Regulation of ion transport by pH and [HCO$_3^-$] in isolated gills of the crab *Neohelice* (Chasmagnathus) granulata

Martin Tresguerres,1,2* Scott K. Parks,1* Sebastian E. Sabatini,3,4 Greg G. Goss,1 and Carlos M. Luquet3,5

1Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada; 2Department of Pharmacology, Weill Medical College of Cornell University, New York, New York; 3Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina; 4Departamento Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina; and 5Asentamiento Universitario San Martín de los Andes, Neuquén, Argentina

Submitted 16 July 2007; accepted in final form 7 January 2008

Tresguerres M, Parks SK, Sabatini SE, Goss GG, Luquet CM. Regulation of ion transport by pH and [HCO$_3^-$] in isolated gills of the crab *Neohelice* (Chasmagnathus) granulata. *Am J Physiol Regul Integr Comp Physiol* 294: R1033–R1043, 2008. First published January 9, 2008; doi:10.1152/ajpregu.00516.2007.—Posterior isolated gills of *Neohelice* (Chasmagnathus) granulatus were symmetrically perfused with hemolymph-like saline of varying [HCO$_3^-$] and pH. Elevating [HCO$_3^-$] in the saline from 2.5 to 12.5 mmol/l (pH 7.75 in both cases) induced a significant increase in the transepithelial potential difference ($V_{te}$), a measure of ion transport. The elevation in [HCO$_3^-$] also induced a switch from acid secretion ($43.7 \pm 22.5$ μequiv kg$^{-1}$ h$^{-1}$) in controls to base secretion ($84.7 \pm 14.4$ μequiv kg$^{-1}$ h$^{-1}$). The HCO$_3^-$-induced $V_{te}$ increase was inhibited by basolateral acetazolamide (200 μmol/l), amiloride (1 mmol/l), and ouabain (5 mmol/l) but not by bafilomycin (100 mmol/l). The $V_{te}$ response to HCO$_3^-$ did not take place in Cl$^{-}$-free conditions; however, it was unaffected by apical SITS (2 mmol/l) or DIDS (1 mmol/l). A decrease in pH from 7.75 to 7.45 pH units in the perfusate also was unaffected by apical SITS (2 mmol/l) or DIDS (1 mmol/l). Other ion-transporting proteins have been molecularly and/or pharmacologically identified in crab gills, but to our knowledge, only their role in ion uptake and not A/B regulation has been directly tested (reviewed in Ref. 7).

A major advantage of the gills of certain crustaceans over other A/B regulatory organs (i.e., fish gill and mammalian kidney) is that they can be easily isolated from the animal and perfused with hemolymph-like saline using a peristaltic pump. This technique allows for the study of the ion transport physiology of the whole organ without the interference of hormonal or nervous factors. Isolated and perfused gills allow for accurate control of the composition of the perfusing and bathing salines, including the addition of specific inhibitors of ion-transporting proteins. These manipulations are impossible to perform in whole animal experiments.

The cellular mechanisms for ion uptake across the posterior gills of *Neohelice granulata* (formerly named *Chasmagnathus granulatus*) are one of the better studied among aquatic animals. The basal (nonstimulated) ion transport mechanism in gills from crabs acclimated to low-salinity water (2 ‰) includes basolateral Na$^+$-$K^+$-$2Cl^-$ cotransporters (NKCC) in parallel with K$^+$ channels (24, 25, 30). In this condition, the apical routes of entry for Cl$^-$ and Na$^+$ are apical Na$^+$-$K^+$-$2Cl^-$ (NKCC) cotransporters in parallel with K$^+$ channels (25, 30). This basal state of ion uptake can be activated by hormonal and nonhormonal factors. For example, dopamine can activate Na$^+$-$K^+$-ATPase via a D1-like receptor-G$_s$ protein-cAMP-PKA pathway (9, 11). A reduction in the osmolarity of the hemolymph-side of the isolated gill also stimulates ion uptake (24, 39), an effect that is partially mediated by cAMP and Na$^+$-$K^+$-ATPase (39). In addition, CA and apical V$^+$-H$^+$-ATPases and Cl$^-$-HCO$_3^-$ exchangers (CBEs) are also recruited during these stimulated conditions (8). Apical V$^+$-H$^+$-ATPases and CBEs working in concert could facilitate Cl$^-$ uptake from a dilute external medium (8), but the combined effect of both transporters is neutral in terms of net A/B transport. Interestingly, the branchial cellular mechanisms for ion transport in *N. granulata* from different salinities resemble those from different segments of the mammalian nephron. This is likely related to similar ionic gradients encountered at the apical membrane in each system.

