J. Membrane Biol. 105, 265–272 (1988)

The Journal of
Membrane Biology

Bicarbonate and Fast-Twitch Muscle: Evidence for a Major Role in pH Regulation

J. Grossie, Carol Collins, and Mark Julian
Department of Physiology, Ohio State University College of Medicine, Columbus, Ohio 43210

Summary. Internal pH (pH_i) was analyzed in rat extensor digitorum longus (Edl) muscle at 30°C with single-barrel liquid ion-selective electrodes. Average pH_i in 284 cells was 7.197 ± 0.006. Increases in CO_2 from nominally 0 to 5% produced an acidification from which recovery took place. In different groups of cells, recovery from the 5% CO_2 acidification was significantly inhibited by 100 μM 4,4′ diisothiocyanatostilbene 2,2′ disulfonic acid (DIDS), Cl removal, Na removal and 2 mM amiloride. Prepulsing with 20 mM NH_4 in the presence of CO_2/HCO_3 typically reduced pH_i to only about neutral, whereas 50 mM reduced pH_i to 6.7–6.8. In the nominal absence of CO_2/HCO_3, 20 mM NH_4 reduced pH_i to about 6.7 from which recovery took place at about 58% of the rate seen in different cells in the presence of CO_2/HCO_3. In the presence of CO_2/HCO_3, cells prepulsed with 50 mM NH_4 had fully recovered to an average pH_i of 7.22 ± 0.04 about 90 min after removal of NH_4. However, 90 min after removal of 20 mM NH_4 in the absence of CO_2/HCO_3, average pH_i was significantly less (7.05 ± 0.03). Intrinsic buffering capacity (β_i) was obtained during pulses of CO_2, acetic acid or after an NH_4 pulse. β_i was significantly reduced in the absence of HCO_3, Cl or Na and HCO_3. The data provide significant support for an important role of HCO_3 in the control of pH_i in fast-twitch muscle.

Key Words fast-twitch muscle · pH regulation · bicarbonate · chloride

Introduction

Internal pH (pH_i) in a variety of cell types is maintained at a level distinctly alkaline to that which would occur if internal and external H activities were distributed in equilibrium with the membrane potential. The mechanism by which this alkaline pH_i is maintained in mammalian soleus (Aickin & Thomas, 1977) and frog skeletal muscle is largely by way of an amiloride-sensitive Na-H exchange. The role of external HCO_3 in the regulation of pH_i in these muscles is minor and in frog semitendinosis appears virtually inoperative if the resting potential is near normal (Putnam, Roos & Wilding, 1986).

Whether this same Na-H exchanger is the prime mode of pH_i regulation in all types of mammalian skeletal muscle remains a matter of conjecture. In an earlier study using DMO to determine pH_i, it was found that removal of Cl from the external solution caused a prominent reduction in acid extrusion in rat diaphragm (Roos & Boron, 1978). This strongly implicates a HCO_3-Cl exchange as an important means of correcting pH_i in this muscle type. With the exception of this study, the literature does not address modes of pH_i regulation in fast-twitch muscle. From well-known differences in types of skeletal muscle, one can easily speculate that fast-twitch muscle might possess a more elaborate means of controlling pH_i. For example, ^{31}P NMR studies by Kushmerick (1987) show that in fast-twitch muscle contraction causes a decrease in pH_i, whereas in slow-twitch muscle contraction causes pH_i to rise. Present interest in the mode of pH_i regulation in this muscle was prompted by several other studies. The resting potential (V_m) in extensor digitorum longus (Edl) muscle from ketoacidotic diabetic rats was significantly depolarized but repolarized to nearly normal upon removal of Cl, (Grossie, 1985) while V_m in soleus muscle from the same type of animal was normal (Paulus & Grossie, 1983). Experiments on normal Edl muscle showed that in the absence of external HCO_3, V_m becomes more positive due in part to increases in internal Na and Cl (Grossie, 1985). The return of HCO_3 reverses these effects. Removal of external Na or Cl also caused repolarization in solutions free of HCO_3. In the course of these studies, it was found that internal Cl activity is less than expected via a passive distribution. The literature shows that at least one other cell, *Aplysia* neurone, has a similar Cl distribution and in this case HCO_3 is likely to have a role in pH_i regulation (Russell, 1978). The present studies were undertaken to ascertain what role HCO_3 might have in the control of pH_i regulation in fast-twitch muscle, and the data strongly suggests that HCO_3 is much more involved in pH_i regulation in this muscle than is the case for mammalian soleus.

Materials and Methods

Adult Sprague-Dawley or Wistar rats of either sex were anesthesized with ether, and the Edl muscle was removed. The muscle was continually superfused with the basic Ringers solution at 20°C during its removal and subsequent placement in the recording chamber. Upon removal of extraneous tissue, the chamber was heated to 30°C and allowed to equilibrate for approximately 20 min before experiments began. The 30°C temperature was used because it has been found that at 37°C, the muscle undergoes a slowly developing contracture after about 2 hr in the bath as evidenced by a wavy appearance of the fibers. This apparent contracture is delayed or prevented at 30°C and is almost never observed at 20°C. All recordings were made from the posterolateral surface on the distal end of the muscle, an area that is composed almost entirely of fast-twitch fibers (Niederle & Mayr, 1978).

