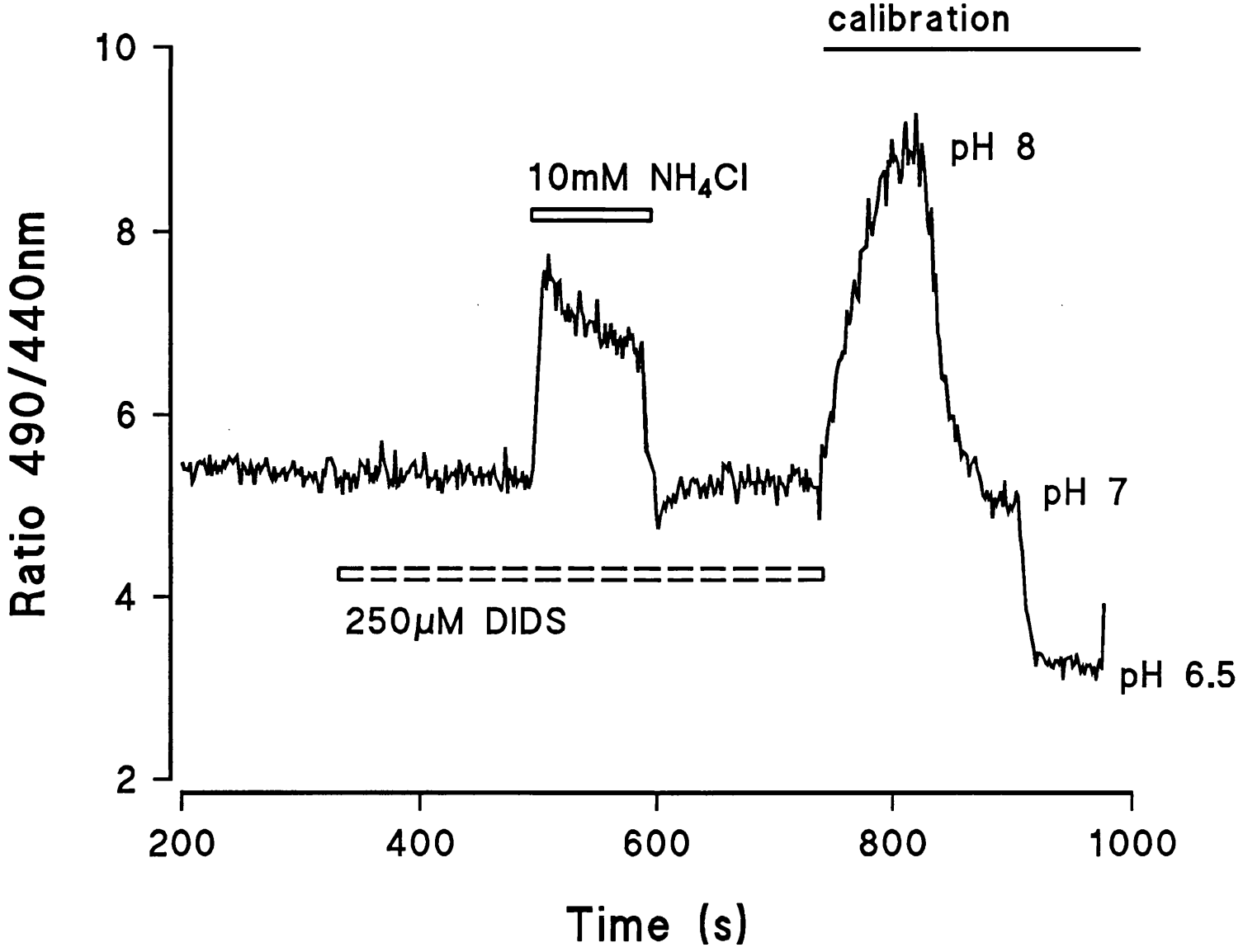

12
10
8
6
4
2
Ratio 490/440nm
10mM TMA
10mM TMA
a
b
c
d
pH 8
pH 6.5
Cl⁻–free CO₂–HCO₃⁻ NaL
0
250
500
750
1000
Time (s)

FIGURE 14. Estimation of $ß_T$ in the presence of the anion exchange inhibitor DIDS (250μM). The record shows a cell with a stable pH_i of 7.1 in CO_2-HCO_3^- NaL. The pH_i was unaffected by the inclusion of 250μM DIDS in the bathing medium. On addition of 10mM NH_4Cl the pH_i changed to 7.7 giving a $ß_T$ estimate of 6.5mM at average pH 7.4, in line with estimates of $ß_T$ made in normal conditions. ($ß_i$ estimated from NH_4Cl removal was 11.25mM at pH 7.23, though this value is not expected to be altered by the presence of DIDS).


calibration
10mM NH_4Cl
pH 8
pH 7
pH 6.5
250μM DIDS
Ratio 490/440nm
Time (s)
10
8
6
4
2
200
400
600
800
1000

Effects of acetazolamide on β_T estimates

If we consider the slowness of the uncatalysed hydration of CO_2 ($\tau_{½}$=5s at 38°C) it is perhaps suprising that Thomas (1976) has been the only author to moot the idea that carbonic anhydrase (CA) is needed if the CO_2-HCO_3^- is to be fully efficacious in proton buffering. Indeed the theoretical power of CO_2 in proton buffering is only applicable if CO_2 and H_2CO_3 are in equilibrium on either side of the membrane. In the absence of carbonic anhydrase the equilibrium states will take some time to achieve and will not fully contribute to instantaneous buffering. If central neurones contain CA then any contribution to β_T is easily testable by estimating β_T in the presence of the membrane-permeant CA inhibitor acetazolamide. I performed such experiments (see figure 15) and the results are displayed in table 11. TMA (10mM) was used to estimate β_T in normal CO_2-HCO_3^- NaL and then in the presence of 20μM acetazolamide. If CA was present and CO_2 hydration is a rate limiting step we should expect acetazolamide to decrease β_T estimates. Table 11 shows estimates of β_T from individual cells in the presence and absence of 20μM acetazolamide.

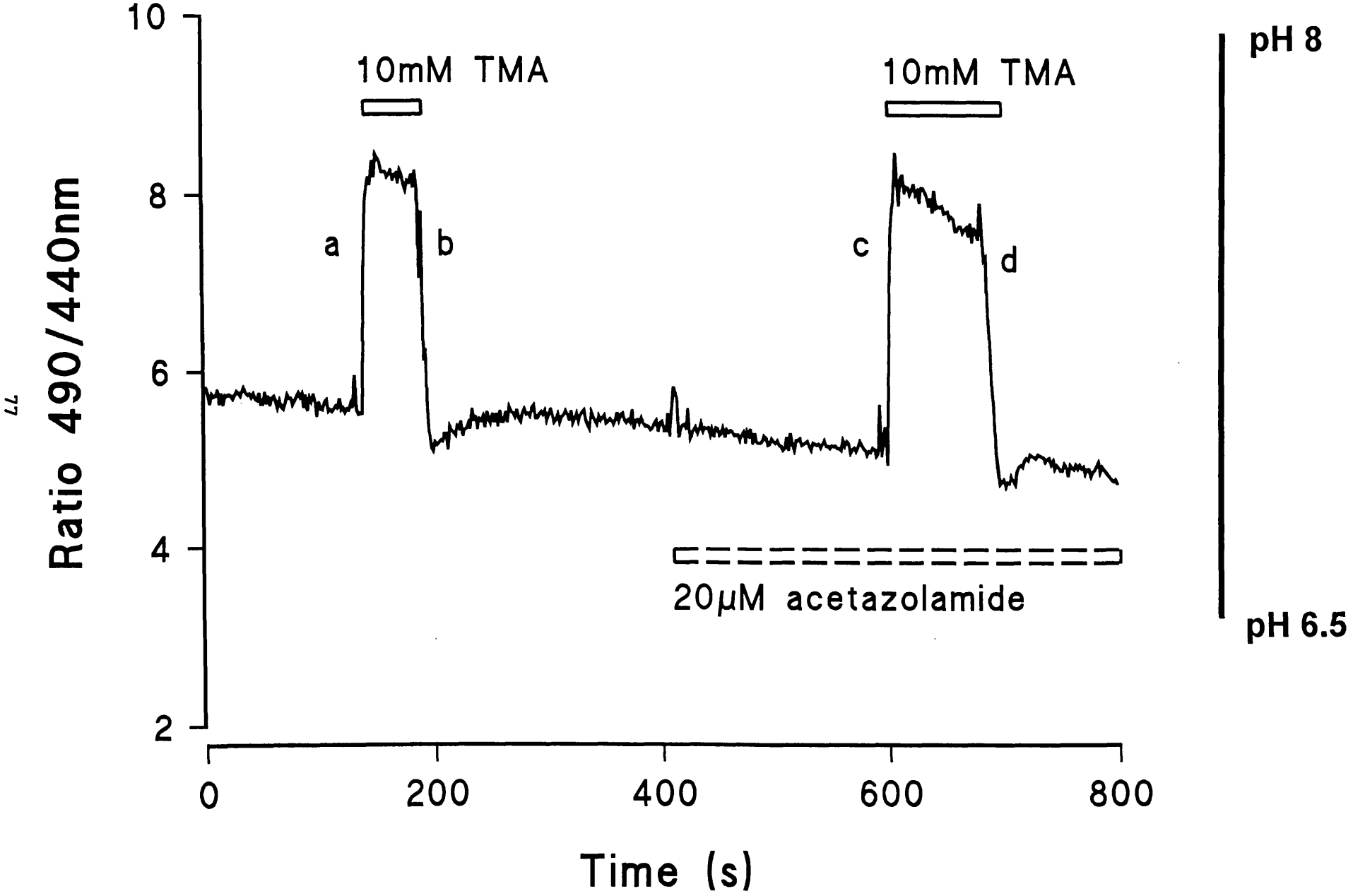
Table 11. *Effect of acetazolamide on β_T estimates.*

Test conditions	β_T	Mean pH_i
Control	8.56±1.58	7.22
Acetazolamide (20μM)	10.6±0.98	7.1

The units of β_T are mmoles/l cytoplasm; n=10 in each case.

The estimates of β_T and β_T+acetazolamide are not significantly different, p=0.29. At 5% CO_2 the mean values of pH_i given correspond to theoretical values of β_{CO2} of 25-30mmoles/l. However, the β_T obtained here are equivalent to previous estimates of intrinsic buffering power (e.g. see tables 1 and 3). This observation that total buffering power is not reduced by acetazolamide may indicate that CA is not necessary for full efficacy of the CO_2-HCO_3^- system in buffering. As the majority of cells demonstrate an apparently uncatalysed hydration of CO_2 (see p33), CA may not be present in these cells.

FIGURE 15. Effect of inhibiting intracellular carbonic anhydrase on $ß_T$ estimates. $ß_T$ estimated in this cell under normal CO_2-HCO_3^- NaL conditions (from pH_i changes *a* and *b*) was 6.1mM at pH 7.35 and 6.9mM at pH 7.3. The inclusion of the membrane permeable carbonic anhydrase inhibitor acetazolamide (20μM) in the bathing medium had no discernible effect on subsequent $ß_T$ estimates. $ß_T$ estimated in the presence of acetazolamide (from *c* and *d*), as shown in the trace, was 6.4mM at pH 7.3 and 8mM at pH 7.2.


10
8
6
4
2
Ratio 490/440nm
10mM TMA
10mM TMA
a
b
c
d
20μM acetazolamide
pH 8
pH 6.5
0
200
400
600
800
Time (s)

The relationship between ß and pH_i

The buffering power of an individual buffer is inherently pH-dependent (Koppel & Spiro, 1914; see Roos & Boron, 1980). The shape of a plot of intracellular proton buffering versus pH_i is due to a number of buffers and will depend on the individual pK_a values and the concentrations of the individual buffers however. The inverse relationship between $ß_i$ and pH_i over the physiological range has been reported for several tissues (Szatkowski & Thomas, 1989, Vaughan-Jones & Wu, 1990, Goldsmith & Hilton, 1992) but not in CNS neurones. Similarly there have been studies showing the increase in $ß_T$ with pH_i (Szatkowski, 1989).

The most satisfactory method for investigating these relationships is to employ small stepwise changes in extracellular acid or base concentration, and TMA has proved the easiest to use in this respect. TMA is the challenge of choice as NH_4Cl will give a pH_i shift that varies with duration of application and butyric acid requires inhibition of monocarboxylate ion transport. The need to minimise the pH_i range over which the measurement is taken is of paramount importance. Estimates of ß are made from each of the step changes in pH_i. The step increases in extracellular [TMA] allow estimation of ß values at progressively higher pH values. This is illustrated in figure 16. Care must be taken in calculations when estimating buffering power when the initial intracellular $[TMAH^+]$ is not zero (as will be the case when increasing [TMA] from 1 to 5.5mM for example). In this case the alkaline load will not be equal to the intracellular concentration of $TMAH^+$ at peak alkaline pH as some $TMAH^+$ is already present in the cell. This must be taken into account. The results obtained from this technique are summarised in figure 17.

Both $ß_i$ and $ß_T$ were found to increase with decreasing pH_i. The effect was most marked with $ß_i$. Intrinsic ß increased by about 60% over the pH range 7.7-6.7. The less steep relationship for $ß_T$ shows a minor contribution from $ß_{CO2}$ at more alkaline pH_i values (ie as $[HCO_3^-]_i$ rises). The large increase in $ß_T$ expected at more alkaline values is not seen.

FIGURE 16. The relationship between buffering power and pH_i. Using sequential increases in weak base concentration (TMA) the relationship between $ß_i$ and pH_i can be investigated in a single cell. In this trace extracellular [TMA] was increased from 0mM to 1, 5.5 and 12.75mM. The estimates of $ß_i$ made from the pH_i changes *a*, *b* and *c* were 7.1, 5.2 and 4.7mM at respective mean pH values of 7.1, 7.38, 7.58. $ß_i$ estimated from TMA removal (*d*) was 5.25 at pH 7.23. This is a clear demonstration of $ß_i$ increasing with decreasing pH_i.

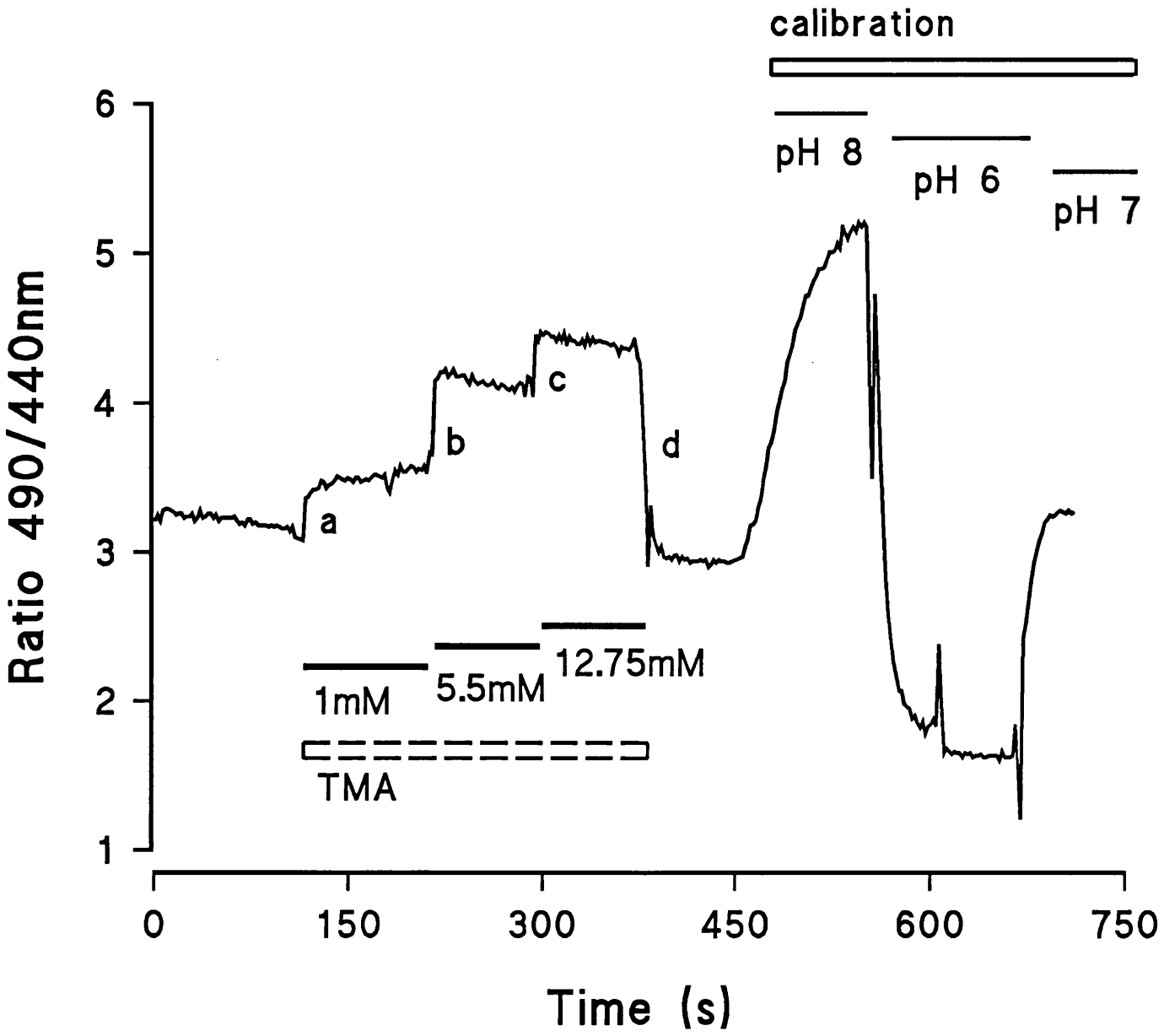
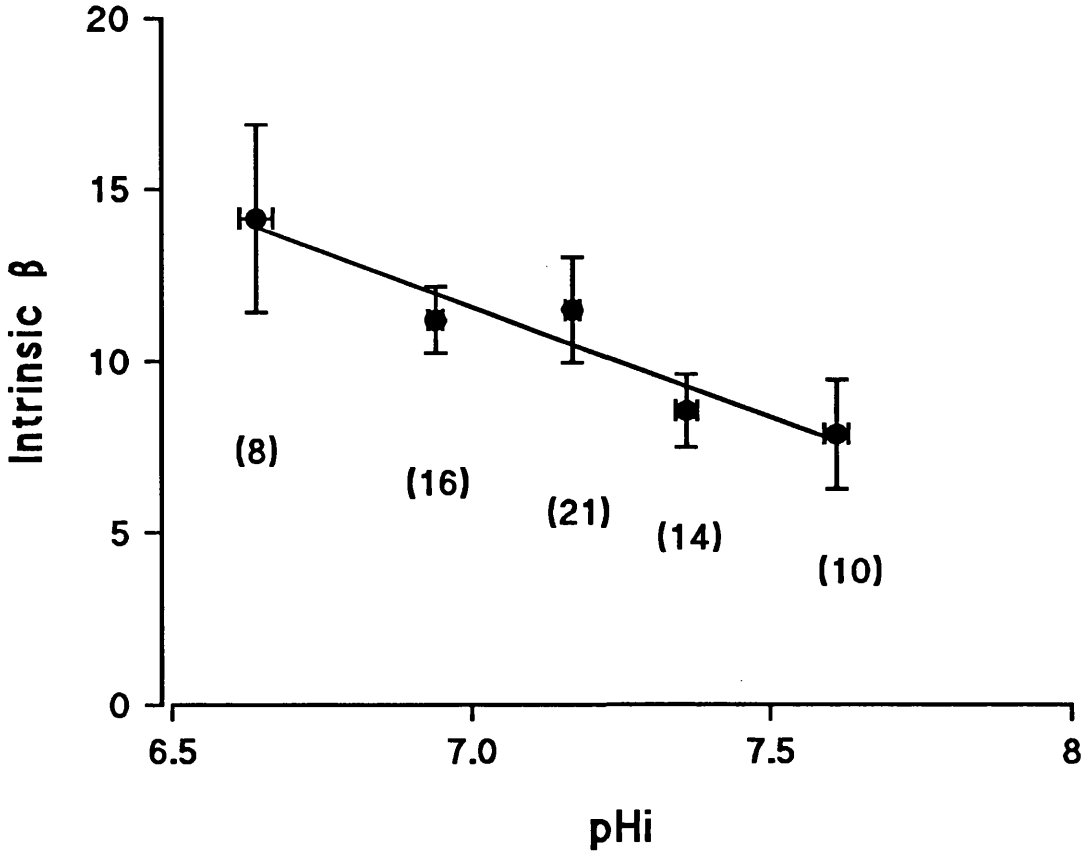
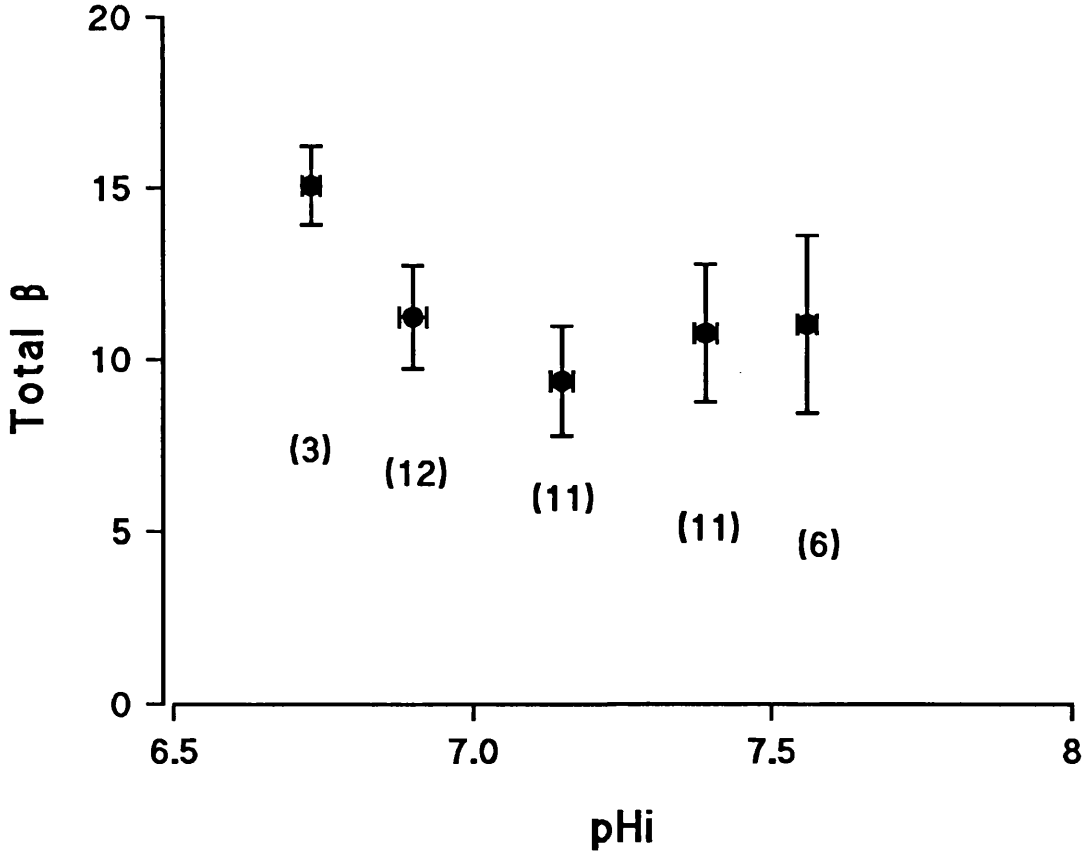