In the current work, we investigated the involvement of the gills of *N. granulata* in A/B-relevant ion transport. We used the...
advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt commercial available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

The Canadian Council for Animal care procedures. Stage C intermolt

pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

The Canadian Council for Animal care procedures. Stage C intermolt

pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.

MATERIALS AND METHODS

Animals. N. granulata (Dana 1851) were collected by hand from a muddy beach at San Antonio Oeste (Rio Negro, Argentina) during the Austral summer of 2006–2007 and spring of 2007. The animals were transported to the Laboratory of Aquatic Ecotoxicology Centro Internacional de Educación para el Desarrollo (CIEDE, San Martin de los Andes, Neuquén, Argentina), where the gill perfusion experiments were performed. Crabs were acclimated in plastic containers with aerated seawater of 2% salinity for at least 1 wk. Water temperature was kept at 18 ± 2°C. The animals were fed twice a week with commercially available pellets of trout food. All procedures followed the Canadian Council for Animal care procedures. Stage C intermolt

Adapted from: Advantages of the isolated gill preparation to test the hypothesis that ion transport can be directly stimulated by [HCO₃] and pH. Our results indicate that ion transport is stimulated by high [HCO₃] and by low pH and that the specific mechanisms are different in each condition.
was fully reversible, since \( V_{te} \) returned to its basal value after reintroducing the original control saline (3.15 ± 0.49 mV). A subsequent application of a saline with control \( [\text{HCO}_3^-] \) but with a higher pH of 7.81 did not have any significant effect on \( V_{te} \) (2.83 ± 0.45 mV, \( n = 6, P > 0.05 \)). However, the saline with high \( [\text{HCO}_3^-] \) and a control pH of 7.75 induced a significant increase on \( V_{te} \), of a similar magnitude to the “high \( [\text{HCO}_3^-] \) pH 7.81” saline (5.17 ± 0.88 mV, \( n = 6, P < 0.05 \)).

These results indicate that an increase in the saline \( [\text{HCO}_3^-] \) and not pH, is the stimulus that ultimately causes the elevations on \( V_{te} \). Consequently, only the “high \( [\text{HCO}_3^-] \) pH 7.75” saline was used for the pharmacological characterization. These results are shown in Fig. 1, A and B.

Gills perfused with the control saline secreted an apparent \( \text{H}^+ \) flux \((J_{\text{H}^+})\) of \(-43.72 ± 22.5 \) μequiv·kg\(^{-1}\)·h\(^{-1}\). Perfusion with “high \( [\text{HCO}_3^-] \) pH 7.81” saline induced the reverse in secretion to an apparent \( \text{HCO}_3^- \) flux \((J_{\text{HCO}_3^-})\) of 84.7 ± 14.4 μequiv·kg\(^{-1}\)·h\(^{-1}\). Therefore, the net change \((J_{\text{ctrol}} - J_{\text{bicarb}})\) in \( J_{\text{HCO}_3^-} \) was of 128.5 ± 31.6 μequiv·kg\(^{-1}\)·h\(^{-1}\) \((P < 0.05; n = 5)\) (Fig. 1C). This indicates that the elevations in \( V_{te} \) are accompanied by net base efflux across the gill epithelium.