Internal hydrogen activity was determined with H-selective electrodes using the ligand prepared by Ammann et al. (1981). Single-barrel pH electrodes were constructed with filamented glass tubing (W-P Instruments). After construction, the micropipette tips were dipped in a 0.1% solution of Dow-Corning 1107 in acetone, heated at 300°C for 6–8 hr and then stored over selica gel until use. The micropipettes were backfilled with the H-sensitive ligand and then backfilled with a buffer identical to that used by Ammann et al. (1981). The microelectrodes were connected to a varactor bridge electrometer (Analog Devices 311J) with the output connected to a digital voltmeter, oscilloscope, polygraph (Grass P7A) and a differential amplifier. The microelectrodes were calibrated in four solutions of known pH ranging from 6.5 to 7.8 determined with a glass pH electrode (Radiometer). These calibrating solutions had the following composition (in mM): NaCl, 129.9; KCl, 10; Na_2HPO_4, 10; $Na_2B_4O_7$, 2.8. HCl was added to adjust pH to approximately 6.5, 7.0, 7.4 and 7.8. The slope-relating voltage to pH was calculated. The average slope was 61 ± 0.8 mV/10-fold change in H activity (n = 114). The mean difference between the pre- and post-recording calibration was 0.042 ± 0.5 mV/10-fold change in H activity. Most of these pH electrodes showed no sensitivity to any ions or drugs used. However, some were found to have an apparent sensitivity to CO_2, since they gave a pH that was always much more acidic than that determined by the glass pH electrodes. However, these pH microelectrodes were discarded.

Membrane voltage was recorded with 3M KCl electrodes (resistance 5–10 MΩ) made with ordinary borosilicate glass tubing (i.e. 1 mm). Filamented glass tubing was also used to make 3M KCl electrodes in which the resistance was about 25–35 MΩ. Either type was connected to an electrometer (W-P Instruments, M4A) that contained a circuit for injection of current through the recording microelectrode. The electrometer output was connected to a digital voltmeter, oscilloscope, polygraph and a differential amplifier. Microelectrodes were mounted on a micromanipulator (Jena). The cell was first impaled with the pH electrode and about 15 sec allowed for the voltage to attain a steady value. Subsequently, the V_m electrode was placed into the same cell 50–100 μM away and a small (5–10 nA) anodal current was passed. A hyperpolarization recorded from the pH electrode confirmed that the two were in the same cell. The anodal current was turned off and pH_i was recorded for at least 1 min before any manipulations were carried out. It was found that when drift occurred in the pH_i record, it was apparent during the first minute, i.e., soon after impalement by the KCl electrode. In fresh preparations, drift in the pH_i record was seldom a problem, but in preparations that had been in the bath for 5–6 hr or more, an alkaline drift was common and frequently resulted in termination of the experiment. This problem was particularly acute in experiments where an NH_4 prepulse was used to acidify the cell.

V_m and pH_i were recorded on different channels of the polygraph at its minimal chart speed of 0.25 mm/sec. Voltage deflections of the pH_i channel pen were read to the nearest 0.25 mm which, with a typical pH microelectrode, represented a pH difference of 0.003 unit. While continually monitoring pH_i and V_m, various test solutions were introduced into the bath chamber by gravity flow via glass tubing at an initial rate sufficient to produce a fourfold change in bath volume in approximately 20 sec. Inflow was then slowed to 8–10 ml/min. Recovery of pH_i during exposure to acidifying agents is due to extrusion of acid equivalents and was expressed as a rate of change of pH_i per minute. Recovery rates in the presence of CO_2 were taken as the change in pH_i from the minimal value to the maximal pH_i at 15 min. Recovery rates following an NH_4 prepulse were determined from the slope of a line drawn tangent to the pH_i record at a pH_i of 6.9.

When cellular acidification was elicited with acetate or CO_2, estimates of buffering capacity (β) were calculated from the difference between the pre-acid pH_i and the lowest pH_i attained during acid exposure. When acetate was used, β was calculated with the following formula

$$\beta = \frac{C \cdot 10^{(pH_i - pK)}}{\Delta pH_i\,(1 + 10^{(pH_o - pK)})} \tag{1}$$

where C is the total concentration of acetate and acetic acid, pK was 4.57 and pH_o is the external pH. In the presence of CO_2/HCO_3 buffers and acetate, the above equation gives the total buffering capacity ($\beta\tau$). Intrinsic (non-CO_2) buffering capacity (β_i) was obtained by subtracting CO_2 buffering capacity (βCO_2) from $\beta\tau$. βCO_2 was determined from pH_i and the internal [HCO_3] (βCO_2 = ln 10 [HCO_3]$_i$). β was also determined from the change of pH_i attendant to the removal of NH_4 by an equation analogous to the above, transposing the pH and the pK of 9.41. In the nominal absence of CO_2, the above formula yields β_i. Intrinsic buffering capacity was also calculated from the change in pH_i induced by increasing CO_2

$$\beta_i = \frac{\delta P CO_2\, 10^{(pH_i - pK)}}{\Delta pH_i}\,. \tag{2}$$

In this formula, the CO_2 solubility coefficient (δ) was taken as 0.0298 (Harned & Bonner, 1945) and the dissociation constant (pK) was taken as 6.05 (Harned & Davis, 1943). Calculations of β_i were done pursuant to the following assumptions: that the carbonic and acetic acid dissociation constants and CO_2 solubility coefficient are the same inside and outside the cell. In the case where NH_4 was used to acidify the cell, it was assumed that all internal NH_4 diffused from the cell as NH_3, although this probably was not the case in the absence of CO_2/HCO_3 (*see* Discussion). It was also assumed that β_i was independent of pH_i.