calibration
pH 8
pH 6
pH 7
Ratio 490/440nm
6
5
4
3
2
1
a
b
c
d
1mM
5.5mM
12.75mM
TMA
0
150
300
450
600
750
Time (s)

FIGURE 17. The relationship between ß and pH_i. The values plotted in the two graphs are mean buffering power (intrinsic or total) ±s.e.m. for mean pH_i values (±s.e.m.). The figures in brackets are the number of observations for each point. In the top trace the estimates of intrinsic buffering power increase with decreasing pH_i. The linear regression fitted shows an approximate 60% increase in $ß_i$ as pH falls from 7.6 to 6.6 (the line has a correlation coefficient of -0.95 and the p-value testing the line fit is 0.01. The equation of the line is y=-6.44x + 56.7).

The the lower plot shows that $ß_T$ also increases with falling pH. The relationship is slightly less steep though and this may represent an increasing contribution from the CO_2-HCO_3^- system as pH_i rises. However, the dramatic increase in $ß_T$ with increasing pH_i expected on the basis of an open buffer system is not seen.


20
15
10
5
0
Intrinsic β
(8)
(16)
(21)
(14)
(10)
6.5
7.0
7.5
8
pHi


20
15
10
5
0
Total β
(3)
(12)
(11)
(11)
(6)
6.5
7.0
7.5
8
pHi

RESULTS II: Effect of glutamate on intracellular pH

Effect of L-glutamate on intraneuronal pH

Application of 25-50μM L-glutamate (2x250ms pulses or 30s bath application) to individual neocortical or hippocampal neurones, demonstrating a stable resting pH_i in HEPES NaL, elicited an intraneuronal acidification (8/11 cells). This pH shift was generally rapid in onset and was invariably maintained after L-glutamate had been washed off (as demonstrated in figure 18). The acidification was usually characterised by the cell having a new stable resting pH_i, acid relative to the original resting pH. The extent of the pH_i shift was 0.125±0.025 units (n=6) from a resting pH_i of 7.2. The time taken to attain a new stable resting pH_i after glutamate application varied widely, the range being 30s-360s.

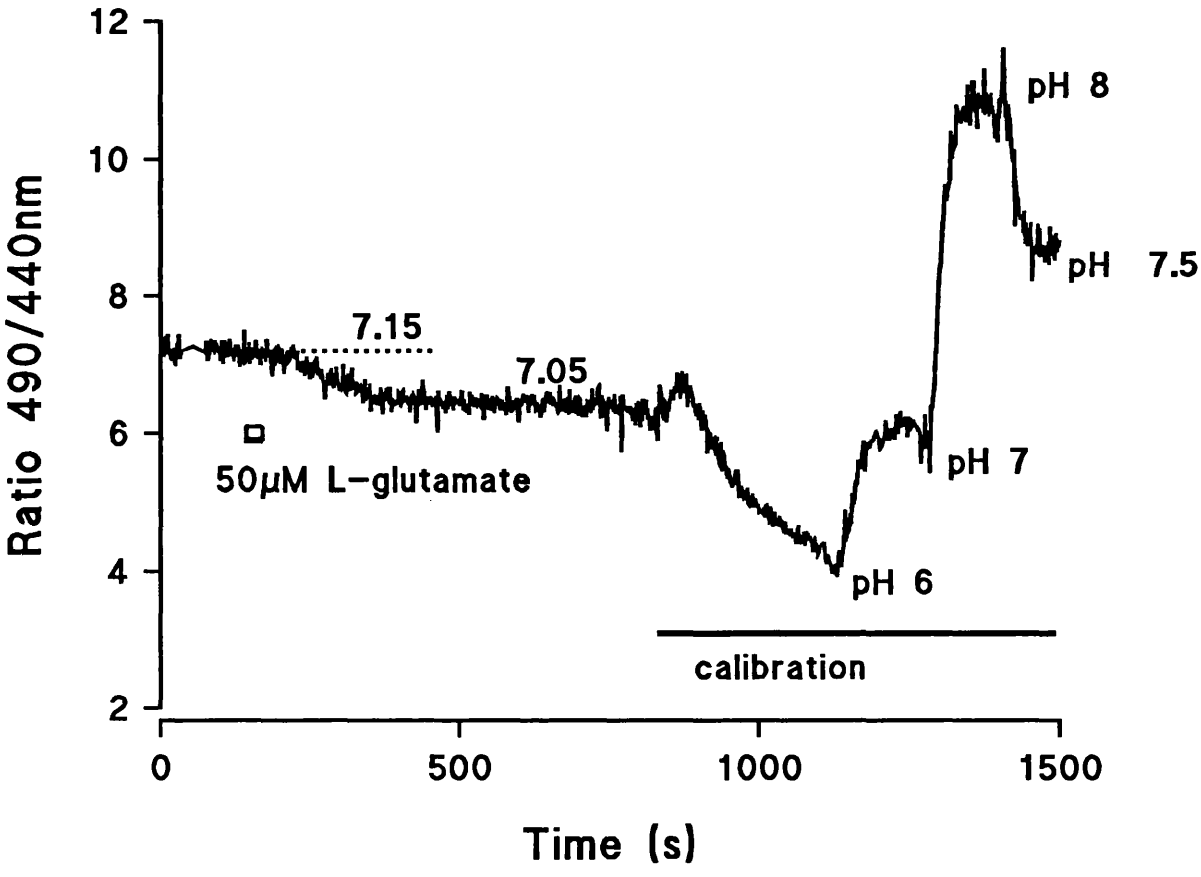
Repeating this protocol in CO_2-HCO_3^- NaL provided different results. L-Glutamate challenges evoked a rapid onset intracellular acidification (3/5 cells) that was continuous and maintained. This is shown in the lower panel of figure 18. The rate of decline of pH_i was $1.68x10^{-4}$ units.s^{-1} (s.e.m.= $3.6x10^{-5}$) from a mean resting pH_i of 6.8. The pH_i continued to decline over the course of the experiments (longest duration= 40 minutes).

Effect of tACPD on intraneuronal pH

The observed action of glutamate on neuronal pH_i was presumably mediated either by glutamate receptors (ionotropic or metabotropic) or a glutamate transporter (Bouvier *et al.* 1992). The sustained nature of the pH_i shifts suggested that glutamate receptors had been activated, possibly metabotropic glutamate receptors (mGluR). This was tested by applying a specific metabotropic glutamate receptor agonist (tACPD) to individual cells. The agonist tACPD (25-50μM) when applied via pressure ejection or bathing solution provoked intraneuronal pH shifts. These shifts were qualitatively similar but quantitatively different to those elicited by L-glutamate application (see figure 19). In HEPES NaL a 30s bath application of 25-50μM tACPD

FIGURE 18. The effect of L-glutamate on intraneuronal pH. Application of 50μM L-glutamate (2x 250ms pressure ejection pulses) to a neurone bathed in HEPES NaL (top panel) causes an intracellular acidification. In this case the pH_i fell by 0.1 units from a resting value of 7.15. The step acidification was still maintained 10 minutes after the glutamate was washed off.

When this protocol is carried out in CO_2-HCO_3^- NaL a steady decline in pH_i ensues (lower panel). This decline continues for the course of the experiment (15 minutes).


Ratio 490/440nm
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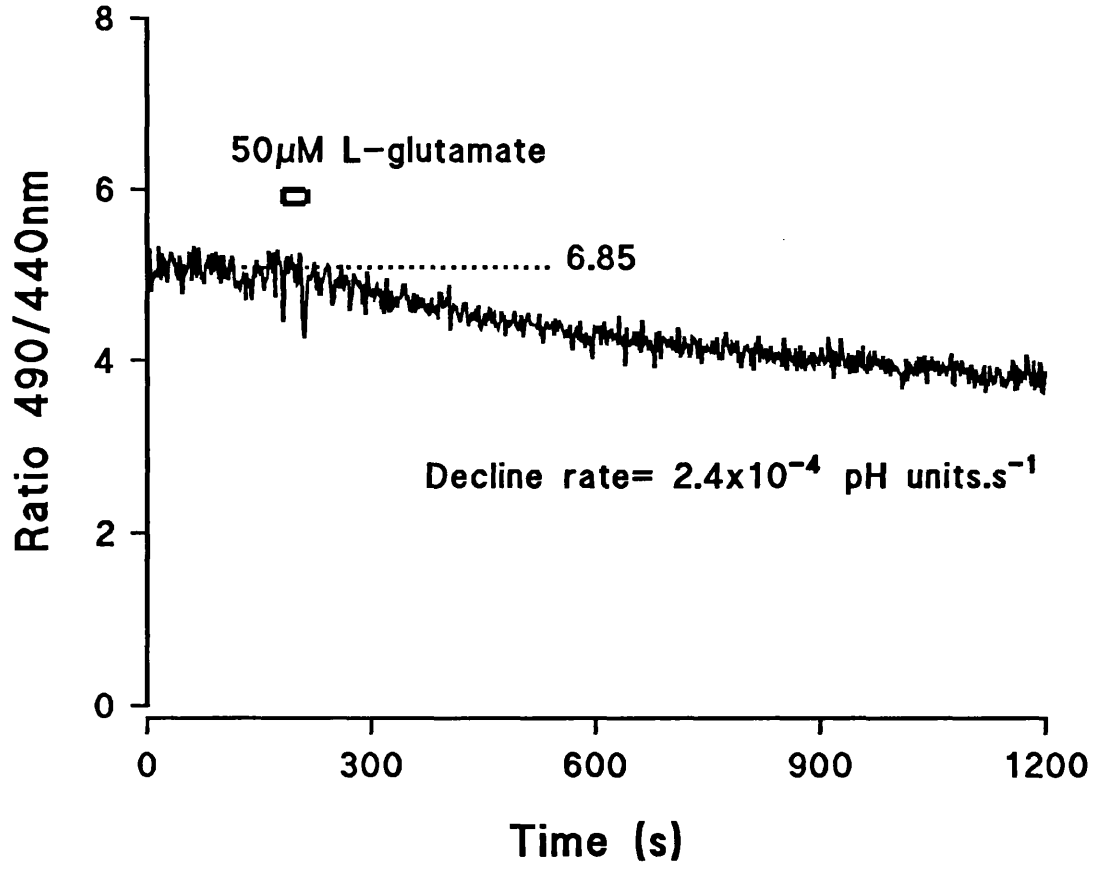
10
8
6
4
2
0
500
1000
1500
Time (s)
pH 8
pH 7.5
pH 7
pH 6
7.15
7.05
50μM L-glutamate
calibration


Ratio 490/440nm
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6
4
2
0
300
600
900
1200
Time (s)
50μM L-glutamate
6.85
Decline rate= 2.4x10^{-4} pH units.s^{-1}

caused a mean fall in baseline pH_i of 0.24 units (9/15 cells) from a mean resting pH_i of 7.2. In CO_2-HCO_3 NaL a matching application of tACPD generally caused a continuous decline in pH_i of $3.3x10^{-4}$ units.s^{-1} (s.e.m.= $9.1x10^{-5}$), 6/10 cells, from a mean resting pH_i of 7.0 . These changes in pH_i were sustained for the duration of the experiment (longest trace= 40 minutes). Table 12 shows comparison of the pH_i changes induced by L-glutamate and tACPD.

Table 12. *The effects of glutamate and tACPD on neuronal pH_i*

Test substance	**CO_2-HCO_3^- NaL**	**HEPES NaL**
L-glutamate (25-50μM)	$1.68x10^{-4}$ units.s^{-1} (n=3)	-0.125 (n=6)
tACPD (25-50μM)	$3.3x10^{-4}$ units.s^{-1} (n=5)	-0.24 (n=9)

The figures given show the rate of decline of pH_i in CO_2-HCO_3^- NaL and the magnitude of the shift in pH_i in HEPES NaL. S.e.m. values give in text.

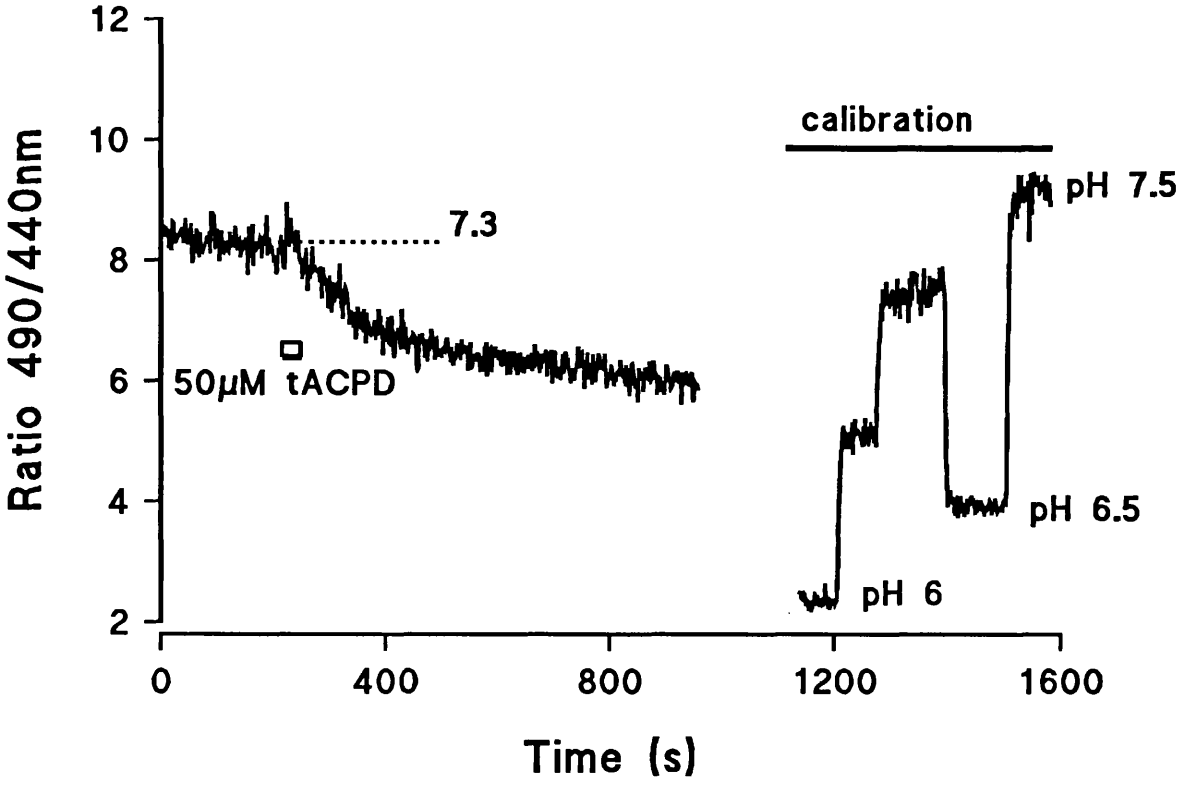
Alternatively, the results can be presented as rate of pH_i decline at set intervals following the initial rapid response. These results are presented in table 13. It is apparent that after the initial pH change (0-100s) both glutamate and tACPD tend to cause a steady decline in pH_i in HCO_3^- NaL whereas in HEPES buffer the rate of pH_i change slows by 600s. With glutamate the rate of decline is not significantly different at (100-300s) or (300-600s) in HCO_3^- NaL (p=0.46). At 300-600s, however, the decline is considerably slower in HEPES NaL compared to HCO_3^- NaL, but not quite significant (p=0.09). Similarly the rate of decline evoked by tACPD in HCO_3^- NaL is no different at 100-300s or 300-600s (p=0.9). However, in HEPES NaL the decline evoked by tACPD is significantly slower at 300-600s compared to 100-300s (p=0.04).