**Pharmacological characterization.** To test whether carbonic anhydrase (CA) is involved in the \( V_{te} \) response to high \( [\text{HCO}_3^-] \), we added 200 μmol/l acetazolamide into the high \( [\text{HCO}_3^-] \) perfusate. Acetazolamide completely and reversibly abolished the increased \( V_{te} \) (Fig. 2, A and B). Bafilomycin is a specific inhibitor of V-\( \text{H}^+\)-ATPases at nanomolar concentrations (5). Basolateral application of bafilomycin (100 nmol/l) did not exert any significant effect on the \( \text{HCO}_3^- \)-stimulated \( V_{te} \), suggesting that V-\( \text{H}^+\)-ATPase is not important in this process (Fig. 2, A and C). On the other hand, ouabain (5 mmol/l) almost completely blocked \( V_{te} \) both during control (not shown) and high \( [\text{HCO}_3^-] \) conditions (Fig. 2D), demonstrating that Na\(^+\)-K\(^+\)-ATPase is the major driving force for the transepithelial transport of ions. To identify the basolateral route of exit of \( \text{H}^+ \) from the cells into the hemolymph space, we tested the effect of basolateral amiloride (1 mmol/l). This treatment completely and reversibly blocked the \( \text{HCO}_3^- \)-stimulated \( V_{te} \), suggesting that basolateral Na\(^+\)/H\(^+\) exchangers (NHE) are critical for the overall transepithelial transport mechanism activated by high \( [\text{HCO}_3^-] \) (Fig. 3, A and B). Importantly, amiloride did not have any effect on the basal \( V_{te} \), and it completely but reversibly blocked the \( \text{HCO}_3^- \)-induced response when added prior to elevating \([\text{HCO}_3^-]\) (Fig. 3C). This indicates that the putative NHE are specifically recruited for the base secreting mechanism. This finding also shows that the concentration of amiloride used does not significantly affect Na\(^+\)-K\(^+\)-ATPase function.

Our last two experimental series were designed to test for the involvement of apical Cl\(^-\)/HCO\(_3^-\) exchangers. Introduction of Cl\(^-\)-free conditions produced a significant decrease in \( V_{te} \), which reflects the importance of Cl\(^-\) for the basal ion transport mechanism (see 24, 30, 40). An increase of \([\text{HCO}_3^-]\) under Cl\(^-\)-free conditions did not result in the typical stimulation of \( V_{te} \), indicating that Cl\(^-\) ions must be present for the \( \text{HCO}_3^- \) stimulation to occur (Fig. 4, A and B). Returning to normal Cl\(^-\)-containing conditions did not restore the original \( V_{te} \), probably because the unnatural Cl\(^-\)-free saline induced a new basal steady state of ion transport. However, the gill epithelium was able to respond with the typical increase in \( V_{te} \) to an elevation in \([\text{HCO}_3^-]\), indicating that it was still healthy and functional (Fig. 4, A and B). Finally, we tested the effect of apical DIDS (1 mmol/l) on the \( V_{te} \) stimulated by \( \text{HCO}_3^- \), but this treatment did not result in any significant changes in \( V_{te} \) (Fig. 4C). Apical SITS (2 mmol/l) was also without any significant effect on \( V_{te} \) (not shown).

**Stimulation of \( V_{te} \) by low \( \text{pH} \) in isolated perfused gills.** A reduction in the \( \text{pH} \) from 7.75 to 7.45 \( \text{pH} \) units in the perfusion conditions induced an immediate and significant stimulation of \( V_{te} \) from 3.68 ± 0.58 to 6.32 ± 0.72 mV \((n = 13)\). The original \( V_{te} \) was restored upon reapplication of the saline with \( \text{pH} \) of 7.75. The low \( \text{pH} \)-stimulated \( V_{te} \) and subsequent wash-out was a repeatable event (Fig. 5A), and it was accompanied by an
increase in net acid secretion from \(-79.1 \pm 18.5 \) to \(-146.9 \pm 19.2 \) \(\mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\) (net increase of \(-67.8 \pm 18.4 \) \(\mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\), \(P < 0.05\); \(n = 5\)) (Fig. 5B). To test whether CA is involved in the low pH-stimulating mechanism, we added 200 \(\mu\text{mol/l}\) acetazolamide into the 7.45 pH perfusate of isolated gills that had already been stimulated by low pH. Acetazolamide completely and reversibly abolished the stimulated \(V_{te}\) (Fig. 6). We also investigated the involvement of V-H\(^+\)-ATPase. Basolateral bafilomycin (100 \(\mu\text{mol/l}\)) inhibited the low pH-stimulated \(V_{te}\), but, unlike acetazolamide, its effect was only slightly reversible (Fig. 7). However, the original resting \(V_{te}\) was restored upon reintroduction of the control pH 7.75 saline, indicating that the inhibitory effect of bafilomycin specifically acts on the mechanism stimulated by low pH. Next, we tested the involvement of apical Na\(^+\) channels and basolateral Na\(^+\)/HCO\(_3\)\(^-\) cotransporters by adding apical phenamil (50 \(\mu\text{mol/l}\)) and basolateral DIDS (1 \(\mu\text{mol/l}\)), respectively, under low-pH stimulating conditions. An inhibitory effect of phenamil was seen in a 3 out of 5 preparations, but its magnitude was highly variable, and thus it is not shown or analyzed. On the other hand, basolateral DIDS totally inhibited the elevated \(V_{te}\), although it first produced an important \((-10 \text{ mV})\) hyperpolarization of \(V_{te}\) in most of the preparations (Fig. 8). The inhibitory effect of DIDS was not reversible. DIDS did not cause any significant changes when applied under control pH conditions (Fig. 8C), indicating that both the initial hyperpolarization and the inhibitory effect observed are related to the low pH-stimulating mechanism.