Solutions

The basic Ringer solution consisted of the following (in mM): NaCl 126; KCl 5; Na_2HPO_4 1; $NaHCO_3$ 24; $MgCl_2$ 1; glucose 11. Oxygen (95%) and CO_2 (5%) were added to the solution to give a pH of 7.37–7.4. Nominally HCO_3-free solutions were made by substituting HCO_3 with 24 mM N-2-hydroxyethylpiperazine N 2 ethane sulfonic acid (HEPES). An appropriate amount of NaOH was added to adjust the pH to 7.4 at 30°C. These solutions were

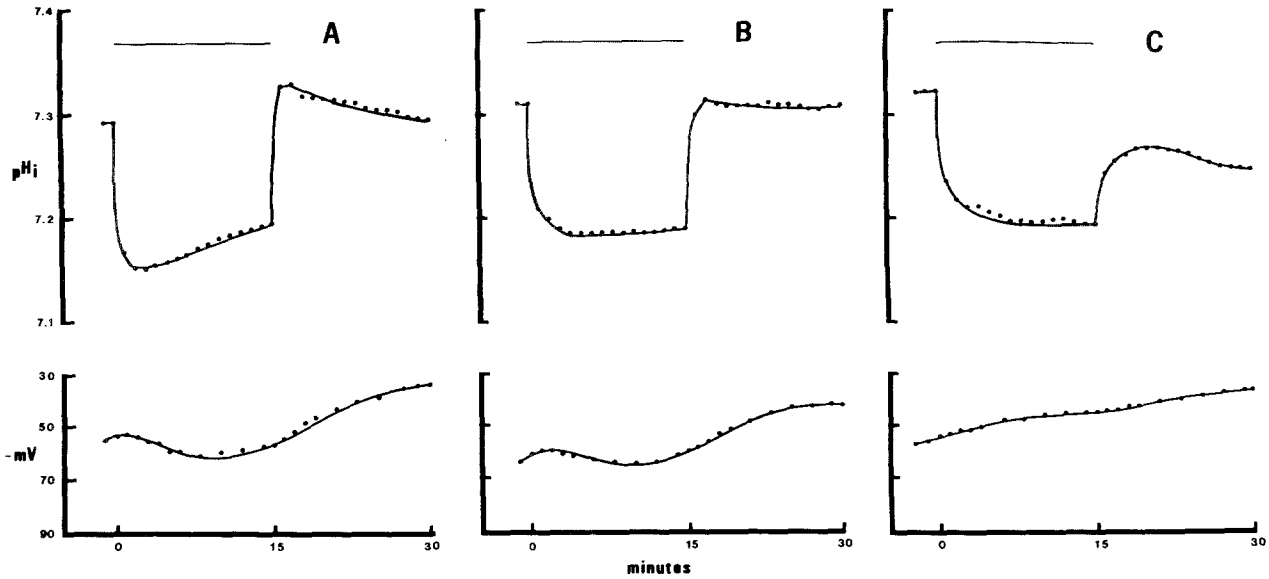

Fig. 1. (*A*) Average pH_i and V_m in Edl muscle cells in response to an increase in CO_2 from nominally 0 to 5%. (*B*) DIDS (100 μM) was added to the CO_2/HCO_3 solution. (*C*) 2 mM amiloride was added to the 5% CO_2 solution. Horizontal bars represent the period of CO_2 application

equilibrated with 100% O_2 with other constituents being the same as the basic Ringer solution. For Na-free, HCO_3-free solutions, 24 mM HEPES was used instead of $NaHCO_3$ and N-methyl-D-glucammonium (NMDG) was supplemented for Na. For Na-free solutions buffered with CO_2/HCO_3, NMDG was substituted for Na and choline HCO_3 was substituted for $NaHCO_3$. Na-free acetate solutions were prepared by adjusting an appropriate concentration of acetic acid to a neutral pH by addition of a known amount of NMDG. These solutions were equilibrated with 100% O_2. When acetic acid was used to reduce pH_i at normal Na concentrations, external Cl was held constant by substituting 20 mM Na gluconate for 20 mM NaCl. Na gluconate was withheld from the Na acetate solution with all other ingredients being the same as for the basic Ringer solution. Cl-free solutions were prepared by substituting gluconate salts and adding 10 mM Ca gluconate to maintain Ca activity (Kenyon & Gibbons, 1977). Abolition of contractile activity in the Cl-free solution was achieved by adding 20 mM of commercially (Aldrich) obtained 2,3 butanedione monoxime (BDM), which appears to inhibit crossbridge function (Mulieri & Alpert, 1984). When an NH_4 prepulse was used as an acidifying agent, 20 or 50 mM NaCl was withheld for equimolar quantities of NH_4. All solutions contained 0.5 mg/ml bovine serum albumin. Amiloride and 4,4' diisothiocyanatostilbene 2,2' disulfonic acid (DIDS) were obtained commercially (Sigma).