Table 13. *Rates of pH_i decline evoked by glutamate and tACPD.*

Time after agonist application:	0-100s	100-300s	300-600s
Glutamate (HCO_3^- NaL) n=3	**8×10^{-4}** $\pm 3.3 \times 10^{-4}$	**3.3×10^{-4}** $\pm 8.3 \times 10^{-5}$	**2.2×10^{-4}** $\pm 5.4 \times 10^{-5}$
Glutamate (HEPES NaL) n=6	**8×10^{-4}** $\pm 2.6 \times 10^{-4}$	**2.5×10^{-4}** $\pm 9 \times 10^{-5}$	**0.7×10^{-4}** $\pm 3.4 \times 10^{-5}$
tACPD (HCO_3^- NaL) n=6	**2×10^{-3}** $\pm 9 \times 10^{-4}$	**2.9×10^{-4}** $\pm 1.1 \times 10^{-4}$	**3×10^{-4}** $\pm 9 \times 10^{-5}$
tACPD (HEPES NaL) n=10	**5.5×10^{-4}** $\pm 5 \times 10^{-5}$	**3.3×10^{-4}** $\pm 7.9 \times 10^{-5}$	**1.1×10^{-4}** $\pm 5.6 \times 10^{-5}$

Agonist doses are 25-50μM and rates of decline are in 'pH units'.s^{-1} ± s.e.m. The pH_i decline at 100-300s evoked by tACPD in HCO_3 NaL is not statistically different from that in HEPES NaL (p=0.96). However, by 300-600s the decline in HEPES NaL is much slower (p=0.09, not quite significant). Similar results were obtained with glutamate in the two buffers, with the difference between HCO_3 NaL and HEPES NaL not being significant at 100-300s (p=0.66) but significant at 300-600s (p=0.04).

FIGURE 19. The effect of tACPD on intraneuronal pH. The top panel shows the effect of 50μM tACPD applied by pressure ejection to a neurone bathed in HEPES NaL. The neurone underwent a large acidification (0.3 units). The lower panel shows the continual decline in pH_i seen with tACPD application in CO_2-HCO_3^- NaL. The individual calibrations of both records are shown.


Ratio 490/440nm
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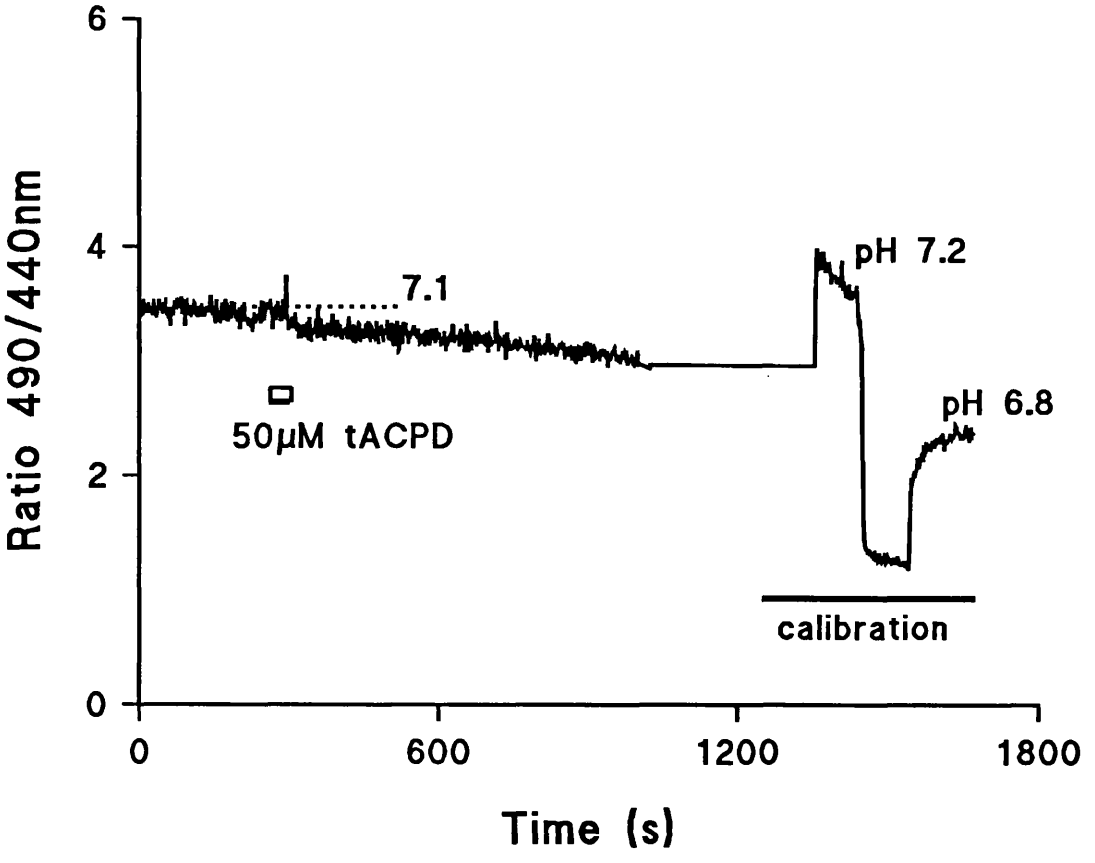
10
8
6
4
2
0
400
800
1200
1600
Time (s)
calibration
pH 7.5
pH 6.5
pH 6
7.3
50μM tACPD


Ratio 490/440nm
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2
0
0
600
1200
1800
Time (s)
pH 7.2
pH 6.8
7.1
50μM tACPD
calibration

Effect of tACPD on pH_i regulating mechanisms

In an initial attempt to establish how activation of mGluRs leads to modulation of neuronal pH_i the possibility of a final action on the normal pH_i regulating mechanisms was considered. It is possible that pH_i could fall due to a stimulation of cell metabolism, a block of carboxylate transport or a block of some other pH_i regulating mechanism. This latter possibility would prove the most easy to investigate. Although pH_i regulating mechanisms may be active at rest the most satisfactory way of investigating any such effects is to displace pH_i and observe the active exchangers attempt to restore resting pH_i (Thomas, 1984). Cells can be acid loaded by the NH_4Cl prepulse technique detailed in Results I. Alkaline loading cells is more problematic as the removal of CO_2 from the bathing medium (which elicits an intracellular alkalinisation for the reasons given above in Results 1; Figure 4) precludes the action of the only exchanger known to contribute to alkaline recovery (the Cl^-/HCO_3^- exchanger). For this reason we explored the action of tACPD on acid recovery mechanisms.

Acid loading in HEPES NaL and CO_2-HCO_3^- NaL.

Cells acid loaded by NH_4Cl challenge in HEPES NaL showed a slow rate of pH_i recovery ($1.2x10^{-3}$ units.s^{-1}, s.e.m.= 0.0003 n=5, half-time= 57.8±8.6s). In the absence of CO_2/HCO_3^- this recovery is largely mediated by Na^+/H^+ exchange. Bath application of 25-50μM tACPD during this recovery stage did not appear to have any effect on the rate of recovery (5 cells). This is illustrated in figure 20. However, in CO_2-HCO_3^- NaL acid recovery was halted or dramatically slowed by tACPD challenges (6/8 cells). This is shown in table 14. Under these conditions pH_i recovery will be mediated by bicarbonate dependent mechanisms also and recovery will therefore tend to be much faster ($2.6x10^{-3}$ units.s^{-1}, s.e.m.= 0.001 n=5, half-time= 28±5.5s; two-tailed t-test p-value for half time recovery in HEPES NaL and HCO_3 NaL is 0.02).

Table 14. *Effect of tACPD on acid recovery half times in HCO_3^- NaL.*

Normal recovery before ACPD challenge	Recovery in 25-50μM tACPD
25	∞
28	∞
19	∞
59	∞
29	∞
17	∞
39	38
8.5	7

Results are paired and show recovery half times (s) in single cells before and after an application of tACPD. The failure of recovery in 6/8 cells is not statistically significant using the paired sample sign-test, the probability of $x \geq 6$ for n=8 and p=0.5 is 0.144, which is not significant at 5% level.

In order to ascertain precisely the type of pH_i regulating mechanism being affected by tACPD application the Na^+/H^+ exchanger was specifically inhibited using an amiloride analogue. Neurones acid loaded in CO_2-HCO_3^- NaL whilst in the presence of 10μM ethyl isopropylamiloride (EIPA) showed a pH_i recovery presumably accomplished solely by bicarbonate dependent mechanisms (rate= 2.6×10^{-3} units.s^{-1}, n=8, half-time=21.2±2.7s). A challenge of 50μM tACPD stopped pH_i recovery in 3/5 cells acid loaded in this way (see figure 21). N.B. although acid recovery rates appear to be no different in the presence and absence of EIPA (p=0.24), Aickin (1994a) has suggested that the Na^+/H^+ exchanger is only invoked when HCO_3^- exchangers are not working.

The apparent irreversible action on pH_i of mGluR activation, coupled with the failure of some cells to show any pH_i recovery after acid loading required demonstration of both normal and inhibited acid recovery in the same cell. This was achieved by acid loading a cell in CO_2-HCO_3^- NaL, observing normal acid recovery, and subsequently acid loading the same cell but this time washing into a CO_2-HCO_3^- NaL containing 50μM tACPD (see figure 21). Of the neurones subjected to this protocol 3/4 showed inhibition of acid

recovery (see Table 15) in the presence of 50μM tACPD. As a control some cells were exposed to two consecutive acid loads in normal CO_2-HCO_3^- buffer and showed normal recovery from both loads.

Table 15. Effect of tACPD on acid recovery half times in HCO_3^- NaL.

First recovery	Recovery in 25-50μM tACPD
45	225
21	∞
74	246
35	40

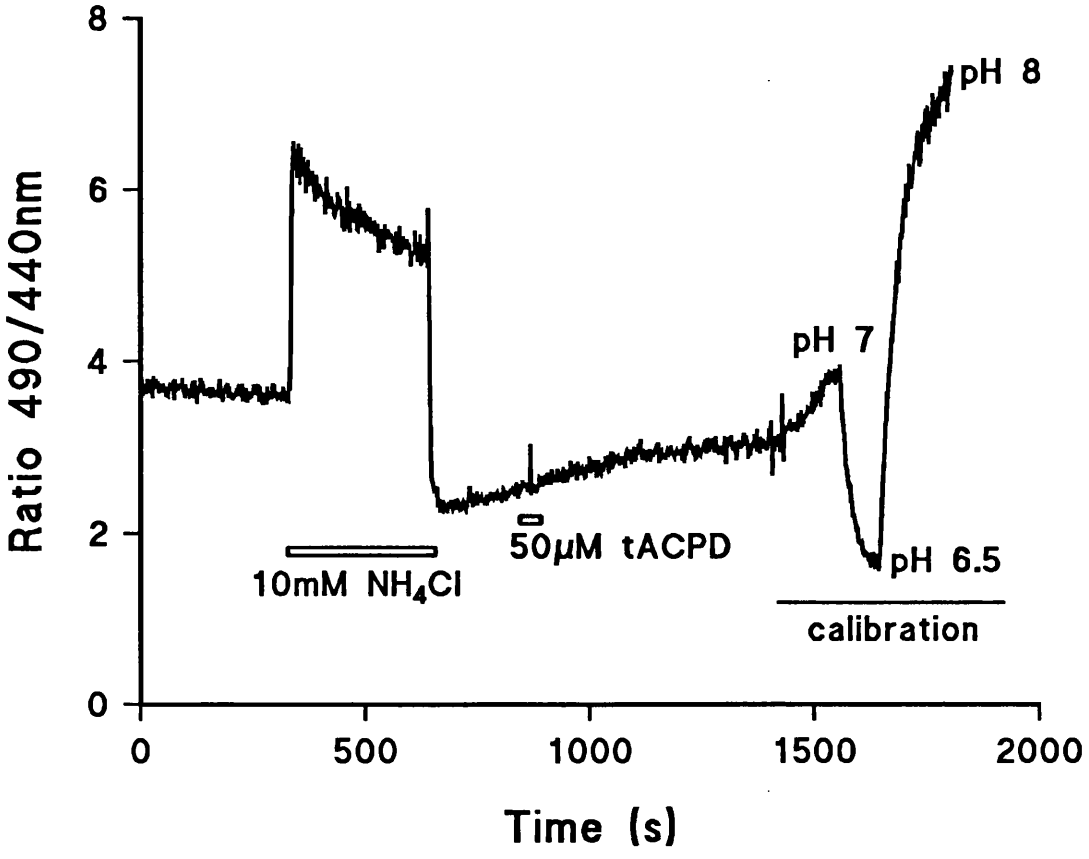
Results are paired and recoveries are expressed as half times (s) (n=4). As the sample is small 4 of 4 cells showing a slowed recovery is not significant tested with a paired sample sign-test (for x=4 when n=4 and p=0.5 is 0.062, not significant).

Involvement of G-proteins

In further experiments to elucidate the observed action of tACPD we investigated a role for G-proteins. Most of the effects of mGluR activation have been reported as G-protein mediated e.g. Manzoni *et al*, 1992, Martin *et al*, 1993 and Birrell & Marcoux, 1993. Cells were incubated for ≈24 hours with 230ng/ml pertussis toxin. This should effectively inhibit processes that depend on G_i and G_o proteins (Ui *et al*, 1985). On repeating the acid loading protocol in CO_2-HCO_3^- buffer we found that 50μM tACPD had no effect on acid recovery in 5/8 cells (c.f. inhibiting 6/8 recoveries in untreated cells though not statistically significant using the sign-test), suggesting that the mode of action of tACPD is pertussis toxin sensitive, (in the remaining three cells the recovery was unmeasureable after the tACPD challenge).

FIGURE 20. The effect of tACPD on pH_i recovery following an acid load. The top trace in this figure shows a cell bathed in HEPES NaL, with a stable resting pH_i of about 6.9, subjected to the NH_4Cl acid-loading procedure. On removal of NH_4Cl from the bathing medium the cell underwent an acidification. The subsequent pH_i recovery was not inhibited by 50μM tACPD.

The lower panel shows the different effect of tACPD on pH_i-recovery in CO_2-HCO_3^- NaL. The cell shown had a stable resting pH_i of about 7.0 and showed the usual pH_i changes when challenged with 10mM NH_4Cl. However, the recovery ceased when 50μM tACPD was added to the bathing medium. Also no subsequent acid-recovery occured.


Ratio 490/440nm
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6
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2
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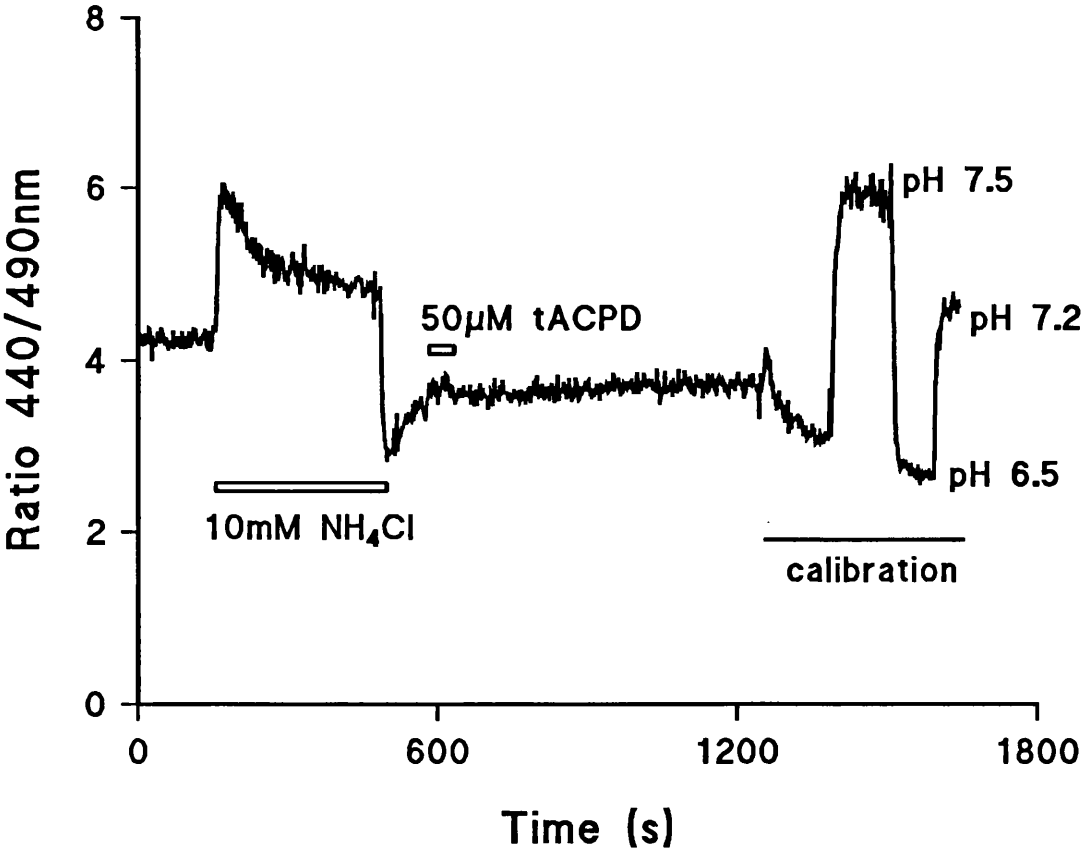
0
500
1000
1500
2000
Time (s)
pH 8
pH 7
pH 6.5
50μM tACPD
10mM NH4Cl
calibration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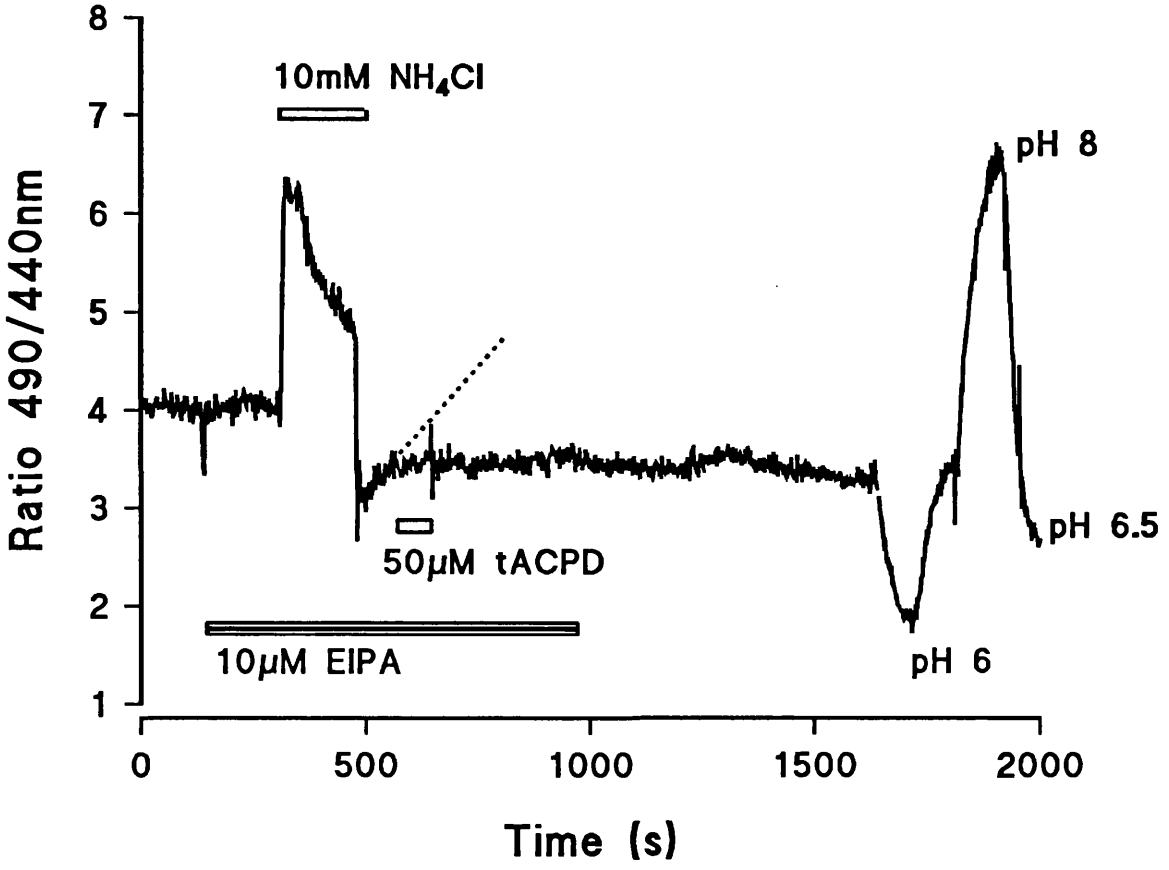
4
2
0
0
600
1200
1800
Time (s)
pH 7.5
pH 7.2
pH 6.5
50μM tACPD
10mM NH4Cl
calibration

FIGURE 21. The effect of tACPD on non-Na^+/H^+ pH_i-recovery. The top trace shows a cell bathed in CO_2-HCO_3^- NaL containing the specific Na^+/H^+ exchange inhibitor EIPA. After the cell was acid loaded using NH_4Cl the pH_i-recovery seen was mediated by non-Na^+/H^+ recovery mechanisms. Application of 50μM tACPD appeared to block any further pH_i-recovery.