Lastly, we tested whether the low-pH stimulated \(V_{te}\) depends on the transepithelial movement of Na\(^+\) and Cl\(^-\) by using solutions with reduced concentrations of these ions. As shown in Fig. 9, these conditions abolished and in some cases even reversed \(V_{te}\). Introduction of Na\(^+\)-free pH 7.45 saline did not elicit an increase in \(V_{te}\), demonstrating that the transepithelial movement of Na\(^+\) (apical channel and basolateral NBC?) is essential for the low pH activation of \(V_{te}\) (Fig. 9, A and B). Conversely, a reduction in saline pH to 7.45 during Cl\(^-\)-free conditions still induced a significant increase of \(V_{te}\) of a magnitude comparable to that in normal, Cl\(^-\)-containing, saline. This effect was only partially reversible (Fig. 9, C and D).

**DISCUSSION**

Our results demonstrate that ion transport across posterior gills of *N. granulata* is stimulated by an increase in [HCO\(_3\)] and also by a decrease in pH. This suggests that the posterior gills are important in detecting and correcting A/B disturbances in the hemolymph of the whole animal.

Gills from crabs acclimated to 2\%e salinity maintain a basal ion uptake activity when perfused with the control saline used in the current study, as estimated from \(V_{te}\), short-circuit current and \(^{22}\text{Na}\) uptake (24, 30, 39). Increasing [HCO\(_3\)] by 10 \(\mu\text{mol/l}\) elevated both \(V_{te}\) and base secretion to the apical medium. An equivalent increase in pH alone did not have any

---

**Fig. 2.** Effects of basolateral acetazolamide, bafilomycin, and ouabain on the HCO\(_3\)\(^-\)-activated \(V_{te}\). A: representative trace showing a trial experiment in which both drugs were applied to the same preparation, but the data shown in B are from independent experiments. B: acetazolamide (200 \(\mu\text{mol/l}\)) summary statistics (\(n = 7\)). C: bafilomycin (100 \(\mu\text{mol/l}\)) summary statistics (\(n = 6\)). D: ouabain (5.00 \(\mu\text{mol/l}\)) summary statistics (\(n = 4\)). Control saline, 2.50 mmol/l HCO\(_3\)\(^-\), 12.5 is the [HCO\(_3\)] in mmol/l, pH of all saline solutions was adjusted to 7.75. The asterisks indicate statistical differences with the control (\(P < 0.05\); one-way repeated-measures ANOVA, Dunnett’s multiple comparison posttest).
effect on $V_{te}$. This indicates that an electrogenic transepithelial base secretion mechanism is activated directly by $[\text{HCO}_3^-]$. The pharmacology profile suggests that the mechanism activated by high $[\text{HCO}_3^-]$ is different from those described in our previous studies (see below). Our $V_{te}$ measurements are not without certain limitations. In particular, $V_{te}$ is a reflection of both current ($I$) and transcellular and paracellular resistance ($R$). Therefore, it is possible that the changes observed were produced by changes in $R$. However, our saline manipulations were always performed at constant osmotic pressure, and thus it is unlikely that paracellular $R$ changes in the dramatic fashion.
that is required to justify the observed changes in $V_{te}$. Similarly, transcellular $R$ would have to increase substantially, which could only happen by a reduction in the conductance of the ion-transporting proteins involved (most likely at the apical membrane). However, this hypothesis would make it difficult to explain the pharmacological inhibition of the stimulated $V_{te}$ by low pH and $\text{HCO}_3^-/\text{H}_2\text{CO}_3$. Therefore, we are confident that the $V_{te}$ measurements are indeed a reasonable estimation of net transepithelial ion transport ($I$) under our experimental conditions.

In addition, $V_{te}$ measurements in open-circuit conditions have certain advantages over short-circuit conditions (i.e., inactivation of V-H$^+$/H$^+$-ATPase, see Refs. 7 and 19).

