Intracellular Alkalination Caused by Chloride Removal in the Smooth Muscle of Guinea-Pig Vena Cava

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Abstract Intracellular pH (pH$_i$) of smooth muscle of guinea-pig vena cava was measured with a pH-sensitive dye, 4',5'-dimethyl-5-(and -6-)carboxyfluorescein. Sustained intracellular alkalination (pH$_i$ 7.46) was produced when Cl$^-$ was replaced with gluconate in the presence of HCO$_3^-$, but not produced in the absence of HCO$_3^-$. Alkalination caused by Cl$^-$ removal was nearly completely blocked by 200 $\mu$M DIDS, a Cl$^-$ transport inhibitor. In the solution containing normal Cl$^-$ concentration, pH$_i$ (7.15) was not affected by DIDS, and pH$_i$ was also nearly the same in the presence and absence of HCO$_3^-$. When full alkalination was developed in Cl$^-$-deficient solution, HCO$_3^-$ removal produced only a weak recovery. A carbonic anhydrase inhibitor, acetazolamide (100 $\mu$M) had no clear effect on pH$_i$ in both normal and Cl$^-$-deficient solutions. Intracellular acidification caused by Na$^+$ removal was much less when NaCl was replaced with sucrose than with N-methyl-D-glucamine chloride. It is concluded that when Cl$^-$ is removed, pH$_i$ can be strongly altered by a Cl$^-$-HCO$_3^-$ exchange mechanism in the guinea-pig vena cava. A contribution of this mechanism to pH$_i$ is, however, probably rather minor under physiological conditions.

Key words: intracellular pH, chloride removal, Cl-HCO$_3^-$ exchange, vascular muscle, smooth muscle.

Na$^+$-H$^+$ exchange is the principal cellular pH regulating system in various tissues, including smooth muscle, but HCO$_3^-$ also seems to play an important role in intracellular pH (pH$_i$) regulation [1]. In some smooth muscles, it is known that pH$_i$ in the steady state is more acidic in the nominal absence than in the presence of CO$_2$ and HCO$_3^-$, probably due to the presence of a HCO$_3^-$-dependent mechanism for acid extrusion [2, 3]. Removal of Cl$^-$ causes alkalination in some smooth muscles (guinea-pig vas deferens [4]; rabbit aorta [5, 6], but not in the guinea-pig ureter [2]). These results strongly suggest the presence of a Cl$^-$-HCO$_3^-$ exchange mechanism in many smooth muscles, as first proposed for guinea-pig vas deferens.

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In the present experiments, we investigated effects of Cl\(^-\) and HCO\(_3\)^- removal on pH\(_i\) in the guinea-pig vena cava by measuring the absorbance of the pH-sensitive dye, 4',5'-dimethyl-5-(and -6-)carboxyfluorescein.

**METHODS**

Guinea-pigs weighing 250–350 g of either sex were stunned and bled, and vena cava was excised. After cutting longitudinally, the internal surface was gently rubbed with wet cotton to remove the endothelium. The preparation was set up in a small organ bath (20×20×3 mm) to record changes in pH\(_i\). The bath was perfused with prewarmed physiological solution (35\(^o\)C) continuously at a rate of 5 ml/min. pH\(_i\) was obtained by measuring the absorption spectrum of the pH-sensitive dye, 4',5'-dimethyl-5-(and -6-)carboxyfluorescein (Me\(_2\)CF) with the multi-photospectrometer (Otsuka Denshi, MCPD-100). Me\(_2\)CF was loaded by exposing the tissue to the membrane-permeable Me\(_2\)CF diacetate (50 \(\mu\)M) for 30–60 min. After correcting the spectrum, as described separately [7], pH\(_i\) was estimated from the ratio of absorbance of the tissue at 513 and 476 nm, based on the calibration curve obtained in the presence of nigericin (10 \(\mu\)M).

Physiological solution contained (mM): NaCl 127, KHCO\(_3\) 6, CaCl\(_2\) 2.4, MgCl\(_2\) 1.2, glucose 12, and HEPES buffer 10 (pH 7.4). The solution was gassed with either 100% O\(_2\) or air, but these made no clear difference in the results. When Cl\(^-\) was removed, gluconate was used as the substitute. NaCl was replaced isoosmotically either with sucrose or N-methyl-d-glucamine (NMDG). When HCO\(_3\)^- was removed, KHCO\(_3\) was replaced with KCl. In some experiments, HCO\(_3\)^- concentration was increased to 24 mM by replacing NaCl with equimolar NaHCO\(_3\), and the solution was gassed with 5% CO\(_2\) and 95% O\(_2\).

Nigericin was obtained from Calbiochem, and acetazolamide and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) were obtained from Sigma. Me\(_2\)CF diacetate was purchased from Molecular Probes.