The lower panel in this figure shows the effect of tACPD on normal acid pH_i-recovery mechanisms. The cell shown was bathed in CO_2-HCO_3^- NaL and had a resting pH_i of 7.05. The cell was challenged with 10mM NH_4Cl and showed normal pH_i-recovery in the face of the usual acid load. However, when the NH_4Cl challenge was repeated, this time washing into CO_2-HCO_3^- NaL that contained 50μM tACPD no attempt at pH_i-recovery by the cell was apparent.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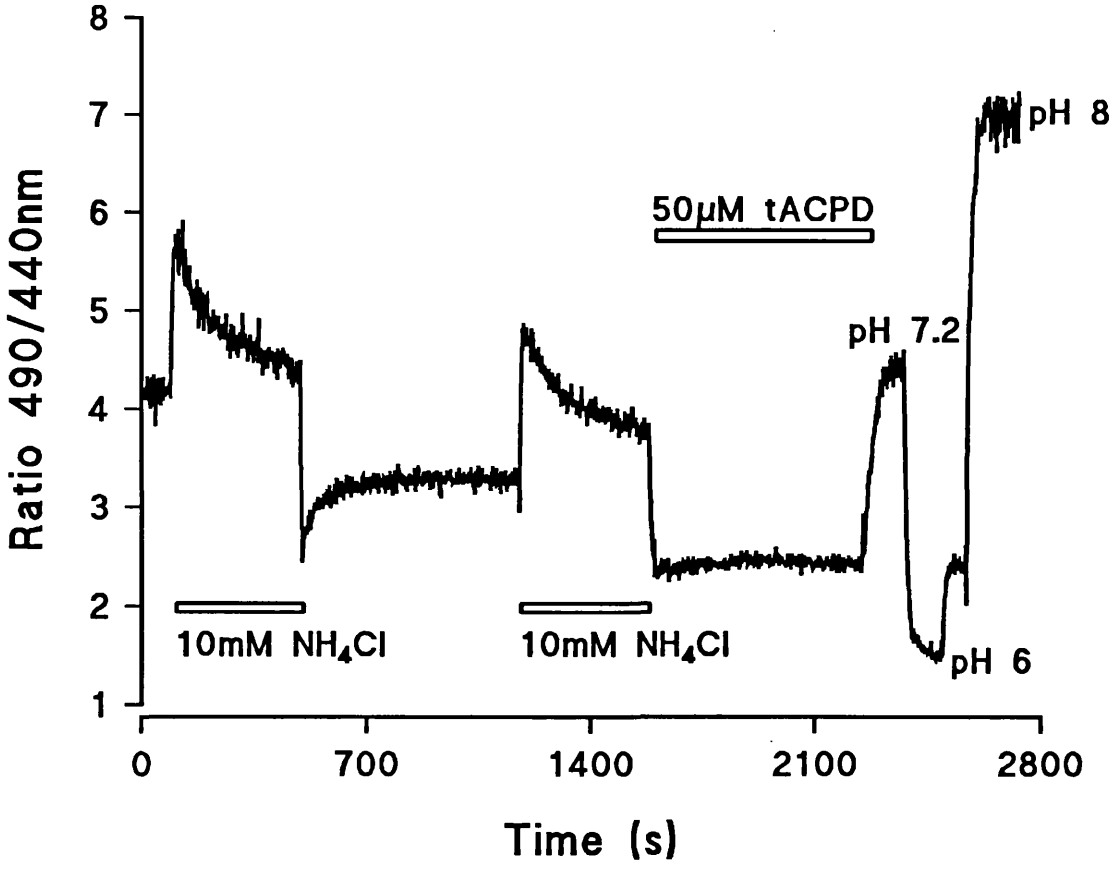
pH 8
pH 6.5
50μM tACPD
10μM EIPA
pH 6
Ratio 490/440nm
Time (s)
0
500
1000
1500
2000
1
2
3
4
5
6
7
8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pH 7.2
10mM NH4Cl
10mM NH4Cl
pH 6
Ratio 490/440nm
Time (s)
0
700
1400
2100
2800
1
2
3
4
5
6
7
8

Effect of metabotropic receptor antagonists on tACPD action

We attempted to block the action of tACPD on HCO_3^--dependent pH_i recovery mechanisms with the antagonists L-aminophosphonopropionic acid (LAP3) and α-methyl carboxyphenylglycine (MCPG). LAP3 has previously been used as a specific metabotropic receptor antagonist but has low potency. It has been shown to block the Ca^{++} mobilizing effect of tACPD in rat cerebellar neurones at concentrations of 1mM (Irving *et al*, 1990). Unfortunately LAP3 will act like any other weak acid and permeate the cell membrane to some extent. At the high concentrations required this will have detectable effects on pH_i. In fact application of 500μM LAP3 evoked a step intracellular acidification as demonstrated in figure 22. This acidification vanished on removal of LAP3 from the bathing medium. Consequently LAP3 was considered unsuitable as an antagonist for the action of tACPD on pH_iin experiments with BCECF.

Another putative metabotropic receptor antagonist, α-methyl carboxyphenylglycine (MCPG), has been shown to be a competitive antagonist at lower concentrations (250μM, Eaton *et al*, 1993, Thomsen *et al*, 1994). This antagonist does not disturb pH_i *per se.* Used at concentrations of 250-500μM MCPG appeared to block the effect of tACPD on resting pH_i, when the antagonist MCPG was co-applied with tACPD (50μM) *or* when cells were incubated with MCPG for 5 minutes an challenged with tACPD. However, on washing out MCPG most cells showed a subsequent decline in pH_i (see figure 23). The mean rate of decline of pH_i was $3.3x10^{-4}$units.s^{-1} $\pm 2.3x10^{-5}$ (n=3).

The ability of MCPG to antagonise the blocking action of tACPD on acid recovery was also explored. Neurones were acid loaded using the NH_4Cl prepulse technique and then washed into a normal CO_2-HCO_3^- NaL bathing solution where they showed normal acid recovery. The cells were subsequently acid loaded again but this time washed into CO_2-HCO_3^- NaL containing 50μM tACPD and 500μM MCPG. An example of this is shown in figure 24. Three of four cells showed normal acid recovery from this second challenge (see table 16) whereas 3/4 cells washed into CO_2-HCO_3^- NaL containing just tACPD (no antagonist) showed *no* recovery. (This values are not statistically significantly different when tested using the sign test)

Table 16. *The effect of tACPD on half times of acid recovery in presence of MCPG.*

Control recovery	Recovery in tACPD and 500μM MCPG
56	119
76	81
72	76
85	87

Results are paired and recoveries are expressed as half times (s).

FIGURE 22. The effect of L-amino phosphonopropionic acid (LAP3) on intracellular pH. The cell had a stable resting pH_i of about 7.05. When 1mM LAP3 was included in the bathing medium the intracellular pH underwent a step acidification, similar in nature to those seen with other weak acid challenges. The acidification (to pH 6.85) was partially reversed when the LAP3 was removed from the bathing medium.

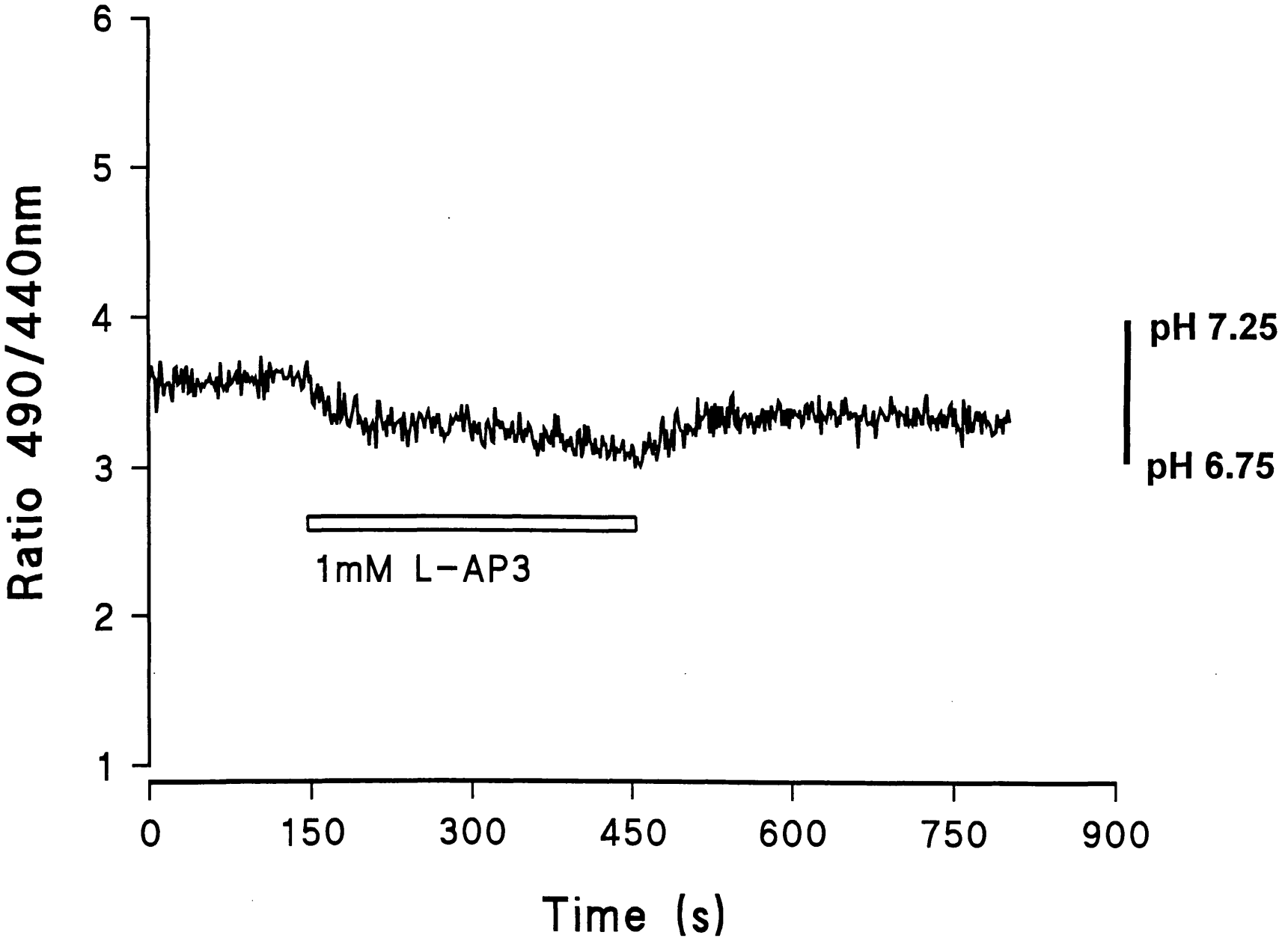

Ratio 490/440nm
6
5
4
3
2
1
0
150
300
450
600
750
900
Time (s)
1mM L-AP3
pH 7.25
pH 6.75

FIGURE 23. The effect of α-methyl carboxyphenylglycine (MCPG) and tACPD on intracellular pH. Application of 500μM MCPG to the CO_2-HCO_3^- NaL bathing this cell elicited a slight alkalinisation (0.05 units). A challenge of tACPD (50μM) appears to have no effect on pH_i. However, on washout of the MCPG a sustained decline in pH_i occurs ($3.75x10^{-3}$ units.s^{-1}). The cause of this decline in pHi is unknown. It is possible that the MCPG is having partial agonist activity. However, MCPG still only causes a pHi decline on washout.

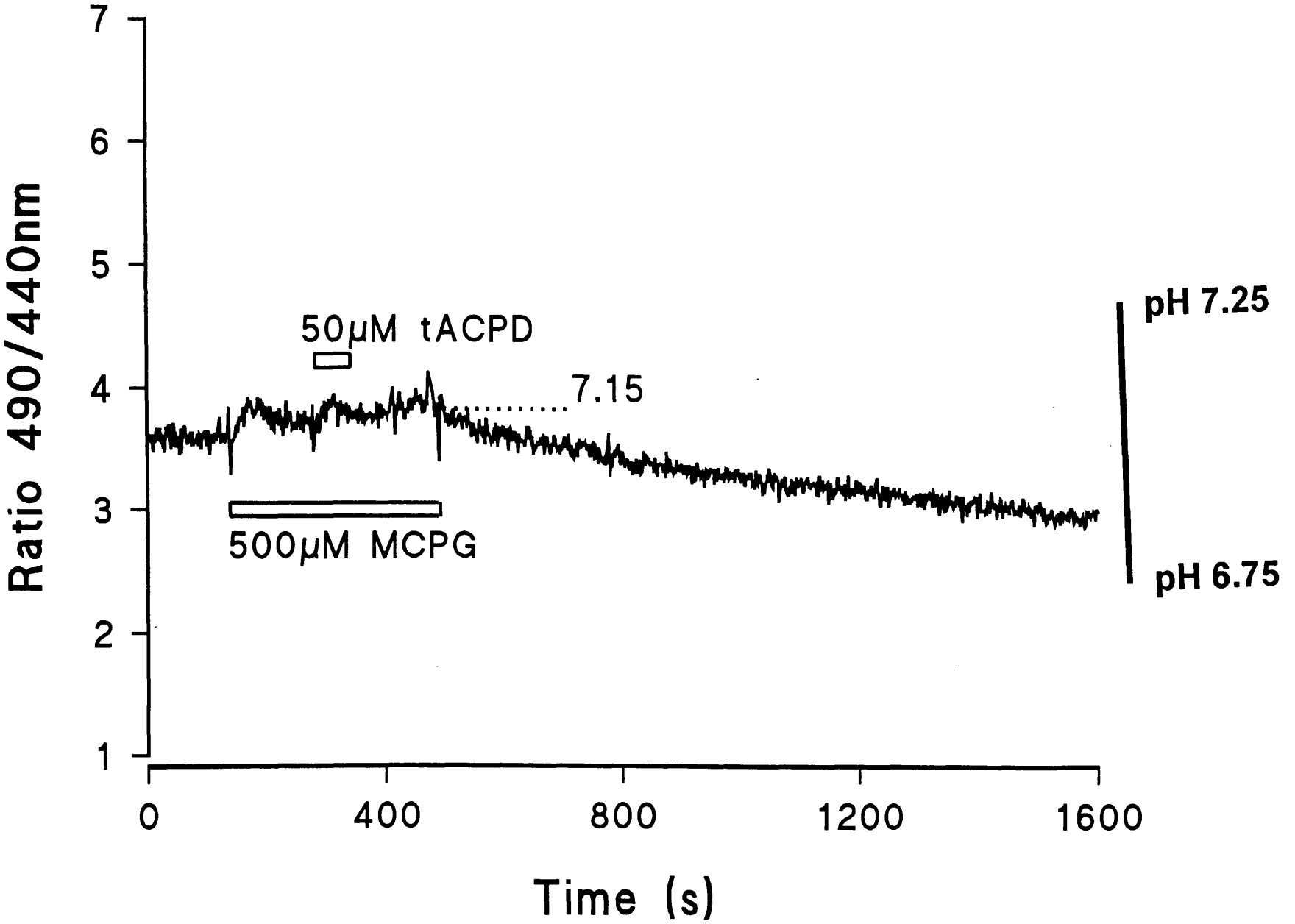
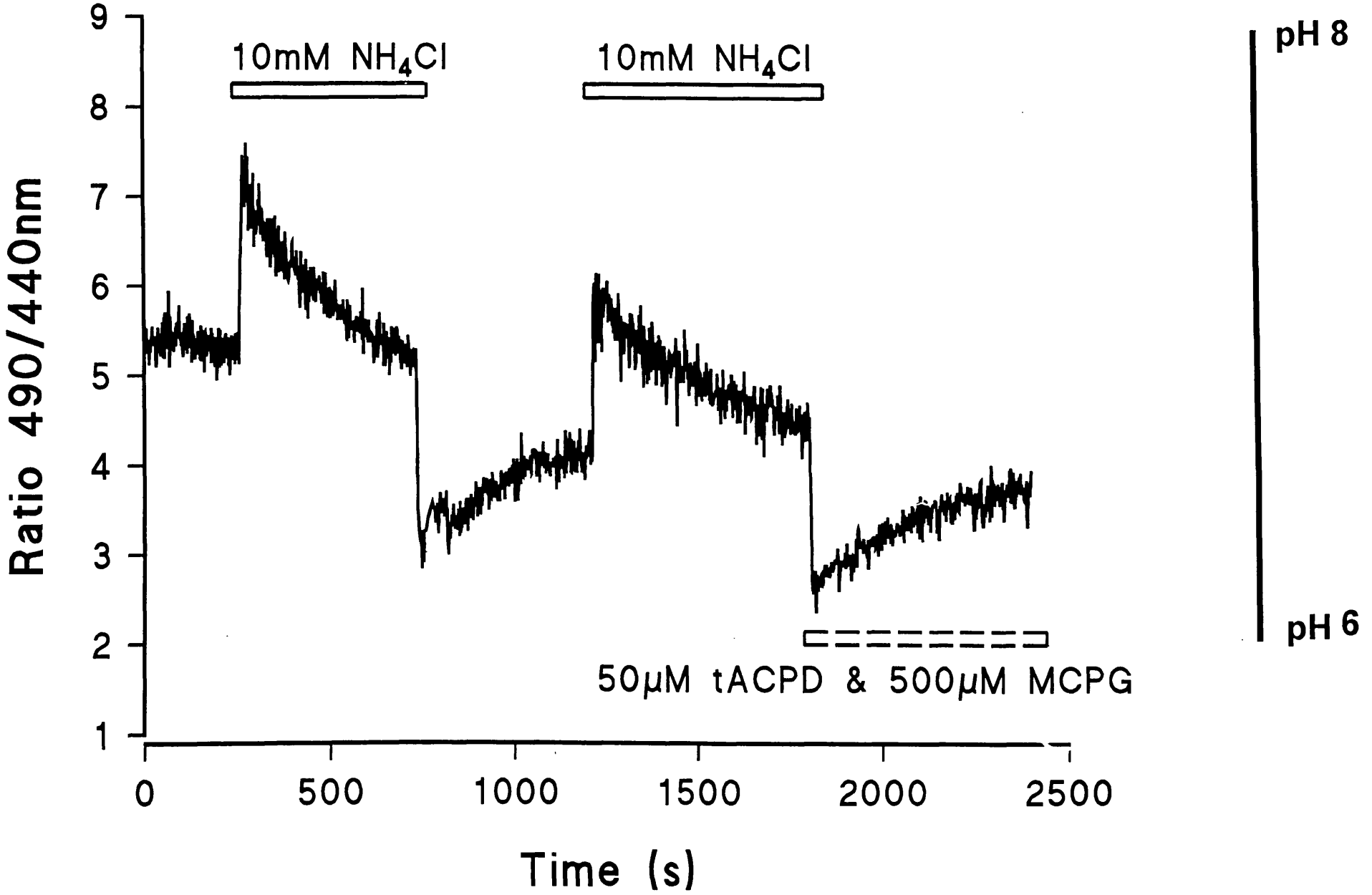

Ratio 490/440nm
7
6
5
4
3
2
1
50μM tACPD
7.15
500μM MCPG
pH 7.25
pH 6.75
0
400
800
1200
1600
Time (s)

FIGURE 24. Antagonism by MCPG of tACPD action on acid recovery. This trace shows a cell that was acid loaded using NH_4Cl in CO_2-HCO_3^- NaL. The cell showed a normal recovery ($1x10^{-3}$ units.s^{-1}) on NH_4Cl removal. The cell was then acid loaded in the same fashion but this time washed into a solution containing 500μM MCPG and 50μM tACPD. Acid recovery was slightly reduced ($0.75x10^{-3}$ units.s^{-1}). Considering the greater buffering power at more acidic pH values (see figure 17) the actual rate of proton pumping was probably not greatly reduced.