Carbonic anhydrase. Our results indicate that CA is essential for the gill responses to both high $[\text{HCO}_3^-]$ and low pH. On the basis of current models for branchial A/B regulation in aquatic animals (33), it is likely that both extracellular and intracellular CA are involved in the response to elevated $[\text{HCO}_3^-]$. In fact, both types of CA are present in the posterior gills of C. granulatus (8). Extracellular CA would dehydrate $\text{H}^+$ and $\text{HCO}_3^-$ into $\text{CO}_2$, which can diffuse inside the ion-transporting cells. Once inside, $\text{CO}_2$ is probably rehydrated into $\text{H}^+$ and $\text{HCO}_3^-$ by intracellular CA (Fig. 12), the main intracellular substrates in the mechanisms stimulated by high $[\text{HCO}_3^-]$ and low pH. Unfortunately, the drug used in our study does not allow us to differentiate between extracellular and intracellular CA, and further studies are necessary to confirm the involvement and specific roles of both CA isoforms. On the other hand, saline $[\text{HCO}_3^-]$ was negligible in the low pH experiments. Therefore, the source of $\text{CO}_2$ for the acid secretion mechanism is unclear. One possibility is that the $\text{HCO}_3^-$ that is reabsorbed into the hemolymph combines with $\text{H}^+$, generating $\text{CO}_2$ that diffuses back into the cell. Yet another interesting option is derived from studies on fish intestinal epithelium, where mitochondria-produced $\text{CO}_2$ is an important substrate for ion transport (reviewed in Ref. 10). The gill ionocytes of N. granulata possess large numbers of mitochondria (22, 23), which supports this model. Furthermore, low pH did not produce any change in gill $V_{te}$ when we attempted the same protocol during the winter months. Although preliminary, these results may indicate that gills from winter crabs are metabolically less active than in summer crabs. Whether winter crabs conform to acidotic conditions or use different mechanisms for recovery is a fascinating topic that we are currently investigating.

Responses to high $[\text{HCO}_3^-]$. A priori, we were expecting that basolateral V-H$^+$/H$^+$-ATPases energized the secretion of $\text{HCO}_3^-$ as suggested for hagfish (42, 44), elasmobranchs (40, 43, 45), and teleost fish (2, 33, 41). However, the $[\text{HCO}_3^-]$-dependent $V_{te}$ was insensitive to 100 nmol/l bafilomycin. Importantly, this...
was in contrast to the low pH-stimulating mechanism, indicating that the dose of bafilomycin used is effective in inhibiting V-H\textsubscript{ATPase} in isolated perfused gills. On the basis of the inhibition of the HCO\textsubscript{3}\textsuperscript{-}-induced V\textsubscript{te} by amiloride, we conclude that the basolateral route of exit for H\textsuperscript{+} in crab gills during HCO\textsubscript{3}\textsuperscript{-} stimulation is via a member of the NHE family. Candidate targets for the amiloride sensitivity are some of the NHEs present in the gills of several crustaceans (18, 37, reviewed in 38). Importantly, at least some crustacean NHE isoforms are electrogenic and transport two Na\textsuperscript{+}/H\textsuperscript{+} for each H\textsuperscript{+} (18, 35). However, it is not clear whether the electrogenic NHE is located in the basolateral or apical membrane from those studies. Moreover, our study clearly demonstrates that the driving force for HCO\textsubscript{3}\textsuperscript{-} secretion and H\textsuperscript{+} reabsorption is Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, as seen in the inhibitory effect of ouabain.

Finally, the lack of HCO\textsubscript{3}\textsuperscript{-} stimulation in Cl\textsuperscript{-}-free conditions suggests the involvement of apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers of some sort (Fig. 12). However, apical application of DIDS and SITS did not have any significant effect in the stimulated V\textsubscript{te}. It is interesting to note that apical SITS produced a small but significant inhibition of 16% under hypo-osmotic stimulating conditions, and of 45% in similar, but Na\textsuperscript{+}-free, perfusion saline (8). This was interpreted as indicative of the involvement of a Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger in Cl\textsuperscript{-}-uptake. Lack of SITS/DIDS inhibition in our experimental conditions indicates that either a different, DIDS/SITS-insensitive, Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger participates in the HCO\textsubscript{3}\textsuperscript{-}-induced V\textsubscript{te} or that these drugs did not cross the cuticle in our experiments.