**RESULTS**

When the external Cl\(^-\) concentration ([Cl\(^-\)]) was reduced by replacing NaCl with Na gluconate in physiological solution containing 6 mM HCO\(_3\)^-, pH\(_i\) increased concentration-dependently (Fig. 1a). The rate of change was rather slow, taking about 10 min to reach a steady state. pH\(_i\) in physiological solution (7.15±0.03, \(n = 15\)) was increased to 7.46±0.05 (\(n = 11\)) 10 min after incubation in 7 mM Cl\(^-\) solution. Intracellular alkalization in Cl\(^-\)-deficient solution was maintained at a relatively constant level and the rate of recovery on Cl\(^-\) reapplication was similar to the rate of alkalization on Cl\(^-\) removal (Fig. 1b).

The presence of HCO\(_3\)^- was necessary for intracellular alkalization caused by Cl\(^-\) removal (Fig. 2). The alkalization was greater with higher concentrations of HCO\(_3\)^- between 1 and 6 mM (with 10 mM HEPES buffer). When HCO\(_3\)^- was
Fig. 1. Intracellular alkalization produced by reducing Cl\textsuperscript{−} concentration of the external solution in guinea-pig vena cava. Intracellular pH was measured with a pH-sensitive dye, Me\textsubscript{2}CF\textsuperscript{−}. NaCl was replaced with equimolar Na gluconate. When NaCl was totally replaced, 7 mM Cl\textsuperscript{−} remained as CaCl\textsubscript{2} and MgCl\textsubscript{2}. The external pH was kept constant at 7.4 with 10 mM HEPES buffer. a and b are from different preparations.

Fig. 2. Effects of HCO\textsubscript{3}\textsuperscript{−} removal on intracellular alkalization caused by Cl\textsuperscript{−} removal. Cl\textsuperscript{−} was reduced to 7 mM Cl\textsuperscript{−} by replacing with gluconate as indicated by horizontal bars underneath. 10 mM HEPES buffer was used, except for the solution containing 24 mM HCO\textsubscript{3}\textsuperscript{−} and 5\% CO\textsubscript{2}. Note no response to Cl\textsuperscript{−} removal in the absence of HCO\textsubscript{3}\textsuperscript{−}. Note also only transient changes in intracellular pH when 24 mM HCO\textsubscript{3}\textsuperscript{−} was applied together with 5\% CO\textsubscript{2}. Different preparations for a and b.

further increased to 24 mM HCO\textsubscript{3}\textsuperscript{−} (with 5\% CO\textsubscript{2}), the response to Cl\textsuperscript{−} removal was the same as that observed in 6 mM HCO\textsubscript{3}\textsuperscript{−}. Removal of HCO\textsubscript{3}\textsuperscript{−} had little effect on pH\textsubscript{i} on its own (Fig. 2a). When the solution was buffered with 24 mM HCO\textsubscript{3}\textsuperscript{−} plus 5\% CO\textsubscript{2}, pH\textsubscript{i} decreased by about 0.1–0.2 unit transiently, but pH\textsubscript{i} at the steady

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Fig. 3. Effects of HCO$_3^-$ removal in Cl$^-$-deficient solution (7 mM Cl$^-$) (a) and of acetazolamide (100 μM) (b). Normal external solution contained 6 mM HCO$_3^-$.

See text for further explanation.

Fig. 4. Inhibitory effects of DIDS on intracellular alkalization caused by Cl$^-$ removal. DIDS (200 μM) was added to perfusing solution before the second application of 7 mM Cl$^-$ solution as indicated above the records. In (a) 6 mM HCO$_3^-$ was present and in (b) 24 mM HCO$_3^-$ (5% CO$_2$) was added to HCO$_3^-$-free solution as indicated.

state did not change significantly (Fig. 2b).

Although removal of HCO$_3^-$ before reducing [Cl$^-$] had a strong inhibitory effect on pH$_i$ changes caused by Cl$^-$ removal (Fig. 2), removal of HCO$_3^-$ after the development of alkalization in 7 mM Cl$^-$ solution produced only a very slow and weak inhibitory effect (Fig. 3a). This result was confirmed in two other preparations.

Acetazolamide (100 μM), the inhibitor of carbonic anhydrase [8], had no clear effect on intracellular alkalization caused by Cl$^-$ removal (Fig. 3b).

Figure 4 shows effects of DIDS, a Cl$^-$ transport inhibitor [9]. In the presence
of normal [Cl\(^-\)], DIDS had no clear effect on pH\(_i\). On the other hand, 200 \(\mu\)M DIDS nearly completely blocked intracellular alkalinization caused by Cl\(^-\) removal in the presence of 6 mM HCO\(_3^-\) (a) or 24 mM HCO\(_3^-\) (b).