10mM NH4Cl
10mM NH4Cl
50μM tACPD & 500μM MCPG
pH 8
pH 6
Ratio 490/440nm
Time (s)
9
8
7
6
5
4
3
2
1
0
500
1000
1500
2000
2500

CHAPTER 4: DISCUSSION

The aims of this study were threefold :

1. To estimate the value of intrinsic buffering in CNS neurones and its variation with pH.
2. To investigate the contribution made by CO_2 to buffering in this system
3. Finally the modulation of pH_i by neurotransmitters was to be investigated.

Two striking results have arisen from these investigations. Firstly, CO_2 makes a much smaller contribution to total intracellular proton buffering power than would be assumed on the basis of an open system. This finding must be considered against a background summarised in a statement by Szatkowski in 1989: "the important role of CO_2 in (intracellular) buffering has been undisputed" (since Henderson's observations in 1908). Secondly, it is apparent that glutamate can affect intraneuronal pH through activation of metabotropic glutamate receptors. This discussion will attempt to put all the results obtained into the context of current knowledge about intracellular pH (pH_i) and suggest possible reasons for unexpected features of the data.

Intracellular pH

Accurate assessment of buffering power requires accurate measurement of pH values. A number of methods have been employed to measure pH_i and the dye method employed in this study has been used in a number of other preparations (e.g. rat hippocampal neurones, Raley-Susman *et al*, 1991, Schwiening & Boron, 1994) allowing comparison of results (see Table 17). From early pH_i measurements of intact cells it soon became apparent that H^+ was not distributed according to a Donnan equilibrium. For example using pH-sensitive microelectrodes Caldwell (1954) found that the pH_i of crab muscle was 6.75 and Roos (1971) determined the mean pH_i of rat brain tissue using weak acid distribution and obtained a value of 7.05. To maintain pH_i at such high values requires some means of removal of protons or acid equivalents from the cytoplasm as the equilibrium potential for H^+ will favour H^+ influx. Thus, in the case of a neurone with a resting pH_i of 7.0 the equilibrium potential for H^+, under typical conditions of pH_{out} 7.3,

is -18mV. If H^+ was passively distributed then with an extracellular pH 7.3 and a resting membrane potential of -90mV the pH_i would be 5.8.

The pH_i of the neurones investigated in the course of this study had a mean value of 7.07±0.05 (n=22) in CO_2-HCO_3^- buffered NaL (pH 7.3). This compares well with values of 7.06 (Gaillard & Dupont, 1990), 6.95 (Irwin *et al*, 1994), 7.27 (Pocock & Richards, 1992) and 7.19 (Raley-Susman *et al*, 1993) found in other studies of cultured central neurones (see Table 17). Cells bathed in HEPES buffered NaL (pH 7.3-7.35) had a resting pH_i of 7.03±0.04 (n=22). This is generally lower than values previously reported; cf. 7.37 (Gaillard & Dupont, 1990) and 7.49 (Richards & Pocock, 1992). It does, however, compare well with the value of 6.81 reported by Schwiening & Boron (1994). These resting pH_i values are lower than those values reported for acutely dissociated adult neurones (7.76, Raley-Susman *et al*, 1993). Similarity of resting pH_i values in HEPES NaL and CO_2-HCO_3^- NaL has also been reported in smooth muscle tissue (7.06 - HCO_3^-, Aickin, 1994, 7.07 - HEPES, Baro *et al*, 1989). The present study agrees with earlier studies showing the resting pH_i of central neurones maintained in culture to be considerably higher than a passive distribution of H^+ would allow and implies that pH_i is actively regulated.

Due to the difficulty of applying other methods to such small isolated cells, the pH_i values reported from vertebrate neuronal preparations have all been measured using fluorescent dyes. Nuclear magnetic resonance spectroscopy has been used in mammalian CNS slices, however, and values of 7.3 are reported (Corbett *et al*, 1992). This is slightly more alkaline than the values obtained here, but it is necessarily an averaged intracellular estimate derived from a number of different cell types. Invertebrate neurones are larger than mammalian CNS neurones which permits measurement of pH_i using microelectrodes. Values of pH_i from microelectrode studies on squid axon (7.28 Boron & DeWeer, 1976) and snail neurones (7.26, Roos & Boron, 1981) show reasonably good agreement with the values obtained for mammalian CNS neurones in this study. Although no microelectrode studies of pH_i in mammalian CNS neurones have been performed, studies in smooth muscle tissue show no large difference between BCECF studies (7.07 Baro *et al*, 1989, 7.09 Eiesland *et al*, 1991) and microelectrode investigations (7.06,

Authors	pHi	Buffer	Preparation	Method
Caldwell, 1954	6.75	HCO_3^-	Crab muscle	Microelectrode
Boron & De Weer, 1976	7.28	HCO_3^-	Squid giant axon	Microelectrode
Aickin & Thomas, 1977	7.07	HCO_3^-	Mouse soleus	Microelectrode
Curtin, 1986	6.82	HCO_3^-	Frog sartorius	Microelectrode
Aickin, 1984	7.06	HCO_3^-	Guinea-pig Vas deferens	Microelectrode
Tolkovsky & Richards, 1987	7.30	HCO_3^-	Rat sympathetic neurones	BCECF
Gaillard & Dupont, 1990	7.06 7.37	HCO_3^- HEPES	Cerebellar Purkinje neurones	BCECF
Vaughan-Jones & Wu, 1990	7.20	HEPES	Cardiac Purkinje fibres	Microelectrode
Eiesland *et al*, 1991	7.09	HEPES	Rat myometrial cells	BCECF
Raley-Susman *et al*, 1991	7.17	HEPES	Rat hippocampal neurones	BCECF
Ikeda *et al*, 1992	7.26	HEPES	Guinea-pig OHC cells	BCECF
Corbett *et al*, 1992	7.30	HCO_3^-	Whole brain (pig)	NMR
Schwiening & Thomas, 1992	7.21	HCO_3^-	Locust neurones	Microelectrode
Pirtillä & Kauppinen, 1993	7.27	HCO_3^-	Guinea-pig brain slice	NMR
Hartley & Dubinsky, 1993	7.00	HEPES	Rat hippocampal neurones	BCECF
Schwiening & Boron, 1994	7.03	HCO_3^-	Rat pyramidal neurones	BCECF

Table 17: Resting intracellular pH values of various preparations. (NMR= Nuclear magnetic spectroscopy)

Aickin, 1984, 7.26 Aickin, 1994). Thus the resting pH_i of mammalian CNS neurones does not appear to be considerably different from invertebrate neurones or mammalian muscle tissue, regardless of the measurement technique (see table 17). Notwithstanding the similarity of values for pH_i found with both microelectrode and with pH-sensitive dyes, it would be helpful to use an alternative. One such method is the null point method (Eisner *et al*, 1989). Th e principle of this method is simply to find a mixture of weak acid and weak base that does not produce a change in pH_i. The absolute value for pH_i can then be calculated from the composition of this mixture. This method make no assumptions regarding the nature of the relationship between fluorescence signal changes and changes in pH and is independent of pH_i-regulation and buffering power. As the pH_i estimates made with the nigericin calibration technique agree well with the null point method (Eisner *et al*, 1989, Pocock & Richards, 1992, in CNS neurones), we may assume that we are measuring pH_i with reasonable accuracy. All these values show that an H^+ gradient is maintained across the plasma membrane with intracellular pH more alkaline than would be expected on the basis of a passive distribution. This suggests the existence of membrane-located transporters. The nature of these transporters has already been discussed in the Introduction and will be further discussed below (page 123).

The observation that stable resting pH_i values were not different in CO_2-HCO_3^- NaL and HEPES NaL ($p=0.53$) contrasts with previously published observations of CNS neurones (see Gaillard & Dupont, 1990, Pocock & Richards, 1992). However, the resting pH_i values given in the Results omit cells that showed a declining pH_i when bathed in HEPES NaL (almost 50% of cells). This suggests that the presence of CO_2/HCO_3^- is critical for pH_i-regulation in many cells, though a substantial subset of the population exists where CO_2 seems to have no role in determining resting pH_i. Neurones bathed sequentially in CO_2-HCO_3^- NaL and HEPES NaL invariably showed a shift in pH_i when bathing solutions were changed. Removal of CO_2 caused an intracellular alkalinisation which can be readily understood in terms of the various carbonic acid equilibria. The efflux of CO_2 from the cell promotes dehydration of carbonic acid and association of H^+ and HCO_3^- ions, thus removing protons from solution. The generally fast rate of alkalinisation suggests high membrane CO_2 permeability. Conversely, replacing HEPES NaL with CO_2-HCO_3^- NaL evoked an acidification due to CO_2 entering the cytosol,

hydrating into carbonic acid and dissociating into H^+ and HCO_3^-. The similarity of resting pH_i values in cells that had been bathed for longer periods (10-15 minutes) in CO_2-HCO_3^- NaL or HEPES NaL, and had showed stable, the similarity resting pH_i values suggests that in many cells CO_2 does not play a critical role in determining resting pH_i.

Buffering power estimates

Although all excess protons not utilised in intracellular processes will ultimately be extruded across the plasma membrane, and removed from the body via the lungs or kidneys, it is important to minimise fluctuations of cytosolic pH changes in the short term. The fixed buffers within a cell, which operate irrespective of the external environment (closed buffers) are critical in reducing pH_i fluctuations. The value of this buffering, known as intrinsic buffering ($ß_i$), is a fundamental cell parameter but has only been measured in a few cell types - mainly muscle and neuronal, in both vertebrate and invertebrate, and kidney.

The estimates of $ß_i$ obtained in this study with TMA, CO_2 and NH_3 are in line with other neuronal studies, both invertebrate and vertebrate (see Table 18). As buffering will be pH-dependent (Koppel & Spiro, 1914; see Roos & Boron 1980) it is important to compare $ß_i$ values at similar pH_i values. The estimate of 9 mmoles/l at pH 7.27 for NH_4Cl addition and 9 mmoles/l at pH 7.34 for TMA challenges are very similar to the values of 8 mmoles/l in cerebellar granule neurones (Pocock & Richards, 1992) and 11 mmoles/l in cerebellar Purkinje cells (Gaillard & Dupont, 1990). These studies do not explicitly state the mean pH_i at which the buffering estimates apply. However, the similar resting pH_i values and challenge size would imply similar mean pH_i values. The estimates in this Thesis are also of the same magnitude as that found by NH_3 and TMA challenges or direct H^+ injection in snail neurones (10.4 mmoles/l pH 7.36, Szatkowski & Thomas, 1989 and 11 mmoles/l pH 7-7.5, Thomas, 1976, respectively). In comparison to striated and cardiac muscle the $ß_i$ of CNS neurones is low (45 mmoles/l in mouse soleus, Aickin & Thomas, 1977, 35 mmoles/l frog sartorius, Curtin, 1986, 25.6 mmoles/l cardiac myocytes , Eisner *et al*, 1989a, 20 mmoles/l cardiac Purkinje fibres, Vaughan-Jones & Wu, 1990). The lower values in neuronal tissue compared with striated muscle

Authors	Preparation	ß estimate (mM)	pH range	Method
Boron & DeWeer, 1976	Squid axon	9	7.3-7.8	NH_3
Thomas, 1974	Snail neurone	25	6.8-7.4	CO_2
Thomas, 1976	Snail neurone	11	7-7.5	H^+-injection
Szatkowski and Thomas, 1989		10.4	7.36	NH_3/TMA
Aickin & Thomas, 1975	Crab muscle	47	7-7.3	CO_2
Boron, 1977	Barnacle muscle	28.5	6.9-7.5	CO_2
Aickin & Thomas, 1977	Mouse soleus	45	6.8-7.2	CO_2
Curtin, 1986	Frog sartorius	35	6.85-7.05	CO_2
Eiesland *et al*, 1991	Rat uterus	12.8	7.09	Butyric acid
Aickin, 1984	Guinea-pig vas-deferens	8.6	_	CO_2
Aickin, 1994	Guinea-pig femoral artery	10.3	_	NH_3
Vaughan-Jones & Wu, 1990	Cardiac Purkinje fibres	20	7.2	NH_3
Pocock & Richards, 1992	Cerebellar granule cells	9.4	_	NH_3
Goldsmith & Hilton, 1992	Rat leucocytes	18	7-7.2	NH_3
Ikeda *et al*, 1992	Cochlear hair cells	15.1	7.26	NH_3

Table 18: Estimated buffering power of various preparations. Estimates are of *intrinsic* ß (in the case of Aickin (1994), Eiesland *et al* (1991) and Pocock & Richards (1992) these values also approximate to *total* ß). Buffering power was estimated using either weak acid (butyric acid or CO_2) or weak base challenges (NH_3 or TMA).

presumably reflects the difference in the types of protein present in the two tissues. Also, striated muscle may be subject to sizeable, rapidly developing acid loads during excercise, e.g. from lactic acid, and buffering has probably evolved to meet this. However, the values for smooth muscle are very similar to those found in verterbrate neurones (12.8 mmoles/l Eiesland *et al*, 1991, 14 mmoles/l Baro *et al*, 1989, 8.3 mmoles/l, Aickin, 1984, 8.5 mmoles/l Aickin, 1994). There is a possibility that the method used to alter pH_i may influence the estimate of ß obtained. In snail neurones good agreement has been found between ß estimates from direct injection of acid (11 mmoles/l, Thomas, 1976) and the weak acid/base method (10.4 mmoles/l, Szatkowski & Thomas, 1989), suggesting no great dissimilarities between the two methods. Although the study here only employs the weak acid/base method, two weak bases and two weak acids have been used. This protocol should show whether the value of ß is dependent on the specific choice of challenge used to measure it. Estimates of mean $ß_i$ using CO_2 were 11mM±2.34 (n=10) at average pH_i 7.14, compared with 9 mmoles/l (pH 7.27) with NH_4Cl and 9mmoles/l (pH 7.34) for TMA. It is clear from the results given that buffering power estimates are more or less independent of the weak acid or base used. However, the weak acid/base method depends on a number of assumptions (see Methods and Introduction). Transport of a charged species violates a key assumption implicit in the weak acid/base method, and must be considered when using butyric acid or NH_4Cl.

The assumption that there is no movement of charged species is violated by NH_4^+ during NH_4Cl challenges (see Roos & Boron, 1981). However, NH_4^+ permeation appears to be much slower than that of NH_3. In this situation the movement of NH_4^+ will not generally interfere estimates of ß as the pH_i changes used to estimate ß are measured as soon as the challenge has been given. Furthermore, the acidification produced by NH_4^+ dissociation described in the Results is useful to the investigator in that it allows estimation of ß at acidic mean pH_i values on NH_4Cl withdrawal. Although estimating buffering power from NH_4Cl withdrawal has provided ß values at acidic average pH_i values, challenges with weak acids (butyric and CO_2) provide a useful alternative. Buffering power estimates made from butyric acid challenges were much larger than ß estimates made by NH_4Cl withdrawal at the same average pH_i . Individual estimates were as high as 40mmoles/l at an average pH of 7.25 whereas with NH_4Cl average buffering

power at pH_i 7.25 would be ≈9mmoles/l. Scrutiny of the records showed that active extrusion of acid appeared to occur during butyric acid challenges. This apparent pH_i-regulation during butyrate challenges has previously been reported in rat myometrial cells (Eiesland *et al* 1991), though the cause was unidentified. If a monocarboxylate ion transporter (MCT) was active during acid challenges it may cause overestimation of buffering power by the following method. On initial application to the bathing medium butyrate ions could be transported into the cell by the MCT, thus reducing the number of C_3H_7COOH molecules needed to dissociate in order to attain equilibrium. Consequently the estimate of $[C_3H_7COO^-]_i$ would not be an accurate indicator of the number of protons released into solution by C_3H_7COOH dissociation. This underestimation of the acid load would lead to an overestimation of buffering power (consider equation 2; $ß=[\Delta acid]/\Delta pH_i$). To overcome this problem buffering power was estimated in the presence of a MCT inhibitor, α-cyano-hydroxy-cinnamic acid (CHC). Estimates made with butyric acid in the presence of 5mM CHC gave $ß_i$ estimates of 7.1±2.5 mmoles/l at mean pH_i 6.45. The interpretation of these results offered here is that estimating ß using butyric acid requires particular caution due to potential for monocarboxylate ion transport. This transport may be blocked pharmacologically allowing more accurate assessment of ß values. Disproportionately high buffering power estimates made with weak acids have previously been reported. Thus, Szatkowski & Thomas (1989) reported disproportionately high ß values measured using acetate as compared to butyrate. They attributed this to the effect a small amount of endogenous acetate coupled with some acetate ion transport, though they found no evidence of MCT. Eiesland *et al* (1991) reported apparent pH_i-regulation during maintained butyrate challenges to rat myometrial cells but were unable to identify the cause. However, the consistency of ß estimates with different bases and acids and the consistency between different preparations (Gaillard & Dupont, 1990, Eiesland *et al*, 1991) gives confidence in the weak acid/base method of estimating buffering power.