We have only found one similar study in the literature, performed in isolated perfused gills of the shore crab Carcinus maenas (36). This study concluded that the gill could detect an elevated pH of 8.10 in the perfusate (hemolymph space) and reabsorb H\textsuperscript{+} to restore a normal pH of ~7.70. However, a
saline with high pH but without HCO₃⁻ did not increase H⁺ reabsorption significantly, which suggests that the actual stimulus was [HCO₃⁻] or at least HCO₃⁻ was necessary to the base secretion mechanism. Siebers et al. (36) tested a variety of ion-transporting protein inhibitors, but only ouabain affected Vₑ and H⁺ reabsorption simultaneously. It is thus possible that C. maenas, unlike C. granulatus, relies on electroneutral ion transport for gill A/B regulation. Nonetheless, both isolated gill epithelia demonstrated an ability to detect and correct A/B disturbances, a feature that might be common to crustaceans and other aquatic organisms.

**Low pH-stimulating mechanism.** The inhibition of the low pH-stimulated Vₑ by bafilomycin is a good indicator of the importance of V-H⁺-ATPase in this response. On the basis of the outside positive Vₑ, we propose that V-H⁺-ATPase is located in the apical membrane and acts to secrete the excess H⁺ into the water covering the gills. Although bafilomycin was applied to the basolateral space, it is a membrane-permeable compound (5) and thus it can inhibit V-H⁺-ATPase located at the apical membrane even if applied at the basolateral space.

Apical V-H⁺-ATPase has been proposed to energize apical Cl⁻ absorption in some strong hyperregulating freshwater crabs (26, 27, 29, 34, 48), and also in C. granulatus (8, 24). However, the Cl⁻ independence demonstrated in our study suggests that the low-pH stimulating mechanism is different from the Cl⁻ uptake mechanism. On the basis of Na⁺-uptake models from certain aquatic organisms (for a review, see Ref. 19), we tested for the putative involvement of apical Na⁺ channels by applying apical phenamil on gills with stimulated Vₑ. Although phenamil did inhibit the stimulated Vₑ in certain preparations, the effect was not consistent, probably because of permeability issues at the apical cuticle (28). Our preliminary model presented here includes apical Na⁺ channels (Fig. 12), although further investigation is required to confirm this component.

---

**Fig. 9.** Acid-induced Vₑ stimulation in Cl⁻-free conditions and lack thereof in Na⁺-free conditions. A: representative trace of a Na⁺-free experiment. B: summary statistics of the Na⁺-free experiments. C: representative trace of a Cl⁻-free experiment. D: summary statistics of the Cl⁻-free experiments. Control saline, pH 7.75. a,b-Differing letters and asterisk (*) indicate statistical significance (P < 0.05; n = 5; one-way repeated-measures ANOVA, Tukey’s multiple-comparison posttest).

**Fig. 10.** Western blot analysis in gill homogenates from C. granulatus using antibodies against V-H⁺-ATPase (VHA) and Na⁺-K⁺-ATPase (NKA). Blots incubated with secondary antibody alone did not show any signal. The dotted line indicates that VHA and NKA antibodies were tested in different blots, and the images were merged for this figure.
We have recently reported the involvement of a basolateral electrogeneric Na\(^+\)/HCO\(_3\)^\(-\) cotransporter (NBC) as the way of exit of HCO\(_3\)^\(-\) and Na\(^+\) in isolated fish gill cells (31). In the current study, basolateral application of DIDS produced an initial further stimulation of the \(V_{te}\) already stimulated by low pH, followed by a rapid, complete, and irreversible inhibition. We tentatively propose that DIDS indeed inhibits basolateral NBCs and that the initial \(V_{te}\) stimulation is due to the pumping of protons by apical V-H\(^+-\)ATPases, which is not totally compensated due to the reduction in the transcellular flux of Na\(^+\) as a result of the inhibition of its basolateral transport. Additionally, a transient increment in Cl\(^-\) uptake through Cl\(^-\)/H\(^+\) exchanger.

![Diagram](image)

**Fig. 11.** VHA (A and C) and Na\(^+\)-K\(^+\)-ATPase (B) immunolocalization in gills of *C. granulatus*. Apical V-H\(^+-\)ATPase labeling is indicated with arrowheads in C. Scale bars = 10 \(\mu\)m (A and B) and 5 \(\mu\)m (C).