Procedure

A practical means of reducing pH_i was via application of CO_2, since recovery could be observed during exposure. pH_i transients in a group of control cells were recorded. Recovery rates and buffering capacities in these cells were then compared to others in the same or different muscles in order to determine effects of drugs or of different solutions. The criteria for a successful recording was a lack of drift in the pH_i record before perturbations were initiated, maintenance of impalement for an appropriate period and the return of the V_m and pH electrode voltages to 2 mV of the initial value.

Results

At 30°C in the basic CO_2/HCO_3 Ringer solution, the average pH_i in 284 cells from 52 Edl muscles was 7.197 ± 0.006 and the average V_m was −76.9 ± 0.24 mV.

Upon switching from nominally CO_2-free to 5% CO_2 solution, the mean pH_i in 12 cells decreased from 7.29 ± 0.028 to 7.15 ± 0.027, and then began to alkalize at an average rate of 0.0044 ± 0.0006 pH unit/min (Fig. 1*A*). Removal of CO_2 after a 15-min exposure resulted in an overshoot, which was followed by an acidification over the subsequent 15-min period. The average V_m in the CO_2-free Ringer was lower than in conditions of normal CO_2/HCO_3. Upon increasing CO_2, V_m became more negative then depolarized upon removal of CO_2/HCO_3.

The pH_i recovery during CO_2 exposure was sensitive to DIDS as shown in Fig. 1*B* where the mean pre-CO_2 pH_i was 7.31 ± 0.06 and decreased to an average of 7.18 ± 0.04 in the presence of CO_2 and 100 μM of DIDS. The recovery rate in three cells average 0.00076 ± 0.0007 and significantly ($P = <0.01$) less than controls. The average V_m was slightly less than controls initially but began to repolarize upon increasing CO_2/HCO_3. In a separate

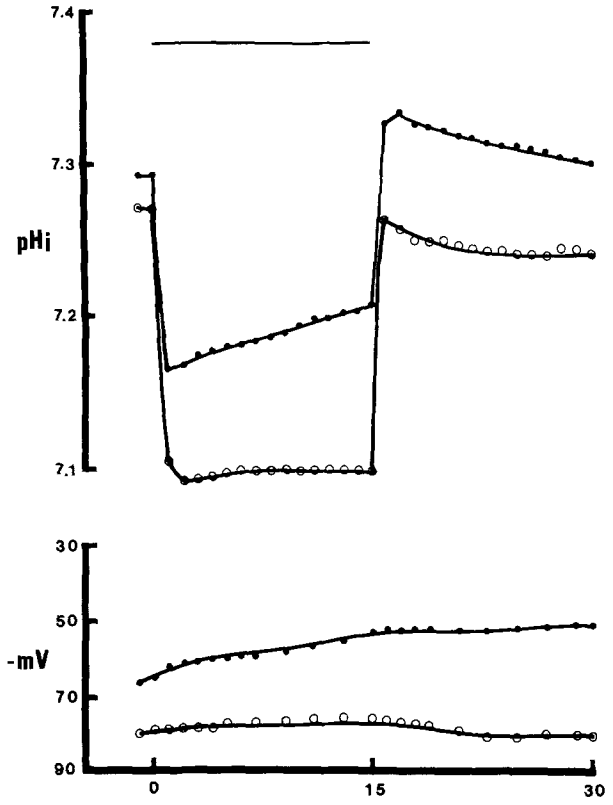

Fig. 2. Average pH_i and V_m in Edl muscle cells in the presence of normal Cl (●) and in a Cl-free (○) condition. Horizontal bar represents the period of CO_2 application. BDM (20 mM) is present throughout in all solutions

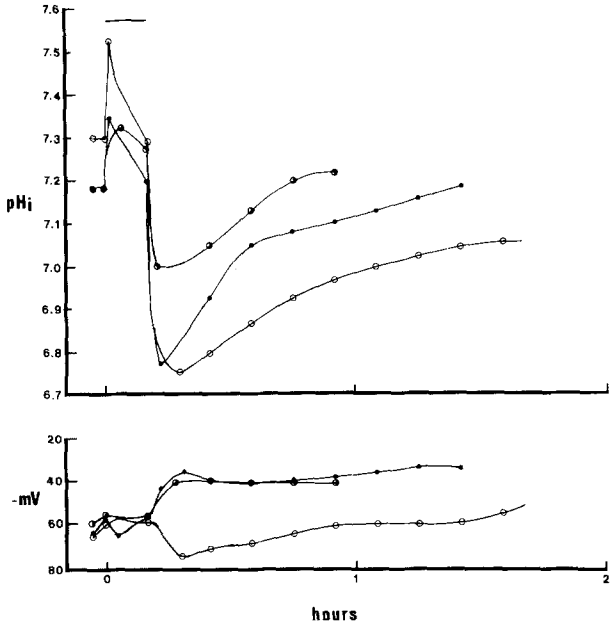

Fig. 3. V_m and pH_i transients in single cells in response to a 10-min pulse (horizontal bar) of 20 mM (◑) and 50 mM (●) NH_4, both in the continuous presence of CO_2/HCO_3, and 20 mM (○) NH_4 in the absence of CO_2/HCO_3

group of five cells (*not shown*), 100 μM of DIDS was added to both the HEPES buffered pre-CO_2 and the CO_2 solution. With continuous exposure to DIDS, the average pre-CO_2 pH_i was 7.19 ± 0.03 and decreased to a mean of 6.97 ± 0.02 in the CO_2HCO_3 solution with no recovery seen during the CO_2 pulse. The mean V_m was initially −66 ± 1.7 mV but no repolarization was seen during CO_2 exposure as was the case in the control condition (Fig. 1*A*).