Relationship between ß and pH_i

Buffering is performed by weak acids or weak bases. Consideration of the Henderson-Hasselbalch equation ($pH=pK+\log[A^-]/[HA]$), as discussed in the Introduction, will show buffering to be most effective when $pH=pK_a$ of the acid or base for a closed buffer

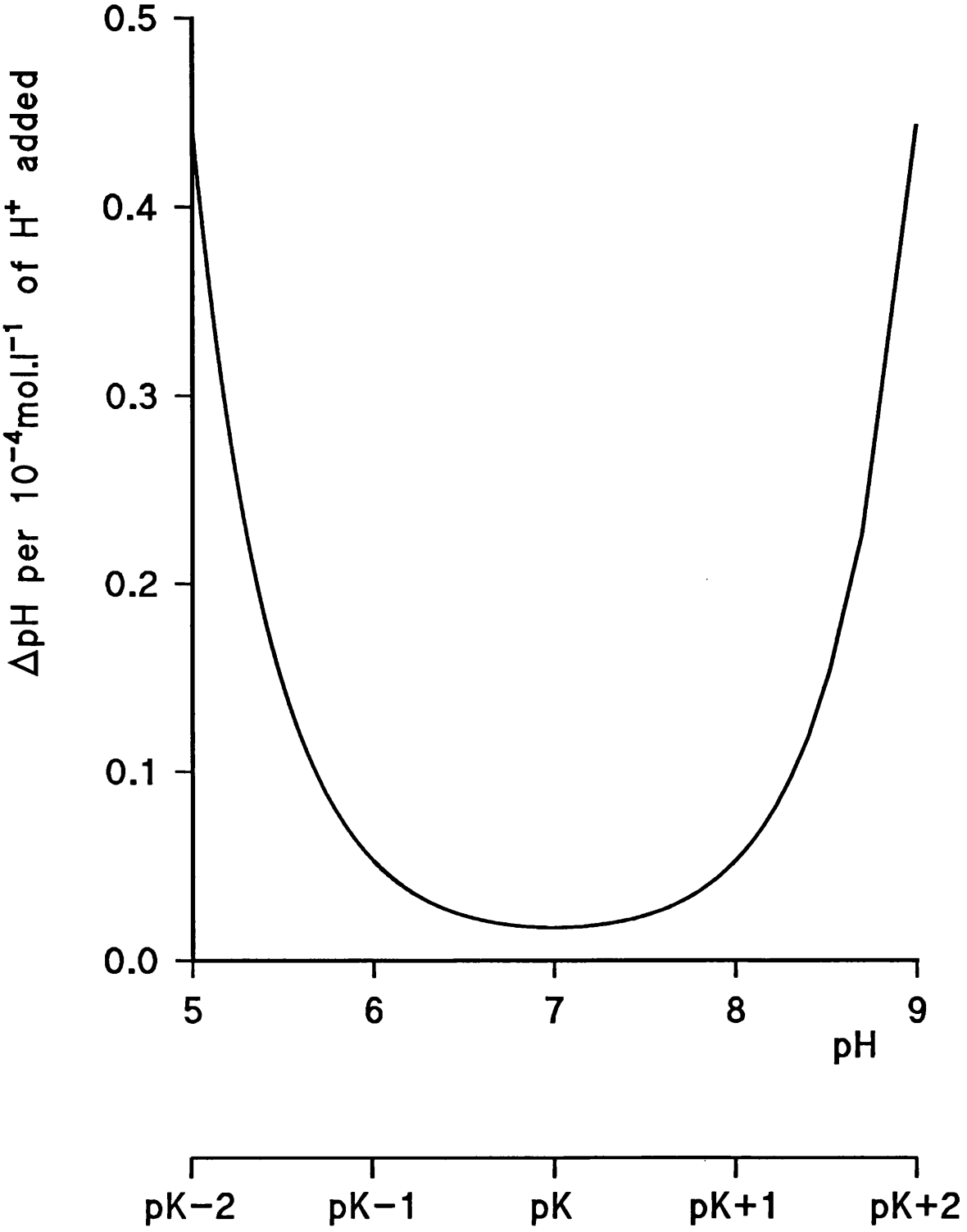
system. At pH values either side of pK_a buffering power will decrease. For a single monoprotic buffer the relationship between buffering strength of a solution and its pH with respect to pK_a is shown in figure 25. As a consequence, buffering is inherently pH-dependent. In trying to characterise this relationship the experimenter will encounter two problems:

Firstly, it is highly unlikely that the buffering capacity of a cell is attributable to a single ionisable group. Buffering is much more likely to be provided by a number of different groups possessing different pK_a values. In turn the individual pK_a values will be affected by the local microenvironment. Consequently, total cellular buffering power may be a complex function of pH and so attempts to fit a smooth curve to ß vs pH_i plots, as has generally been done (Szatkowski & Thomas, 1989, Wenzl & Machen, 1989, Vaughan-Jones & Wu, 1990, Cooper & Hunter, 1994) over-simplifies the true relationship.

Secondly, the measurement of buffering power of a solution inevitably involves a change in the pH of that solution. Therefore buffering power must be measured over a pH range. This inherent pH dependency means that we must strive to estimate ß using the smallest reasonable pH change. The extent of the pH change used is primarily limited by the resolution of the pH-recording system. In these experiments the typical ΔpH_i was about 0.25 units and the estimates of ß are given for an average pH_i value. In this study the relationship between $ß_i$ & pH in single cells was investigated using sequential step changes in the extracellular concentration of a weak acid or a weak base. Figure 16 clearly shows a decrease in $ß_i$ as pH becomes more alkaline in a single cell. This relationship is plotted in Figure 17 and is seen as a ≈60% increase in $ß_i$ as pH drops from 7.6 to 6.6. The inverse relationship between $ß_i$ & pH has been found in other tissues (e.g. Giant Barnacle muscle, Boron, 1977, snail neurone, Szatkowski & Thomas, 1989, cardiac Purkinje fibres, Vaughan-Jones & Wu, 1990, frog distal tubule, Cooper & Hunter, 1994). Also the increase in intrinsic buffering at pH values acid relative to normal resting pH results from the pK_a values of the buffering groups. These results suggest that intrinsic buffering within the physiological range is dominated by buffers whose pK_a values lie below normal resting pH_i. Under normal and pathophysiological conditions neurones are more likely to encounter acid loads than alkaline, and the pattern of cellular buffering is

FIGURE 25. Effectiveness of a monoprotic buffer solution at different pH values with respect pK. This is a plot of the equation derived by Attwell in Chapter 1 of Acid-base Balance (also see Appendix 3) and shows the change in pH of a buffered solution on addition of 10^{-4} moles of acid per litre of solution. The buffer concentration is taken to be 10mM and the pK of the buffer is 7.

At pH 7.0 (10^{-7} moles H^+) the pH of the solution = pK of the buffer. Here the buffer is at its most powerful and the change pH_i for 10^{-4} H^+ added is very small. In contrast when the solution is at pH 5 (2 units away from the buffer pK) the same aliquot of added acid evokes a much larger change in pH.


0.5
0.4
0.3
0.2
0.1
0.0
ΔpH per 10^{-4}mol.l^{-1} of H^+ added
5
6
7
8
9
pH
pK−2
pK−1
pK
pK+1
pK+2

adapted to meet this. From studies of the estimated intraneuronal concentrations of ionisable amino acids and inorganic & organic phosphate groups (McIlwain & Bachelard, 1971, Clarke et al 1989, Salford *et al*, 1973), and from knowing the pK_a' values of these groups we can attempt to model intrinsic buffering. The principal titratable groups in rat CNS neurones are carboxyl groups as free amino acids and carboxyl residues on proteins ($pK \approx 4.5$) at 100mEq, phosphate groups as inorganic phosphate, adenosine phosphates and creatine phosphate ($pK \approx 6.25$) at 20mEq and α-aminos as free NH_2- ($pK \approx 8$) at 10mEq. Theoretical proton buffering power provided by carboxyl, phosphate and α-amino groups as a function of pH is plotted in Figure 26. This predictive study fits well with the actual data obtained (figure 17). The nature and value of β_i over the physiological pH range can seemingly be explained by the contributions of three major ionisable groups within neurones.

FIGURE 26. Plot of theoretical intrinsic proton buffering power of a cell based on carboxyl, phosphate and α-amino groups. From the known concentrations of these groups in whole rat brain (100mEq, 20mEq- including inorganic- and 10mEq respectively), as well as their pK_a' values expected for amino acid residues in proteins (Fersht, 1985 and McIlwain & Bachelard, 1971), a plot of $ß_i$ versus pH_i can be constructed. (See Clarke *et al*, 1989 and Salford *et al*, 1973 for amino acid and organic phosphate estimated from whole rat brain).

As discussed, each buffer works best at pH values close to its pK_a'. The sum of the individual buffering powers is the intrinsic buffering power, represented by line $ß_i$. The inherent pH-dependency and magnitude of intrinsic buffering power shown in this theoretical plot is very close to the experimental data obtained in cultured CNS neurones in this study- marked on the graph as data points with error bars.

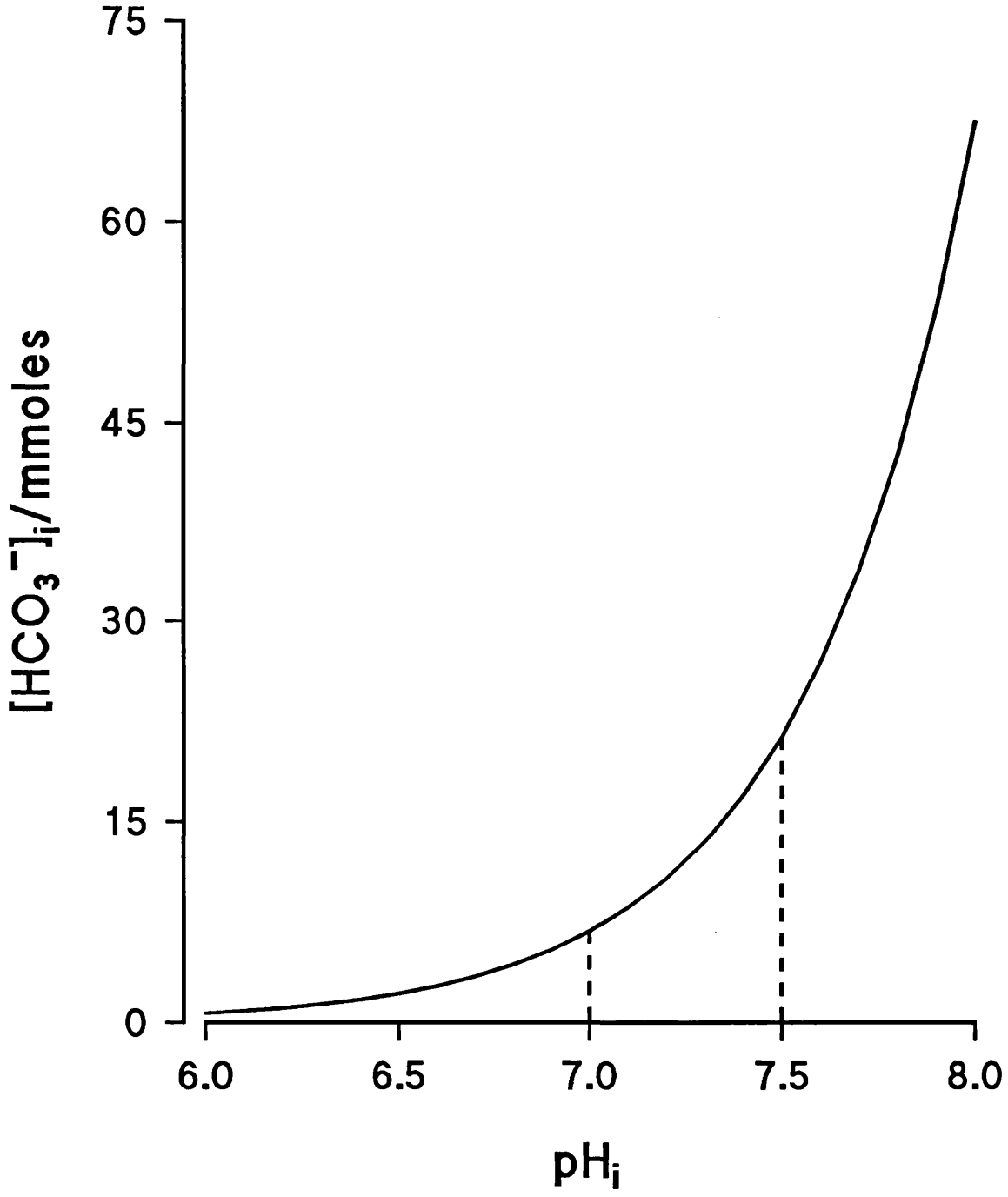
Buffering power (mmoles/l)
0
5
10
15
20
pH
6.0
6.5
7.0
7.5
8.0
R-COO⁻
PO4 2-
βi
α-NH2

Effect of CO_2 on buffering power

As discussed in the Introduction, the total buffering power of a cell is comprised of intrinsic buffering plus buffering from open buffers. In most physiological cases this second component is wholly due to CO_2/HCO_3^- and is designated $ß_{CO2}$. This extra buffering provided by CO_2 has been demonstrated in snail neurones (Thomas, 1976 and Szatkowski & Thomas, 1989) and mouse soleus muscle (Aickin & Thomas, 1977). Other studies have presumed the theoretical increase of buffering power with CO_2 and subsequently calculated proton flux rates using the values of $ß_T$ (e.g. Gaillard & Dupont, 1990). However, the presence of a fixed PCO_2 has failed to increase total buffering power in CNS neurones (Pocock & Richards, 1992), in vascular smooth muscle (Baro *et al*, 1989, in guinea-pig ureter (Aickin, 1994b), in femoral artery smooth muscle (Aickin, 1994) or in guinea-pig vas deferens (Aickin, 1988). The first two studies used BCECF to measure pH and the latter three employed microelectrodes, suggesting that the dye method is not reason itself for the low $ß_{CO2}$ measured. The value of $ß_{CO2}$ is most simply measured in CNS neurones using the weak acid/base method on cells bathed in CO_2-HCO_3^- NaL. Mean ß estimates under these conditions are of $ß_T$. Subtraction of mean $ß_i$ will give $ß_{CO2}$ ($ß_T=ß_i+ß_{CO2}$). Alternatively, paired estimates of $ß_i$ and $ß_T$ can be made in the same cell by bathing sequentially in HEPES NaL and CO_2-HCO_3^- NaL.

The most striking aspect of the data obtained in this study is the observation that total buffering power ($ß_T$) is no greater than intrinsic buffering power ($ß_i$). The contribution of CO_2 to proton buffering power (equal to $2.3[HCO_3^-]_i$ in an open system) should mean that $ß_T$ is some 30 mmoles/l greater than $ß_i$ at pH_i 7.25 (see Figure 27). In fact $ß_i$ and $ß_T$ are not significantly different for NH_4Cl addition or removal ($p=0.33$ for addition and 0.61 for removal). This is in contrast to results obtained by Szatkowski (1989) and Thomas (1976) who found that the presence of CO_2 significantly increases buffering power in snail neurones. Was this apparent ineffectiveness of CO_2 in proton buffering an artefact of estimating ß with NH_4Cl? Estimates were made of $ß_T$ and $ß_i$ using trimethylamine challenges. The response of pH_i to TMA addition is qualitatively different to that obtained with NH_4Cl. This is probably due to the slow permeation of NH_4^+ ions during NH_4Cl challenges which dissociate intracellularly, thus acid loading the cell and giving a characteristic shape to the pH_i changes (see figure 2). With TMA, however, no

FIGURE 27 Relationship between pH and HCO_3^- when PCO_2=35.6mmHg. At constant PCO_2 there is a dramatic increase of $[HCO_3^-]_i$ with increasing pH_i. The value of $[HCO_3^-]_i$ can be estimated from manipulation of the Henderson-Hasselbalch equation: $[HCO_3^-]_i = \alpha . PCO_2 . 10^{(pHi-pKa)}$, where α=solubility coefficent of CO_2 ($0.03 mmol.l^{-1}$ per mmHg) and pK_a="overall" dissociation constant for CO_2. For an open buffer system at a constant PCO_2 of 35.6mmHg with $ß_{co2} = 2.3[HCO_3^-]_i$ (for derivation see Appendix 2) we would expect $ß_{co2}$ to increase from 19.5mM to 61mM as pH_i rises from 7.0 to 7.5.


75
60
45
30
15
0
$[HCO_3^-]_i$/mmoles
6.0
6.5
7.0
7.5
8.0
pH_i

such permeation of the ionic species is thought to occur and so a 'flat-top' pH_i response is seen (see Figure 7, lower panel). Despite these different responses to different bases, the buffering power estimates made using either base tally. Estimates of ß made with TMA, shown in Table 3, concur with those estimated using NH_4Cl; $ß_T$ was no greater than $ß_i$.

The possibility that the expected difference was obscured by the very large natural variation in ß between neurones was investigated by estimating $ß_i$ and $ß_T$ in the same cell. Measuring $ß_i$ and $ß_T$ in the same cell also failed to provide evidence of any significant contribution of CO_2 to proton buffering power (paired t-test p=0.53 for NH_4Cl addition and 0.57 for removal). Also, when $ß_T$ and $ß_i$ were measured consecutively in the same cell with TMA, no increase in buffering power was seen when CO_2 was present. Furthermore, $ß_T$ estimated with TMA in the same cell at two different $[HCO_3^-]_i$ values shows no increase when PCO_2 & $[HCO_3^-]_i$ are increased.

The apparent lack of contribution to $ß_T$ by CO_2 is all the more suprising when we are estimating ß using weak bases. This is because at alkaline pH values (that estimating ß with bases entails) we would expect the greatest contribution from CO_2 as the concentration of $[HCO_3]_i$ increases dramatically with increasing pH_i at constant PCO_2 (see Figure 27). Since the contribution of CO_2 to buffering power is calculated to be $=2.3x[HCO_3^-]_i$ (Woodbury, 1964 and Appendix 2) then $ß_{CO2}$ should increase accordingly, if the cell is acting as an open system. The plot of $ß_T$ vesus pH_i (Figure 17 lower, panel) shows that $ß_T$ does not dramatically increase as mean pH approaches 7.5 as would be expected in an open system. That the relationship between $ß_T$ and pH_i is not as steep as that for $ß_i$, may reflect a minor contribution from $ß_{CO2}$ at alkaline pH values. The failure of $ß_T$ to increase significantly above $ß_i$ in the presence of a constant PCO_2 calls into question the validity of assuming the CO_2-HCO_3^- system to be a major intracellular buffer. Possible reasons for the shortfall of $ß_{CO2}$ from the theoretical value are discussed below (see page 127).

pH_i-regulating mechanisms

It is important to know the nature of pH_i-regulating mechanisms present in these cells if we are to estimate buffering power accurately. Any pH_i regulation may cause the experimenter to underestimate pH_i changes on weak acid/base addition and thus overestimate buffering power (Vaughan-Jones & Wu, 1990). Knowing the variety of mechanisms present we can take steps to negate the effects of pH_i regulation, if any, on buffering power estimates. In the last five years investigations into the pH_i-regulating mechanisms of central neurones have shown the existence of a Na^+/H^+ antiporter (Gaillard & Dupont, 1990, Raley-Susman *et al*, 1991, Pocock & Richards, 1992) a Na^+-dependent Cl^-/HCO_3^- antiporter (Schwiening & Boron, 1994) a Na^+-independent Cl^-/HCO_3^- antiporter (Pocock & Richards, 1992) and a Na^+/HCO_3^- symporter (Pocock & Richards, 1992) which mediate recovery from acid and alkali loads. Identification of these transporters in the cells under test was carried out by appropriate ion replacement and pharmacological inhibition.

Equimolar replacement of external sodium with the impermeant ion NMDG, (which would disrupt the action of the Na^+/HCO_3^- symporter and Na^+-dependent Cl^-/HCO_3^- antiporter and the Na^+/H^+ antiporter), led to a rapid onset declining pH_i in 4/4 cells bathed in CO_2-HCO_3^- NaL. These results show that extracellular Na^+ is critical for maintenance of a stable pH_i when these neurones are maintained in culture. Sodium replacement in HEPES NaL evoked a pH_i decline in only half the cells tested. This may appear to be at odds with the result that sodium replacement in CO_2-HCO_3^- NaL *always* causes a pH_i decline (4/4 cells). However, these experiments were only performed on cells initially exhibiting a stable resting pH_i in HEPES NaL. Consequently those cells with a bicarbonate dependency for stable resting pH_i are specifically excluded from the results in HEPES NaL. The results must therefore be read as 50% cells *not dependent* on CO_2/HCO_3^- for stable resting pH_i (ie a subset of the normal population) show a pH_i decline on Na^+ replacement. These cells most likely require the activity of the Na^+/H^+ antiporter to maintain a stable pH_i. This conclusion is reinforced by the observation that in 4/11 cells there was an acidic pH_i drift when 1mM amiloride was applied to the bathing medium. Thus, besides apparent Na^+/HCO_3^- activity regulating resting pH_i in some cells, others show a necessity solely for Na^+/H^+ antiport to maintain a stable resting

pH_i. The cells showing no dependency on sodium for stable pH_i maintenance may employ a mechanism similar to that previously reported in mammalian CNS neurones. Richards & Pocock (1989) reported acid load recovery in the absence of both Na^+ & HCO_3^- although the underlying mechanism remains unknown.

Replacing extracellular Cl^- with methanesulphonate did not generally lead to a change in intracellular pH_i (no effect in 4/5 cells). The anion exchange inhibitor DIDS (250μM) had no effect on resting pH_i (4/5 cells). This suggests that when cells show a dependency on CO_2-HCO_3^- for stable pH_i the Na^+/HCO_3^- symporter is the disrupted pH_i-regulating mechanism rather than Cl^-/HCO_3^- exchange.