Removal of Na\(^+\) is known to cause intracellular acidification in vascular smooth muscle, probably due to impairment of Na\(^+\)-H\(^+\) exchange [10–12]. An example of the acidification on Na\(^+\) removal observed in the guinea-pig vena cava is shown in Fig. 5. In this experiment, sucrose and NMDG were used as Na\(^+\) substitute. In the absence of HCO\(_3^-\) both substitutes produced a similar degree of acidification, whereas in the presence of 6 mM HCO\(_3^-\) the sucrose substitution produced much less acidification than the NMDG substitution. Applications of NH\(_4\)Cl (20 mM) produced expected changes in pH\(_i\) [13]; i.e., alkalinization during application and transient acidification on removal of NH\(_4\)Cl. These pH\(_i\) responses were very similar in the presence and in the absence of HCO\(_3^-\), except for slightly faster recovery from acidification following NH\(_4\)Cl washout in the presence of HCO\(_3^-\) than in the absence of HCO\(_3^-\).

**DISCUSSION**

As already reported for some smooth muscles [4–6], removal of Cl\(^-\) produces an increase in pH\(_i\) in the presence of HCO\(_3^-\), but not in the absence of HCO\(_3^-\) in the guinea-pig vena cava. This alkalinization is likely to be mediated through a Cl\(^-\)-HCO\(_3^-\) exchange mechanism, which can be blocked by DIDS. Weak intracellular acidification by DIDS treatment has been reported for cultured vascular muscles [6, 10] and rat mesenteric artery [14] in the presence of HCO\(_3^-\). In the present experiments, however, DIDS applied for more than 10 min did not alter pH\(_i\) in normal solution, even at a concentration enough to block alkalinization in
Cl⁻-deficient solution nearly completely. This negative effect of DIDS may be related to the fact that HCO₃⁻ removal did not modify pHᵢ in the solution containing normal [Cl⁻] in the vena cava. Thus, it is unlikely that Cl⁻-HCO₃⁻ exchange contributes significantly to the pHᵢ regulation under physiological conditions at least in this muscle.

Alkalization in Cl⁻-deficient solution can be maintained for more than 30 min. In the guinea-pig vas deferens, intracellular [Cl⁻] is reduced relatively rapidly in Cl⁻-free solution with the time constant of about 6 min [15]. A decrease in the activity of Cl⁻:HCO₃⁻ exchange is expected to occur, as the cellular Cl⁻ content decreases in Cl⁻-deficient solution. Thus, a constant influx of HCO₃⁻ does not seem necessary to keep a prolonged alkalization caused by Cl⁻ removal. A very weak effect of HCO₃⁻ removal after full development of the effect of Cl⁻-deficient solution supports this idea.

pHᵢ at the steady state is higher in the presence of HCO₃⁻ in some smooth muscles (guinea-pig vas deferens [4]; cultured vascular muscle [3, 6, 10]), but in the present experiments on the guinea-pig vena cava, removal and reapplication of HCO₃⁻ did not alter pHᵢ in the solution containing normal [Cl⁻], except for a transient change. The lack of the effect of HCO₃⁻ on steady state pHᵢ has also been reported for guinea-pig ureter [2] and rat mesenteric artery [14]. The activity of Cl⁻:HCO₃⁻ exchange under normal conditions may differ in different smooth muscles. As observed in guinea-pig ureter [2], acetazolamide has no effect on the steady state pHᵢ and also on the alkalization induced by Cl⁻ removal in the vena cava. This is probably due to the fact that these smooth muscles contain little carbonic anhydrase, as reported for other smooth muscles [8, 16]. The relationship between the activities of Cl⁻:HCO₃⁻ exchange and carbonic anhydrase should be further investigated.

Since the recovery from acidification upon NH₄Cl washout is not significantly affected by HCO₃⁻ removal, Cl⁻:HCO₃⁻ exchange may not be involved in the pHᵢ recovery from acidification induced by NH₄Cl removal, as reported for cultured vascular muscle [6]. In this recovery process, an Na⁺-H⁺ exchange mechanism is probably playing a major role [12].

Na⁺ removal causes intracellular acidification and this can be explained by impairment of Na⁺-H⁺ exchange [10-12, 14]. When Cl⁻ is simultaneously removed, by substituting NaCl with sucrose, however, the degree of acidification becomes clearly less in the presence of HCO₃⁻. Therefore, acidification is stronger for NMDG replacement (normal [Cl⁻]) than sucrose replacement (low [Cl⁻]) for NaCl. In the absence of HCO₃⁻, this difference in acidification was greatly reduced. The acidification caused by Na⁺ removal seems antagonized by alkalization caused by simultaneous Cl⁻ removal which activates Cl⁻:HCO₃⁻ exchange.

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