In a third group of four cells, amiloride (2 mM) was added to the CO_2/HCO_3 solution (Fig. 1*C*). The average initial pH_i in this group was 7.35 ± 0.03. No recovery was seen as the mean pH_i gradually acidified in the presence of CO_2. Removal of CO_2 was followed by an undershoot of the original pre-CO_2 pH_i. Initially V_m was similar to controls, but there was no repolarization in the presence of CO_2.

Separate groups of cells were first exposed to the HCO_3-free Ringer solution that also contained 20 mM BDM (Fig. 2). In five cells, the average pH_i decreased from 7.29 ± 0.02 upon application of CO_2 also containing 20 mM BDM. The subsequent average recovery rate was 0.0038 ± 0.0004 pH unit/min and not significantly different from controls (Fig. 1*A*). CO_2 removal was followed by an overshoot and then a return to the pre-CO_2 pH_i. Average V_m in this group was initially more negative than other controls but the repolarization normally seen in the presence of CO_2 was not apparent. Separate muscles were exposed to a Cl-free, CO_2/HCO_3-free Ringer plus 20 mM BDM. Upon switching to a Cl-free solution with normal CO_2/HCO_3 concentrations, pH_i fell from 7.27 ± 0.029 to 7.09 ± 0.029. Recovery during CO_2 exposure was very slight (0.00084 ± 0.0006 pH_i unit/min) and significantly ($P = <0.001$) less than normal or BDM-treated controls with normal Cl.

Three cells were analyzed in response to CO_2 acidification in the absence of Na in both the pre-CO_2 and the CO_2 solution. No recovery was observed during CO_2 application in any cell. These studies were not vigorously pursued, however, since they would not distinguish between a Na-H antiport and a Na-dependent HCO_3/Cl transport.

An increase in CO_2 consistently failed to produce a substantial decrease in pH_i. Indeed, the lowest mean pH_i attained in the presence of CO_2 was only 7.15. In an effort to reduce pH_i to lower levels, several muscles were exposed to 20 or 50 mM NH_4. Examples of these responses are seen in Fig. 3. In the presence of normal CO_2/HCO_3, a 10-min prepulse of 20 mM NH_4 consistently failed to reduce pH_i much beyond neutral with complete recovery occurring in about 40 min. In order to consistently

Table. Apparent intrinsic buffering capacities (β_i) of Edl muscle measured by various methods

Acidifying		Ion/drug (Added+/omitted−)	β_i (meq H/pH$_i$/liter SE)	n
Agent	Buffer			
0–5% CO_2	HEPES/HCO_3	—	101 ± 11	12
Acetic	HCO_3	—	95 ± 15	11
0–5% CO_2	HEPES/HCO_3	+DIDS[b]	120 ± 6	3
0–5% CO_2	HEPES/HCO_3	+DIDS[c]	40 ± 2	5
0–5% CO_2	HEPES/HCO_3	+Amiloride	93 ± 5	4
0–5% CO_2	HEPES/HCO_3	+BDM	108 ± 7	5
0–5% CO_2	HEPES/HCO_3	+BDM,−Cl	64 ± 12	5
0–5% CO_2	HEPES/HCO_3	+NMDG,−Na	67 ± 10	3
Acetic	HEPES	−HCO_3	52 ± 4	8
Acetic	HEPES	−HCO_3,−Na	44 ± 3	13
NH_4[a]	HCO_3	—	97 ± 18	8
NH_4[a]	HEPES	−HCO_3	87 ± 10	6

[a] β_i measured from the fall in pH$_i$ occurring upon NH_4 removal.
[b] DIDS was applied only during CO_2 acidification.
[c] DIDS was applied before, during and after CO_2 acidification.

decrease pH$_i$ to lower levels in the normal CO_2/HCO_3 solution, muscles were exposed to 50 mM NH_4 for 10 min. This decreased pH$_i$ to about 6.8. However, full recovery from the 50-mM prepulse typically required almost 90 min. In four cells in which impalement was maintained to near completion, recovery rates at a pH$_i$ of 6.9 averaged 0.015 ± 0.0009 pH unit/min. V_m became more positive upon NH_4 removal.

In the nominal absence of CO_2/HCO_3, five cells were prepulsed with 20 mM NH_4. In this condition, this concentration of NH_4 was sufficient to reduce pH$_i$ to 6.7–6.8, but typically, complete recovery required considerable time. In these cells, the mean recovery rate at pH$_i$ 6.9 was 0.0087 ± 0.0012 and significantly (P = <0.01) less than that of cells in the normal CO_2/HCO_3 solution. V_m underwent repolarization upon NH_4 removal. Attempts to record pH$_i$ over these extended periods proved quite difficult. However, measurements of pH$_i$ in individual cells gave support to the slow recovery seen in the absence of CO_2/HCO_3. At an average of 95 ± 6 min after a 10-min prepulse of 20 mM NH_4, the average pH$_i$ in 10 cells was 7.05 ± 0.027. However, at a similar period (96 ± 3 min) in other muscles exposed to a 10-min prepulse of 50 mM NH_4 in the presence of CO_2/HCO_3, the average pH$_i$ in 7 cells was 7.22 ± 0.038 and significantly (P = <0.01) higher than those in the CO_2/HCO_3-free condition. Thus, there appears to be little doubt that removal of CO_2/HCO_3 slows recovery to a significant degree.