The cellular diversity of neocortical and hippocampal cultures means we can only generalise as to the pH_i-regulating mechanisms expected in a single cell. Diversity of pH_i-regulating mechanisms has even been shown within the same neuronal cell type (cerebellar granule neurones, Pocock & Richards, 1992). The resting pH_i of cells in this study is acidified by extracellular sodium removal, and half the cells tested also show in decline in pH_i in the absence of CO_2/HCO_3^-. A third of cells exhibit a declining pH_i on amiloride application. The resting intracellular pH of most cells is apparently independent of extracellular Cl^- and unaffected by DIDS application. The results are best explained by the presence of Na^+/H^+ and Na^+/HCO_3^- symport. Cl^-/HCO_3^- antiport activity appears to be expressed in a small population of cells also. The critical dependence on CO_2 for a stable resting pH_i in some cells may arise from a dependency on the Na^+/HCO_3^- and/or Cl^-/HCO_3^- transporters. The lack of effect of removing extracellular Cl^- on the pH_i of most cells, however, (in contrast to the inevitable fall in pH_i seen when removing extracellular Na^+) suggests only limited Cl^-/HCO_3^- exchange.

All of these transporters mentioned had previously been identified in central neurones as well as other tissue types. This study points to the activity of another pH_i-regulating mechanism which appears to be active at rest in some neurones, a monocarboxylate ion transporter (MCT). The MCT has previously been identified in human erythrocytes (Halestrap, 1976) and rat spinal roots (Schneider *et al* 1993). Application of α-cyano-hydroxy-cinnamate (CHC) (an inhibitor of monocarboxylate ion transport) caused a pH_i

decline in 4/6 cells. Why monocarboxylate ion transport should be significant under well oxygenated conditions is unclear. The apparent pH_i decline on inhibition of the MCT may partly be due to the acidic consequences of CHC entry into the cytosol however. The MCT has been demonstrated in brain tissue by Assaf *et al* (1990), and its action may cause over-estimation of buffering power when using weak acids such as butyric acid (see Results I).

The transporters discussed above move pH-changing ions across the *plasma membrane.* Any role that transport of H^+ across organellar membranes may play in pH_i-regulation is, as yet, undetermined. Unfortunately central neurones are too small to measure cytosolic pH with conventional microelectrodes, and dye measurements on whole cells give a general intracellular pH signal which fails to provide information about the specific role of organelles in pH_i-regulating processes.

Effects of pH_i-regulation on ß estimates

Previous workers have shown that ß estimates could be adversely affected by the action of pH_i-regulating processes that masked the true extent of pH_i change on addition of weak acid or base (Szatkowski & Thomas, 1989, Vaughan-Jones & Wu, 1990). This problem can be minimised by back-extrapolation of the pH change to the point of addition of weak acid/base (eg Thomas, 1976) but is clearly unsatisfactory where large extrapolations have to be made. The system used in this study changed the solutions so quickly that back-extrapolation was often unnecessary as the pH_i change occured almost instantaneously (i.e. within 1s). However, any underestimation of ΔpH_i would cause overestimation of ß. Consequently some experiments were performed to estimate ß in conditions where pH_i-regulating transport processes would be inhibited. The contamination of ß estimates by monocarboxylate ion transport was dealt with as described. As all the remaining pH_i-regulating exchangers thus far identified in neurones are Na^+ or Cl^- dependent, $ß_T$ was estimated in solutions free of these ions. Comparisons with $ß_T$ estimates from the same cells in normal bathing media showed no statistically significant difference between the two estimates ($p=0.13$ for Na^+-containing and Na^+-free media and $p=0.19$ for Cl^--containing and Cl^--free media). Similarly, estimating ß in the

presence of amiloride had no effect on the values obtained (p=0.75). Also when estimating $ß_T$ in the presence of DIDS (250μM) no difference was found between mean values of $ß_T$ as compared to control. Finally, the significance of omitting CO_2/HCO_3^- from the bathing media must be considered. In media nominally free of CO_2 the action of the Na^+/HCO_3^- symporter and the Cl^-/HCO_3^- antiporter will be significantly impaired if not totally abolished. The marked similarity of $ß_T$ and $ß_i$ values therefore suggests that HCO_3^--dependent pH_i-regulating transport mechanisms have little effect on ß estimates.

This study differs from much of the previous published work (where pH_i-regulation was shown to be a problem) in the time course of the change in concentration extracellular weak acid or base. Scrutiny of the records shown in papers by Szatkowski & Thomas (1989), Szatkowski (1989), and Vaughan-Jones & Wu, (1990) reveals that their systems required up to 2-3 minutes for maximal pH_i changes to occur. This may reflect an equally slow change in extracellular weak acid/base concentration. These very slow changes provide ample opportunity for pH_i-regulating mechanisms to operate and so reduce the apparent pH_i changes evoked by application of weak acid or weak base. Not suprisingly, in these experiments blocking pH_i-regulation was found to have a profound effect on the value of ß estimates.

The design of the system used in this study is such that extracellular acid or base concentrations can be changed with a time course of less than 1 second. This minimises the effect that any pH_i-regulating transport processes can have on the pH_i change. It also therefore reduces the need for back-extrapolation which will be prone to possibly significant errors. Consequently ß values estimated when pH_i-regulating transport processes were blocked were found to be no different to ß values estimated under conditions were pH_i-regulation was permitted. In conclusion, pH_i-regulating transport processes have a minimal effect on ß estimates in this system and so we may be confident that the ß estimates obtained are as accurate as the method allows.

Reasons for shortfall of $ß_{CO2}$

Although CO_2 has not been found to increase ß in a number of preparations (Aickin, 1988, Baro *et al*, 1989, Pocock & Richards, 1992, Aickin, 1994), no study has previously proposed a reason for these observations. In order to account for the CO_2 system not acting as expected in an open system we need to consider the following options:

(i) The Henderson-Hasselbalch equation and hence Law of Mass Action are not valid in this situation.
(ii) Uncharged TMA and NH_3 are accumulated by the cells.
(iii) The CO_2-HCO_3^- system is too slow to contribute to immediate buffering.
(iv) The pK_a of the indicator (and the estimated pH_i values) is significantly affected by HCO_3^-.
(v) The system is not at equilibrium.
(vi) The CO_2 system is not acting as an open buffer.

These are considered in turn.

The Henderson-Hasselbalch equation and hence law of mass action are not valid in this situation. Since consistent $ß_i$ estimates were obtained with different weak acid/base challenges, or when doubling PCO_2 from 20 to 40mmHg, there is no reason to suspect the equation does not hold.

Uncharged TMA and NH_3 are accumulated by the cells. This would mean more $TMAH^+$ and NH_4^+ produced than estimated. Consequently the actual alkaline load would be greater than calculated, and we would underestimate ß. It is difficult to conceive why this should only occur in CO_2-HCO_3^- NaL.

The CO_2-HCO_3^- system is too slow to contribute to immediate buffering. This may well be the case in the absence of carbonic anhydrase. In attempting to explain the shortfall of $ß_{CO2}$ from its theoretical expected value, it is useful to consider the nature of the CO_2 system. Unlike the simple ionisation/ deionisation processes that occur when phosphates, carboxyl and α-amino groups buffer protons, the CO_2 system requires a chemical step

as well as transmembrane fluxes. In order for the system to buffer excess OH^-, for example, CO_2 must enter the cell and hydrate to form H_2CO_3 which then subsequently ionises to H^+ and HCO_3^-. This introduces a time dependency to the buffering (at odds with the classification of buffering by Roos & Boron (1981)). Although the ionisation of the carbonic acid is to all intents instantaneous, the hydration of CO_2 is comparatively slow. In many cases *in situ* the hydration of CO_2 or dehydration of H_3CO_3 is speeded by the presence of the enzyme carbonic anhydrase (CA). This makes the overall reaction of $CO_2+H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ very fast. In the absence of CA, however, this reaction will be much slower, perhaps limiting its ability to contribute to immediate proton buffering. The CO_2/HCO_3^- system will only be able to contribute as an open buffer if CO_2 and H_2CO_3 are fully in equilibrium across the membrane. Indeed, it has already been suggested that carbonic anhydrase may be necessary for full efficiency of the CO_2 system in proton buffering in snail neurones (Thomas, 1976).

A potential role for CA in buffering was investigated by estimating $ß_T$ in the presence and absence of acetazolamide (a membrane permeant CA inhibitor). If the time course of the CO_2 hydration were a limiting factor, then $ß_T$ estimates should be smaller in the presence of acetazolamide as $ß_{CO2}$ will be reduced. CNS neurones maintained in culture showed no difference between $ß_T$ estimates in normal conditions and those where 20µM acetazolamide was present ($p=0.29$). This result is hardly suprising however if the cells do not contain CA. In the rat CNS carbonic anhydrase seems to reside mainly in glia and the interstitium. Standard methods for showing the distribution of CA have failed to show its presence in rat central neurones (Trachtenberg & Sapirstein, 1980) though carbonic anhydrase activity has been reported in rat hippocampal neurones (Pasternack *et al*, 1993). A lack of CA in these neocortical neurones is suggested from the slow time course of many CO_2 induced acidifications. Also pH_i changes from increasing PCO_2 tended to take about 30s to reach completion, suggesting an uncatalysed reaction (e.g. see Results, Figure 3)

As we fail to see a reduction in $ß_T$ in the presence of acetazolamide, and CA seems to be localized to non-neuronal sites in the brain, then this may appear to offer a simple explanation as to the shortfall of $ß_{CO2}$ from its expected value. i.e. The contribution of

CO_2-HCO_3^- to proton buffering in these neurones is impaired by the relatively slow speed of the CO_2 hydration/H_2CO_3 dehydration reaction. However, it is unclear why no time dependent buffering provided by the CO_2 system is apparent. This would manifest itself as a slow decrease in the pH_i change evoked by a given amount of weak acid/base. When we expose a cell to TMA it should show an immediate alkalinisation, the extent of which will be determined by the intrinsic buffering power of the cell. This would be followed by a slow decrease in the magnitiude of the alkalinisation as the CO_2 system buffers by uncatalysed CO_2 hydration and subsequent ionisation of H_2CO_3. This is not seen in experimental traces, at least not enough to account for the theoretical deficit of $ß_{CO2}$. A lack of carbonic anhydrase activity has also been reported in smooth muscle (Mühleisen and Kreye, 1985). However, Aickin (1994b), has found that constant PCO_2 fails to increase buffering even when the preparation has been left long enough for CO_2 hydration to reach equilibrium without being catalysed. The results presented here do not disagree with this. It is not sufficient to propose that the lack of CA is the cause of CO_2 failing to increase $ß_T$ in smooth muscle tissue or cultured neurones.

These considerations bring into question the validity of considering the CO_2 system as an intracellular buffer at all, rather than a pH_i-regulating mechanism. In the absence of CA the process is slow (especially when buffering alkaline challenges) compared to intrinsic buffering and, more significantly, it involves transmembrane fluxes of CO_2 for completion. In respect of these two attributes it is perhaps more akin to pH_i-regulating transport processes than conventional buffers.

The pK_a of the indicator (and the estimated pH_i values) is significantly affected by HCO_3^-. There is no evidence that this is the case. A change in the pK_a of the dye BCECF in HCO_3^- buffer could lead to an incorrect estimate of total buffering power. A change could be measured by titrating a solution of the dye (the unconjugated free acid form) with H^+ in a fluorimeter cuvette. Duplicating this procedure in HEPES NaL and CO_2/HCO_3^- NaL would show if the pK_a changed. However, the pK_a should be constant for a given microenvironment, and considerable changes in ionic strength are required for pK_a changes of sufficient magnitude to account for apparent buffering loss. Furthermore, the studies performed with microelectrodes in muscle tissue (Aickin, 1988, 1994 and 1994b)

would suggest that the observation of $ß_T \equiv ß_i$ is not an artefact of the dye method.

The system is not at equilibrium. The stability of pH_i seen during the majority of TMA challenges in CO_2/HCO_3^- NaL would suggest that this is not the case.

The CO_2 system is not acting as an open buffer. This is a possibility; two arguments are now proposed which are not mutually exclusive:

(i) To act as an open buffer the concentration of one of the buffering species (in this case H_2CO_3) must be kept constant *during* buffering and there must be a change in the total concentration of the buffering species (i.e. $[H_2CO_3]_i + [HCO_3^-]_i$). For the bicarbonate buffer system to act as an open buffer during an alkaline load, CO_2 must diffuse into the cell and be hydrated to form H_2CO_3 and $[HCO_3^-]_i$ will increase. This takes a finite time (see p 33 and Figure 10). Consequently, CO_2 cannot contribute to instantaneous buffering ($t < 5$ s) in these cells and bicarbonate /CO_2 will behave as a closed buffer system. Assuming full equilibration of the bicarbonate buffer system, at pH_i of 7.2 and PCO_2 of 40 mm Hg, intracellular $[HCO_3^-]$ will be about 10 mM and the bicarbonate buffer would contribute only about 2 mmoles.l^{-1} to total buffering.

(ii) Another explanation is proposed that requires viewing the $[H^+]$ of a solution as a dependent variable, as has been argued most persuasively by Stewart (1978). In this treatment the $[H^+]$ of a solution is determined by various independent variables such as weak acid dissociation, total weak acid present, ionic product of water and conservation

of electroneutrality in the bulk fluid. The theory and its adaptation to the consideration of intracellular buffering is presented in Appendix 4. $[H^+]$ is a dependent variable and is determined by factors such as PCO_2, total quantity of weak acids present and their K_a values and the strong ion difference (SID). The value of $[H^+]$ in itself cannot determine the SID, the total quantity of weak acid etc. The case of CO_2 is treated separately from other weak acids due to its special nature.

As shown in the theory, there exists an electroneutrality constraint on the behaviour of HCO_3^-. For example, other factors remaining constant (PCO_2, total acid and SID) the rise in HCO_3^- that would arise from CO_2 buffering in order to counter an alkaline load is limited by the amount of BH^+ generated inside the cell. The concentration of HCO_3^- may only rise by as much as BH^+ rises, in order to maintain electroneutrality. Hence in figure 5b the addition of 10mM TMA causes pH_i to rise from 7.2 to 7.95 with the generation of 3mM $TMAH^+$. Assuming PCO_2 remains constant at 5%, then $[HCO_3^-]_i$ must rise from ≈13mM to ≈76mM in order to satisfy the Henderson-Hasselbalch equation. This is not possible unless the SID has changed because only 3mM of $TMAH^+$ has been added to the cytoplasm. The law of electroneutrality will be violated unless the increase is concomitant with the efflux of another anion (or influx of a cation). This *will* be the case in cells where considerable Cl^-/HCO_3^- exchange occurs, for example in red cells. Such is the case in snail neurones where CO_2 has been shown to be effective in increasing buffering power (Thomas, 1976). The presence of Cl^-/HCO_3^- exchange is not critical, however, merely a mechanism that allows flux of strong ions during an acid or alkaline load when CO_2 is present in solution. For example a HCO_3^-/H^+ activated ion channel. Thus the observation of constant PCO_2 not increasing buffering power in guinea pig ureter smooth muscle even though Cl^-/HCO_3^- exchange has been reported (Aickin, 1994b).

Szatkowski (1989) has shown the efficacy of open buffers in snail neurones. Challenging cells with HCl injections in the presence of weak bases such as NH_4Cl or TMA he found that the system reacted as would be predicted for an open system (in this case the buffer is NH_4^+/NH_3 or $TMAH^+/TMA$ rather than CO_2). This requires that other open system buffers should be tested to see if the inability of CO_2 to contribute to buffering power is

specific to CO_2 only. Buffering power could be estimated using NH_4Cl challenges in the presence of a constant [TMA] in the bathing medium. TMA can freely permeate the cell membrane and theoretically should contribute to buffering in the same way that a constant PCO_2 is predicted to. The extra theoretical buffering power provided by a constant [TMA] will be equal to $2.3[TMAH^+]$. If we consider the electroneutrality constraints presented in Appendix 4, then in order for these bases to be effective as open buffers considerable flux of strong ions would have to occur. In the case of the NH_4^+/NH_3 system buffering an acid load H^+ would combine with NH_3 to form NH_4^+. The system should be particularly effective as $[NH_3]$ is constant. The rise in $[NH_4^+]_i$ would be compromised however unless cation efflux or anion influx occured concomitantly. Furthermore, these fluxes would have to occur with all the weak bases that showed open buffer system behaviour. The slow time course of the experiments performed by Szatkowski may allow such flux of strong ions, through H^+ sensitive ion channels for example.

One final point should be made regarding the effects of CO_2 on buffering power. All the previous studies showing a contribution of $ß_{CO2}$ to $ß_T$ exhibit slow time course of pH_i changes used to estimate buffering power ($\approx$1 min. or more) and/or do not block pH_i-regulation (Thomas, 1976, Aickin & Thomas, 1977 and Szatkowski, 1989). As some pH_i-regulating mechanisms are HCO_3^--dependent, and slow changes in pH_i allow time for these mechanisms to act, then the buffering power estimates obtained must be treated with caution.

Further experiments

Had time allowed a number of further experiments would have been performed.

1) Firstly the effects of other possible open buffers on total buffering power (such as TMA or NH_3) would have been investigated. If these buffers were found to increase $ß_T$ this would show the failure of CO_2 to be a specific case, not a rebuttal of the open buffer theory.

2) Measurement of intracellular PCO_2 would allow us to assess if it is high enough inside to cause CO_2 to act as a closed buffer.

3) Voltage clamp of cells challenged with alkaline loads would allow flux of ions to relieve the electroneutrality constraint. This would theoretically allow $ß_{CO2}$ to reach its expected value.

4) The previously unreported phenomenon of monocarboxylate ion transport at rest in central neurones needs to more fully characterised.

Conclusions

The main conclusions from this study of buffering power are:

1) The proton buffering power of rat neocortical and hippocampal neurones maintained in culture is of the order 11mmoles/l at a pH_i of 7.0.

2) The value stated applies to both intrinsic and total buffering, indicating that it is unwise to assume that the presence of a constant PCO_2 necessarily increases buffering power. Although the reported absence of carbonic anhydrase in rat central neurones may slow the CO_2-system in proton buffering to such an extent that it is excluded from immediate buffering procesess this does not appear to explain the shortfall of $ß_{CO2}$ in these cells.

3) The value of buffering is pH-dependent and increases with decreasing pH_i.

4) The magnitude of $ß_i$ and its relationship to pH may be modelled by considering the three main titratable groups within CNS tissue.

5) The neurones examined exhibit varying dependency on pH_i-regulating mechanisms that utilise Na^+ and HCO_3^-, with Cl^--dependent mechanisms being less evident. Significant monocarboxylate ion transport also appears to occur at rest, a previously unreported phenomenon. These mechanisms will generally not interfere with ß estimates if intracellular acid/base changes are sufficiently rapid, as is the case in this study. However, with butyric acid challenges it is necessary to block any transport of the weak acid/base in order to make accurate ß estimates.

6) The failure of CO_2 to increase buffering power may result from CO_2 acting as a closed buffer. This may be due to electroneutrality constraints preventing $[HCO_3^-]_i$ rising to its predicted value during alkalinisations

H^+ ions and intracellular signalling

In contrast to the mechanisms present which resist deviations in pH_i, there are mechanisms which specifically shift pH_i away from its resting value. The data presented in the second Results section provide evidence for glutamate evoked acidifcations in neurones. Modulation of pH_i might play a role in intracellular signalling directly as a second messenger or by its effects on other second messenger systems.

There is evidence from a number of cell types that protons may have a second messenger action. Whether these actions are secondary to, or are the cause of, changes in $[Ca^{++}]_i$, varies with the system studied. In *Xenopus* eggs an increase in intracellular pH is secondary to, and dependent on, a Ca^{++} transient (Grandin & Charbonneau, 1992). In cultured cortical neurones decreasing pH_i elicits a rise in intracellular calcium (OuYang *et al*, 1994). The intracellular calcium binding protein calmodulin increases its affinity for calcium with increasing pH (a pH increase of 6.5 to 7.5 changes the K_d from 0.25μM to 0.02μM, Busa & Nuccitelli, 1984). Intracellular pH transients follow spontaneous contractions in rat uterine smooth muscle (Taggart & Wray, 1993). The inotropic effects of $[H^+]$ in mesenteric vascular smooth muscle (Austin & Wray, 1994) would suggest a role for pH changes in intracellular signalling. More appropriate to this study is the demonstration of cytoplasmic H^+-sensitivity of various ion channels (e.g. L-type Ca^{++}-channels in chick dorsal root ganglion cells (DRG cells), Mironov and Lux, 1991). Glutamate has already been shown to modulate high voltage activated (HVA) currents in catfish horizontal bipolar cells by changing pH_i (Dixon *et al*, 1993). Thus there is direct and indirect evidence that H^+ may play a role as a second messenger. The insensitivity of many processes and proteins to small pH_i changes (Busa & Nuccitelli, 1984) is also supportive of a signalling role for H^+.

In the CNS, glutamate-induced pH_i changes have been demonstrated in mouse hippocampal neurones (through NMDA receptor activation, Irwin *et al*, 1994) and rat hippocampal cells (through the activity of a glutamate uptake transporter Bouvier *et al*, 1992), as well as catfish horizontal bipolar cells (Dixon *et al*, 1993). Also kainate has been shown to induce intracellular acidifications in leech glial cells (Deitmer & Munsch, 1992). In the case of glutamate these pH_i shifts are transient, brief applications (1 min.)

of 50μM glutamate eliciting a 0.3 unit acid shift that recovers within about 5 minutes (Dixon *et al,* 1993). Similarly, NMDA (25μM) gives a 0.2 unit acidification that recovers within 10 minutes (Irwin *et al,* 1994). The action of the glutamate uptake transporter has been implicated as one cause of these pH_i shifts (Dixon *et al,* 1993). The effects of 20μM kainate are also transient (Deitmer & Munsch, 1992). These findings should be considered together with the established effects of GABA on neuronal pH_i. Kaila *et al* (1993) have shown that activation of $GABA_A$ receptors in rat hippocampal neurones causes an intracellular acidification due to HCO_3^- efflux through anion permeable channels. Thus besides the direct evidence from Dixon *et al* (1993) there is also circumstantial evidence that protons may act in the CNS as second messengers. It is now apparent that $[H^+]_i$ is not a constant parameter and the proven activity-induced changes in brain interstitial pH (eg Chen & Chesler, 1992) may be accompanied by concomitant changes in neuronal pH_i.

Action of glutamate receptor agonists on pH_i

The data presented in Results II show that L-glutamate can evoke acidifications in rat neocortical and hippocampal neurones maintained in culture. These shifts occur in both HEPES and CO_2-HCO_3^- buffer but tend to have a different character under the two conditions. In HEPES buffer 50μM L-glutamate elicits a rapid fall in pH_i of 0.125±0.025 units (n=6). In CO_2-HCO_3^- buffer the same challenge of glutamate generally evokes a steady decline. This decline is $1.68x10^{-4}\pm3.6x10^{-5}$ units.s^{-1} (n=3). Considering the buffering power of these neurones (eg Amos *et al,* 1993 and data presented in Results I of this Thesis) these changes represent substantial fluxes of H^+ or acid equivalents. At a typical ß of 10mmoles/l the increase in $[H^+]$ induced by glutamate in HEPES NaL is 1.25mM. Similarly the pH_i decline in CO_2-HCO_3^- NaL is $\approx1.7x10\mu M.s^{-1}$.

Published studies of L-glutamate action on pH_i in HEPES buffered media have not defined the mechanism by which glutamate achieves these changes, though speculate that the glutamate uptake transporter is involved. Here it has been demonstrated that at least one pathway by which this acidifying effect occurs is through metabotropic receptor (mGluR) activation. Application of the specific mGluR agonist tACPD (25-50μM) causes qualitatively similar changes in pH_i to those seen with L-glutamate. The tACPD induced

changes are of greater magnitude though; in HEPES buffer a shift of 0.24±0.08 units (n=9) is seen, and the mean rate of pH_i decline in CO_2-HCO_3^- buffer is $3.7x10^{-4}$ units.s^{-1} (n=6). As with glutamate, the drop in pH_i in HEPES buffer has slowed considerably by 600s after the challenge. In CO_2-HCO_3^- NaL, however, the decline of pH_i is maintained with time (see Table 13).

As with most previously reported effects of mGluR activation, this effect may be G-protein mediated (see Manzoni *et al*, 1992, Martin *et al*, 1993, Birrell & Marcoux, 1993). Cells incubated with an appropriate dose of pertussis toxin (PTX) for 24 hours beforehand are insensitive to the effects of tACPD, suggesting the involvement of G_i or G_o in signal transduction after receptor activation. In CO_2-HCO_3^- NaL application of tACPD (50μM) had no effect on acid recovery in 5/8 PTX treated cells compared with stopping recovery of 6/8 untreated cells. (These results are not significant when subjected to the sign-test, clearly the number of observations must be increased).

Mechanism of action of pH_i change

The effects of tACPD appear, at least in part, to be due to an inhibition of a bicarbonate dependent pH_i regulating mechanism. This conclusion arises from the study of tACPD challenges on neurones recovering from an acid load. In HEPES NaL the main mechanism of pH_i recovery from an acid load in these neurones is the Na^+/H^+ antiporter. Application of tACPD (25-50μM) appeared to have no effect on the rate of recovery in HEPES NaL (5 cells). The Na^+/H^+ antiporter in these neurones is apparently insensitive to the effects of tACPD. However, in CO_2-HCO_3^- NaL, where recovery from acid loads is also mediated by HCO_3^--dependent mechanisms, 25-50μM tACPD halted or severely impaired acid recovery (6/8 cells). One target of mGluR action is therefore presumed to be either the Na^+/HCO_3^- symporter or the Na^+-dependent Cl^-/HCO_3^- antiporter, both of which are reported to mediate pH_i recovery from acid loads in central neurones (Pocock & Richards, 1992 and Schwiening & Boron, 1994). The effect of mGluR activation by tACPD on bicarbonate dependent acid recovery is most dramatically shown in figure 21. Here a cell demonstrating normal pH_i recovery in standard CO_2-HCO_3^- NaL fails to show any recovery of pH_i from an acid load when washed into a solution containing 50μM tACPD. Also, when cells are acid loaded in CO_2-HCO_3^- NaL that contains 10μM EIPA

(which is a potent inhibitor of Na^+/H^+ antiport activity eg Andreeva *et al*, 1992) pH_i recovery ceases when the cells have been challenged with of tACPD (25-50μM).

In order to ascertain the end target of the HCO_3^--dependent acidification evoked by tACPD we could perform experiments in CO_2-HCO_3^- NaL that was free of any Na^+. However, removal of extracellular Na^+ by itself causes a fall in pH_i. Most cells exhibit a decline in pH_i when $[Na^+]_o$ is replaced by equimolar NMDG, as detailed in Results I, Chapter 3. Consequently, for most neurones it is impossible to assess the effects of tACPD on acid recovery in Na^+-free solutions. Similarly we might hope to differentiate between Na^+/HCO_3^- symport and Na^+-dependent Cl^-/HCO_3^- exchange by removal of Cl^-. However, in acid pH_i recovery the exchanger is dependent on *internal* Cl^- and it is difficult to remove intracellular Cl^- by washing cells in Cl^--free solution (Schwiening & Boron, 1994). Thus, although we are unable to ascribe an action of tACPD on pH_i to a specific HCO_3^--dependent pH_i regulating mechanism, one of these regulators is the most likely target. Alternatively, there is a possibility that tACPD activates a channel permeable to HCO_3^- ions which in turn would cause an intracellular acidification due to an electrochemical gradient favouring HCO_3^- efflux.

The observation that tACPD-induced pH_i shifts can also occur in media which contains negligible CO_2 suggests at least one more mechanism by which pH_i shifts might occur. These pH_i shifts are sustained following removal of the glutamate receptor agonist, and involvement of the glutamate uptake transporter (Bouvier *et al*, 1992) is therefore unlikely. The nature of the pH_i shifts is sufficiently different to previous reports of glutamate receptor agonists changing pH_i (Irwin *et al*, 1994, Dixon *et al*, 1993) to warrant further investigation.

Consequences of intracellular acidification

The specific mGluR involved in this response is hard to pinpoint due to the unavailability of selective agonists and antagonists. The apparent antagonism of the response by MCPG suggests involvement of mGluR1 or mGluR2 (Hayashi *et al*, 1994). These two receptors respectively increase phospholipase C activity and down-regulate cAMP activity. Characterisation of the pathway involved in pH_i changes beyond mGluR

activation by use of membrane permeant signalling molecule analogues will require more research. The acidification induced by LAP3 might suggest partial agonist activity; though the effect is transient - unlike that seen with tACPD.

The physiological consequences and significance of a mGluR mediated change in neuronal pH_i are open to speculation. In immediate consideration is the sensitivity of various ion channels to cytoplasmic pH changes (e.g. L-type Ca^{++}-channels in chick DRG cells, Mironov and Lux, 1991). A long term change in pH_i as demonstrated in these neurones maintained in culture might cause concomitant changes in ion channel activity. Glutamate has already been shown to modulate HVA currents in catfish horizontal bipolar cells by changing pH_i (Dixon *et al*, 1993). Application of 1μM glutamate causes a 15% decrease in peak HVA calcium current. The mGluR dependent changes in pH_i may be important when considering the sensitivity of HVA calcium currents to pH (Umbach, 1982, Mironov & Lux, 1991, Takahashi *et al*, 1993) and to mGluR agonists (Rothe *et al*, 1994). Could some of the long term effects dependent on mGluRs (such as long term depression, Hartell, 1994) arise directly from changes in pH_i or changes in other ion levels caused by pH_i shifts? Furthermore, the H^+-sensitivity of proteins such as calmodulin may allow direct interaction with calcium second messenger systems.

Further Experiments

A number further experiments need to be performed to complement the discovery of metabotropic receptor induced changes in pH_i:

1) The effects of glutamate agonists (specifically ACPD) should be more thoroughly examined. A dose-response curve for ACPD should be constructed for HCO_3^- and HEPES buffer.

2) The response of different parts of the neurone to metabotropic receptor activation should be examined. Preliminary results from imaging of neurones suggests the acidifcation observed may be more marked in neurites.

3) The possible partial agonist activity of LAP3 and MCPG should be further investigated. This may help identification of the specific glutamate receptor involved in the response.

4) Any effects of extracellular [Ca^{++}] on the agonist induced pH_i changes should be investigated as most previous reported such effects are $[Ca^{++}]_o$ dependent (Hartley & Dubinsky, 1993, Irwin et al, 1994 and Dixon et al, 1993).

5) Elucidation of the second messenger pathway involved in supression of the bicarbonate dependent pH_i regulating mechanism could be aided by use of membrane permeant signalling molecule analogues. The possibility of G-protein mediation of the effect should be further investigated using pertussis toxin.

Conclusions

1) Application of low concentration of glutamate and glutamate agonists has been shown to affect the intracellular pH of mammalian CNS neurones.

2) Through metabotropic receptor activation, glutamate can elicit an intracellular acidification.

3) The acidification is qualitatively different in HEPES and CO_2-HCO_3^- buffer.

4) This acidification may result from inhibition of a HCO_3^- dependent pH_i-regulating mechanism and may indicate a novel second messenger action of H^+.

CHAPTER 5: APPENDICES

APPENDIX 1

Derivation of Henderson-Hasselbalch equation

Consider a weak acid, HA, in equilibrium with its salt, A^-. The equilibrium between the two species can be represented by the equation:

$$HA \overset{k1}{\underset{k2}{\rightleftarrows}} A^- + H^+$$

Where k_1 and k_2 are rate constants. According to the law of mass action, at equilibrium:

$$k_1[HA]=k_2[A^-][H^+]$$

or $$[H^+]=K_a\frac{[HA]}{[A^-]}$$

Where $K_a=k_1/k_2$=acid dissociation constant

Taking logarithms to the base 10:

$$\log_{10}[H^+]=\log_{10}K+\log_{10}\frac{[HA]}{[A^-]}$$

Multiplying both sides by -1:

$$-\log_{10}[H^+]=-\log_{10}K-\log_{10}\frac{[HA]}{[A^-]}$$

As $-\log_{10}K_a=pK_a$ and $-\log_{10}[H^+]=pH$ then

$$pH=pK-\log_{10}\frac{[HA]}{[A^-]}$$

or $pH=pK+\log_{10}\frac{[A^-]}{[HA]}$ Henderson-Hasselbalch equation

APPENDIX 2

Buffering power in an open system

Consider the case of the CO_2-HCO_3^- system as an open system. If we add H^+ to a solution containing HCO_3^- and at a constant PCO_2 then the H^+ will combine with HCO_3^- and thus be removed from solution. Alternatively an excess of alkali will be countered by dissociation of $H_2CO_3^-$ into H^+ and HCO_3^-. In each case the change in H^+ is equal to the change in HCO_3^-. Consequently the buffering power ($ß_{CO2}$) is represented by $\Delta HCO_3^-/\Delta pH$.

$$[A^-] = \frac{K_a.[HA]}{[H^+]} \qquad (A^- \equiv HCO_3^-)$$

or $\log_e[A^-] = \log_e K_a + \log_e[HA] - \log_e[H^+]$

$\log_e[A^-] = \log_e K_a + \log_e[HA] - 2.3\log_{10}[H^+]$

$\log_e[A^-] = \log_e K_a + \log_e[HA] + 2.3pH$

so $\frac{1}{[A^-]} . \frac{d[A^-]}{dpH} = 2.3$

or $\frac{d[A^-]}{dpH} = 2.3[HCO_3^-]$

Since $\Delta A^- \approx \Delta S^+$, the amount of base added to determine ß,

$$ß = \frac{d[A^-]}{dpH} = 2.3[HCO_3^-]$$

APPENDIX 3

Equation for describing change of solution pH per acid added

In order to show how a buffer works with respect to its pK it is necessary to derive an equation stating how much the pH of a buffer solution changes when we add a certain amount of acid. This derivation is clearly explained by Attwell in chapter 1 of Acid-Base Balance (Ed. R.Hainsworth).

First we consider the total amount of buffer, B in a solution of weak acid:

$$B=[HA]+[A^-]$$

Then we consider the total concentration of protons, C:

$$C=[HA]+[H^+]$$

Rearranging:

$$[HA]=C-[H^+]$$

and

$$[A^-]=[H^+]+B-C$$

Therefore, describing the dissociation of HA in these terms and solving for $[H^+]$:

$$[H^+]=\frac{K(C-[H^+])}{[H^+]+B-C}$$

or

$$[H^+]^2+B[H^+]-C[H^+]=KC-K[H^+]$$

To be a reasonable buffer $B>>[H^+]$ and $B>>K$, so removing the appropriate terms from the equation:

$$B[H^+]-C[H^+]=KC$$

or

$$C= \frac{BH}{K+[H^+]}$$

This describes $[H^+]$ in terms of the total buffer concentration C. If we differentiate this equation with respect to $[H^+]$ we can see by what small amount dC we must increase the total acid concentration to increase $[H^+]$ by the small amount $d[H^+]$.

$$\frac{dC}{d[H^+]} = \frac{BK}{(K+[H^+])^2}$$

If we invert this equation we get $d[H^+]$, the small amount by which $[H^+]$ changes for a small change, dC, in total acid C.

$$\frac{d[H^+]}{dC}=\frac{(K+[H^+])^2}{BK}$$

To convert this to terms of pH rather than $[H^+]$ we must take logarithms to base 10:

$$\frac{d[pH]}{dC}=\frac{d(-\log_{10}[H^+])}{dC}=\frac{-1}{[H^+]}\frac{d[H^+]}{dC}\frac{1}{\ln_{10}}$$

Where $\ln_{10}=\log_e 10$.

Therefore:

$$d(pH)/dC=\frac{-(K+[H^+])^2}{BK[H^+]\ln_{10}} = \frac{-(K+[H^+])^2}{BK[H^+](2.303)}$$

For non-infinitesimal changes of pH and C we can rewrite this differential as:

$$\Delta pH = \frac{-(K+[H^+])^2 \cdot \triangle C}{BK[H^+]2.303}$$

Where ΔpH is the change in the pH of the buffer solution when an amount of acid $\triangle C$ is added. This equation is plotted in figure 26.

APPENDIX 4 (THEORY)

[H+] as a dependent variable

The hydrogen ion concentration of any solution may be viewed as a variable that is dependent on other *independent* variables. These independent variables do not affect each other but will determine the ratio of $[H^+]$:$[OH^-]$. This dependency arises from the law of conservation of mass, the law of electroneutrality and the constraints resultant from mass action equations. This approach is most fully dealt with by Stewart, 1978.

In a simple physiological solution of weak acids and strong ions which is in equilibrium with CO_2 at a constant partial pressure the value of $[H^+]$ must satisfy the following equilibria:

1) $[H^+][OH^-]=K'_w$ water dissociation
2) $[H^+][A^-]=K_A[HA]$ weak acid dissociation
3) $[T_A]=[HA]+[A^-]$ weak acid conservation
4) $[H^+][HCO_3^-]=K_1[H_2CO_3]$ carbonic acid dissociation
5) $[H^+][CO_3^{2-}]=K_3[HCO_3^-]$ bicarbonate dissociation

To conserve electroneutrality the sum of cations in solution must equal the sum of anions. The concentration difference between strong cations and anions is conveniently expressed as the strong ion difference, [S]. ie Σ[Cations]-Σ[Anions]. As there can be no net charge to the bulk solution then:

6) $[S]+[H^+]-[OH^-]-[CO_3^{2-}]-[HCO_3^-]-\Sigma[A^-]= 0$

Where $\Sigma[A^-]$=sum of acid anions excluding those derived from CO_2.
The values of these are described by equations 1-5, so:

$$7)\ [S] + [H^+] - \frac{K_1[H_2CO_3]}{[H^+]} - \frac{K_1K_3[H_2CO_3]}{[H^+]^2} - \frac{K'_w}{[H^+]} - \frac{\sum K_A[T_A]}{[H^+]+K_A} = 0$$

From this equation we can see that $[H^+]$ is determined by the dissociation constant for water, the weak acids present and the amount of CO_2 dissolved in solution.

Now, the buffering power of a solution is determined from the pH change following the addition of acid or base. It is easiest to consider the addition of a strong acid or base such as HCL or NaOH. The addition of either of these will cause a change in the strong ion difference. Moreover, the increase in [anion] from the addition of HCl, for example, represents the amount of acid added. After addition of the acid/base the solution will have a new [S] value, [S']. Therefore, in order to satisfy electroneutrality:

$$8)\ [S']+[H^+]-[OH^-]-[CO_3^{2-}]-[HCO_3^-]-\Sigma[A^-]= 0$$

Subtracting equation 6) from 8) we get:

$$9)\ \Delta[S]+\Delta[H^+]-\Delta[OH^-]-\Delta[CO_3^{2-}]-\Delta[HCO_3^-]-\Sigma\Delta[A^-]= 0$$

Where $\Delta[S]=[S]-[S']$ etc. Since $[HCO_3^-]$,$[A^-]$ and [S] are very much greater than $[H^+]$,$[OH^-]$ and $[CO_3^{2-}]$ within the pH range 6-8 then equation 9) simplifies to:

$$10)\ \Delta[S] \approx \Sigma\Delta[A^-] + \Delta[HCO_3^-]$$

As $\Delta[S]$ is a direct measure of the acid/base added used to displace pH then the buffering power (in this case total buffering power as pCO_2 is present) then:

$$\mathbf{11)}\ \beta_T = \frac{\Delta[S]}{\Delta pH} = \frac{\Delta[HCO_3^-]}{\Delta pH} + \frac{\sum\Delta[A^-]}{\Delta pH}$$

From equation 10 it is clear that the rise in $[HCO_3^-]$ that would occur at constant pCO_2

on the addition of a base cannot be more than the $\Delta[S]$ unless another there is a change in the concentration of another anion from the solution (ie cytoplasm). Similarly the fall in $[HCO_3^-]$ on addition of acid is also limited by $\Delta[S]$. In other words unless movement of other strong ions occurs when alkali is added to the cytoplasm then changes in $[HCO_3^-]$ are limited by electroneutrality constraints.

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