The apparent buffering capacity was obtained with CO_2 or acetic acid pulses in the presence and absence of CO_2/HCO_3 and in Cl-free conditions (Table). The average β_i obtained upon increasing CO_2 from 0 to 5% was 101 ± 11 meq H/pH$_i$/liter. This average was not significantly altered in the presence of BDM. DIDS and amiloride had no effect on apparent β_i when both were applied only during the CO_2 pulse. However,. when DIDS was applied to both the HEPES and the CO_2 solutions, the average β_i was 40 ± 2 meq H/pH$_i$/liter (Table) and significantly (P = <0.001) less than the control average. The removal of Cl produced a significant (P = <0.01) reduction in apparent β_i. In the presence of CO_2/HCO_3, β_i calculated with a 20-mM pulse of Na acetate was comparable to that found with CO_2/HCO_3 pulses. Exposure to the same concentration of Na acetate in the absence of CO_2/HCO_3 resulted in an apparent β_i significantly (P = <0.05) less than that of either CO_2/HCO_3 pulses in normal Cl or Na acetate in the presence of CO_2/HCO_3. However, the apparent β_i for Na acetate in the absence of CO_2HCO_3 was not significantly different from that obtained with a CO_2HCO_3 pulse in the absence of Cl. In the absence of both Na and CO_2/HCO_3, β_i measured with 20 mM NMDG acetate gave an average similar to those obtained with Cl-free CO_2/HCO_3 pulses and for those measured with Na acetate in a CO_2/HCO_3-free state. The removal of Na also significantly (P = <0.05) reduced β_i.

In the presence of CO_2/HCO_3, the apparent β_i calculated with a 50-mM NH_4 prepulse was similar to that calculated with a CO_2 pulse. However, in the absence of CO_2/HCO_3, a prepulse with 20 mM NH_4 produced an apparent β_i similar to that obtained in the presence of CO_2/HCO_3. For reasons to be discussed, the latter result is probably unreliable.

Discussion

As shown previously (Grossie, 1985), the removal of CO_2/HCO_3 is followed by a depolarization which is enhanced at 30°C and expectedly tends to be aggravated by dual impalement. The lower V_m was considered a complicating factor for interpretation of a subsequent acid pulse. As a result of this concern, it was deemed more appropriate to pursue these analyses in cells not previously impaled where maximum voltages could be obtained, then comparing the average pH_i, V_m and recovery rates by simple statistics.

The data shows that Edl muscle responds to CO_2 in a manner similar to that seen in squid axon (Boron & De Weer, 1976) and snail neurone (Thomas, 1977). Rat diaphragm appears to recover from CO_2 acidification (Roos & Boron, 1978) whereas frog sartorius and semitendinosis (Bolton & Vaughan-Jones, 1977; Abercrombie, Putnam & Roos, 1983; Putnam et al., 1986) show no recovery from this form of acidification. Similarly, rat soleus does not recover during CO_2 acidification (Vanheel, Hemptinne & Leusen, 1986). The present data shows a rather rapid recovery in response to CO_2, but followed by an overshoot which is attentuated in comparison with that of depolarized frog muscle (Putnam et al., 1986). Reacidification followed the post-CO_2 overshoot. It may be argued that the alkalizing response seen during CO_2 exposure is merely electrode drift. However, it is highly improbable that the electrode would drift in an alkaline direction during CO_2 exposure, then upon withdrawal of CO_2 suddenly reverse and drift in an acid direction. Presently we cannot fully explain the mild overshoot, although it must be influenced by the inability to instantly and completely remove CO_2. It is possible that an alkaline-recovery system may also exist which would blunt the overshoot. However, it is concluded that the alkalizing response seen during CO_2 application is an actual loss of H or equivalent gain in OH or HCO_3.

The recovery from CO_2 acidification is strongly curtailed in the absence of Cl and was almost abolished when DIDS was applied only during CO_2 exposure as well as when the drug was applied before, during and after CO_2 application. These significant reductions in recovery rates strongly implicate a dependence on HCO_3 and Cl for the recovery process at least over this upper range of pH_i values.

The application of 2 mM of amiloride served to convert the usual recovery into an acidification. Taken alone this segment of data would strongly indicate that recovery from CO_2 acidification is entirely due to a Na-H exchange, but this is difficult to reconcile with the clear effects of DIDS and Cl removal and provides no explanation for the effects of low Cl on acid recovery in rat diaphragm (Roos & Boron, 1978). The employed dose of amiloride is rather large and, therefore, may exert nonspecific effects. Amiloride is known to inhibit both a Na-conductive pathway and the Na-H antiport systems in various epithelial cells and it is important that, generally, much higher concentrations are required to inhibit the Na-H exchange pathway than the conductive pathway (Benos, 1982). In Edl muscle, Cl is passively driven inward (Grossie, 1985) while HCO_3 is directed outward. Therefore, an alkalizing HCO_3-Cl exchange is not possible unless an active transport system exists. On the other hand, a system that exchanges external $NaHCO_3$ for internal Cl could be amiloride sensitive. In renal collecting tubules, an amiloride-sensitive $NaHCO_3$ transport has been reported (McKinney & Burg, 1978). Therefore, a possible explanation for the present data is that the drug can inhibit both types of acid recovery. Given the differential effects of amiloride dose on the conductive and Na-H pathways, experiments with smaller doses of amiloride would still be inconclusive. If a reduced rate of (or no effect on) recovery were observed with, for example, 0.1 or 1 mM amiloride, one might conclude that the dose was too low to inhibit the Na-H exchanger and experiments with higher doses would be indicated. An additional disturbing feature of the amiloride data was that the removal of CO_2 and amiloride elicited an increase in pH_i to a level that was still lower than the pre-CO_2 value. A similar transient was shown with 1 mM amiloride applied to normally polarized frog muscle (Putnam et al., 1986), but currently we can offer no explanation for the observation.

In mouse soleus, removal of 10 or 20 mM NH_4 consistently reduces pH_i well below 7.0 in CO_2/HCO_3 (Aickin & Thomas, 1977) or 20 mM HEPES (Vanheel et al., 1986). In these reported experiments, intracellular H activity peaked several minutes after NH_4 removal and recovery was complete in about 10 min. In the present experiments, however, 20 mM NH_4 frequently failed to produce an acidification lower than about neutral if HCO_3 was present. To consistently decrease pH_i to lower levels in the presence of CO_2/HCO_3, $[NH_4]$ was raised to 50 mM. This reduced pH_i to about 6.7, but time for complete recovery was about four-fold that of mouse soleus (Aickin & Thomas, 1977*b*). Although many recordings failed, usually because of a loss of impalement, a sufficient number of cells were followed to obtain an average recovery rate at a pH_i of 6.9 with a 6% SEM. In the absence of CO_2/HCO_3, however, a similar level of acidity was achieved with only 20 mM NH_4 with an even greater amount of time necessary for recovery as reflected in the

significantly lower mean recovery rate. This slow rate was confirmed in other cells at various times following NH_4 removal. Although the pH_i recording was not sufficiently stable for continuous measurement, their pH_i, obtained upon initial impalement, showed a significantly lower value than cells in other muscles exposed to an $[NH_4]$ 2.5 times higher in the normal CO_2/HCO_3 environment. While the data shows that recovery rate in the nominal absence of CO_2/HCO_3 is only about 58% of that seen in the presence of CO_2/HCO_3, the actual total buffering capacity of the cell is reduced in the nominal absence of CO_2/HCO_3. Therefore, the average H extrusion rate following an NH_4 prepulse is far less than in the presence of HCO_3. These experiments strongly suggest a prominent role for HCO_3 in the recovery process in this muscle.

An analysis of the apparent β_i also suggests an involvement of HCO_3 in pH_i regulation. Meaningful assessments of β_i can only be obtained in conditions in which all forms of H or OH transport are eliminated. Most likely this qualification is not fully attained except in the absence of Cl, Na or $NaHCO_3$. The presence of these ions will facilitate H transport either by the Na-H or the putative $NaHCO_3$-Cl exchange, thus reducing the ΔpH_i and increasing the apparent β_i. It is also noteworthy that the apparent β_i was greatly reduced when DIDS was applied both before and during CO_2 exposure. When applied in this manner, DIDS reduced β_i to a value similar to that seen in the absence of $NaHCO_3$. The larger apparent β_i values seen in control conditions are taken as further evidence that not only the Na-H antiport but an additional HCO_3 transport system serves to raise pH_i in this muscle while acidification is being induced. However, when this transport is eliminated by removal of Na, Cl or HCO_3 or by appropriate application of DIDS, the calculated intrinsic buffering capacity of Edl muscle is similar to that reported for soleus muscle (Aickin & Thomas, 1977*a*).

When β_i was calculated from the decrease in pH_i occurring upon NH_4 removal, the absence of CO_2/HCO_3 appeared to have no effect. In the absence of CO_2/HCO_3, the influx of NH_3 usually caused a greater rise in pH_i, followed by a more rapid acidification. A possible explanation for the enhanced "plateau acidification" in the HCO_3-free state is an increased permeability of the sarcolemma to NH_4 coupled, perhaps, with the absence of an inward transport of HCO_3. As a result of a higher permeability, a portion of the internal NH_4 leaves the cell as the ion instead of the NH_3 form, which leaves H in the cell. These events would lead to a reduction in the pH_i change seen upon NH_4 removal.

In summary, the presented data shows that Edl muscle undergoes recovery from a CO_2 acidification. This recovery is significantly reduced by DIDS and Cl removal. Recovery from acidification produced by an NH_4 prepulse is reduced by about 40% in the absence of HCO_3. The absence of HCO_3 also appears to prevent pH_i from attaining a value of about 7.2 normally found in the CO_2/HCO_3 condition. Therefore, while HCO_3 has but a limited role in the regulation of pH_i in soleus (Aickin & Thomas, 1977*b*), the present data shows that HCO_3 has considerable importance in the complete recovery of pH_i in Edl muscle and in this regard is similar to barnacle muscle (Boron, McCormick & Roos, 1979), smooth muscle (Aickin & Brading, 1984), snail (Thomas, 1977) and crayfish (Moody, 1981) neurones. The proposed model in fast-twitch muscle is that regulation is accomplished by both a Na-H exchanger and a second system in which external Na and HCO_3 is exchanged for internal Cl. Each system is responsible for removal of about equal amounts of acid equivalents from the cell until pH_i rises to about 7.0–7.1. Above this range, the Na-H exchanger operates at a rate so low as to be negligible, whereas the Na/HCO_3-Cl exchanger raises pH_i to maintain a value of about 7.2. This model explains most of the observed pH_i transients as well as providing a physiological basis for the finding that internal Cl activity in Edl muscle is less than that predicted by a passive distribution and definitely appears to be controlled, in part, by external HCO_3 (Grossie, 1985).

The authors express genuine appreciation to Professor W. Simon, Zurich, for the H exchanger and to the Ohio State University Graduate School for partial support.

References

Abercrombie, R.F., Putnam, R.W., Roos, A. 1983. The intracellular pH of frog skeletal muscle: Its regulation in isotonic solution. *J. Physiol. (London)* **345:**175–187

Aickin, C.C., Brading, A.F. 1984. The role of chloride-bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *J. Physiol. (London)* **349:**587–606

Aickin, C.C., Thomas, R.C. 1977*a*. Microelectrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibres. *J. Physiol. (London)* **267:**791–810

Aickin, C.C., Thomas, R.C. 1977*b*. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *J. Physiol. (London)* **273:**295–316

Ammann, D., Lanter, F., Steiner, R.A., Schulthess, P., Shijo, V., Simon, W. 1981. Neutral carrier based hydrogen ion selective microelectrodes for extra and intracellular studies. *Anal. Chem.* **53:**2267–2269

Benos, D.J. 1982. Amiloride: A molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242:**C131–C145

Bolton, T.B., Vaughan-Jones, R.D. 1977. Continuous direct measurement of intracellular chloride and pH in frog skeletal muscle. *J. Physiol. (London)* **270:**801–833

Boron, W.F., DeWeer, P. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. Gen. Physiol.* **67:**91–112

Boron, W.F., McCormick, W.C., Roos, A. 1979. pH regulation in barnacle muscle fibers: Dependence on intracellular and extracellular pH. *Am. J. Physiol.* **237:**C185–C193

Grossie, J. 1982. Contractile and electrical characteristics of extensor muscle from alloxan-diabetic rats: An in vitro study. *Diabetes* **31:**194–202

Grossie, J. 1985. Effect of bicarbonate on resting potentials in mammlian skeletal muscle. *J. Cell. Physiol.* **125:**115–121

Harned, H.S., Bonner, F.T. 1945. The first ionization of carbonic acid in aqueous solutions of sodium chloride. *J. Am. Chem. Soc.* **67:**1026-1031

Harned, H.S., Davis, R. 1943. The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions for 0 to 50°. *J. Am. Chem. Soc.* **65:**2030–2037

Kenyon, J.L., Gibbons, W.R. 1977. Effects of low chloride solutions on action potentials of sheep cardiac Purkinje fibers. *J. Gen. Physiol.* **70:**635–660

Kushmerick, M.J. 1987. Energetics studies of muscles of different types. *Basic Res. Cardiol. (Suppl. 2)* **82:**17–30

McKinney, T.D., Burg, M.B. 1978. Bicarbonate absorption by rabbit cortical collecting tubules in vivo. *Am. J. Physiol.* **234:**F141–F145

Moody, W.J. Jr. 1981. The ionic mechanism of intracellular pH regulation in crayfish neurones. *J. Physiol. (London)* **316:**293–308

Mulieri, L.A., Alpert, N. 1984. Differential effects of 2,3-butanedione monoxime (BDM) on activation and contraction. *Biophys. J.* **45:**47a

Niederle, B., Mayr, R. 1978. Course of denervation atrophy in type I and type II fibres of rat extensor digitorum longus muscle. *Anat. Embryol.* **153:**9–21

Paulus, S.F., Grossie, J. 1983. Skeletal muscle in alloxan-diabetes: A comparison of isometric contractions in fast and slow twitch muscle. *Diabetes* **32:**1035–1039

Putnam, R.W., Roos, A., Wilding, T.J. 1986. Properties of the intracellular pH regulating system of frog skeletal muscle. *J. Physiol. (London)* **381:**205–219

Roos, A., Boron, W.F. 1978. Intracellular pH transients in rat diaphragm muscle measured with DMO. *Am. J. Physiol.* **235:**C49–C54

Russell, J. 1978. Effects of ammonium and bicarbonate-CO_2 on intracellular chloride levels in *Aplysia* neurones. *Biophys. J.* **22:**131–137

Thomas, R.C. 1977. The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *J. Physiol. (London)* **273:**317–338

Vanheel, B., Hemptinne, A. de, Leusen, I. 1986. Influence of surface pH on intracellular pH regulation in cardiac and skeletal muscle. *Am. J. Physiol.* **250:**C748–C760

Received 10 March 1988; revised 27 June 1988