![Diagram](image)

**Fig. 12.** Tentative model for acid/base (A/B) regulation in the gill epithelium of *Neohelice granulata*. The left side of the diagram includes transporters involved in the low pH stimulation of \(V_{te}\); the right side describes the response to high [HCO\(_3\)^\(-\)]. Both systems are depicted in a single cell because we have no evidence supporting the existence of different cell types for A/B regulation in this crab. Mitochondria-produced CO\(_2\) might be an additional source of substrate for CA (see text). The molecular identity and exact stoichiometry of the transporters is unknown, and a question mark (?) indicates that no direct evidence exists for the involvement of the transporter. This model is based on the results from the current paper complemented by previous studies in this crab (21, 24, 30, 38) and other aquatic organisms (31). AE, anion exchanger; CA, carbonic anhydrase; NBC, Na\(^+\)/HCO\(_3\)^\(-\) cotransporter; NHE, Na\(^+\)-H\(^+\) exchanger; NKA, Na\(^+\)-K\(^+\)-ATPase; VHA, V-type H\(^+-\)ATPase.
HCO$_3^-$ exchange, favored by the activation of the V-H$^+$-ATPases and by the intracellular accumulation of HCO$_3^-$, could be an alternative cause of this peak in $V_{ie}$. In the longer term, the apical V-H$^+$-ATPase probably shuts down because the HCO$_3^-$ accumulation inside the cells reduces the availability of H$^+$, and then $V_{ie}$ is inhibited. Lastly, DIDS penetrating into the cell and affecting transporters located at the apical membrane cannot be ruled out. In addition, the results from the Na$^+$ and Cl$^-$ substitutions indicate that the low pH-induced increase in $V_{ie}$ depends on transepithelial Na$^+$ transport. This leaves only the possibility of an NBC and/or Na$^+$-K$^+$-ATPase on the basolateral side. The NBC would be more likely as the HCO$_3^-$ produced via CA hydration of CO$_2$ would need to be transported across the basolateral surface to maintain the charge distribution, as the H$^+$ is pumped out apically.

V-H$^+$-ATPase and Na$^+$-K$^+$-ATPase immunolocalization. Although V-H$^+$-ATPase and Na$^+$-K$^+$-ATPase have been previously detected in $C$. granulatus by pharmacological, biochemical (8, 11, 24, 39), and molecular biology techniques (25), this is the first immunolocalization report of these transporters in this crab. Na$^+$-K$^+$-ATPase is present in both principal and pillar cells, being restricted to the basolateral area in both cell types. A basolateral localization is consistent with the literature (reviewed in Ref. 20) and with the role of Na$^+$-K$^+$-ATPase energizing ion uptake in basal and stimulated conditions (8, 9, 24, 25, 30, 39). A basolateral Na$^+$-K$^+$-ATPase is also important for the high [HCO$_3^-$] and low pH stimulatory mechanisms reported in this study (Fig. 12).

The apical V-H$^+$-ATPase localization in some pillar and principal cells is consistent with its role in acid secretion derived from our perfusion experiments. The V-H$^+$-ATPase labeling found throughout the majority of cells could be due to V-H$^+$-ATPase stored in vesicles. These vesicles might insert into the apical membrane for enhanced acid or ammonia secretion (reviewed in Ref. 49) or when ion uptake is stimulated by hypo-osmotic shock. Similar V-H$^+$-ATPase immunolabeling patterns have been recently reported in 13 other species of crabs (46).

Na$^+$-K$^+$-ATPase and V-H$^+$-ATPase are present both in pillar and principal cells. Therefore, it is possible that the gill epithelium of $N$. granulata has only one cell type for A/B regulation, which could alternatively perform acid or base secretion depending on the physiological status of the animal. This would match hagfish gills, in which Na$^+$-K$^+$-ATPase, V-H$^+$-ATPase, and NHE are all located in the same cells (32, 42, 44). However, it is also possible that the cytoplasmic pool of V-H$^+$-ATPase from the principal and pillar cells differentially insert into the apical membrane during acidosis. Alternatively, it is possible that V-H$^+$-ATPase is present in the basolateral membrane of certain gill cells, but the labeling looks cytoplasmic due to the deep infoldings of the basolateral membrane (22, 23) (see Ref. 41 for a similar situation in teleost fish).

**Perspectives and Significance**

The low-pH and HCO$_3^-$ stimulation of $V_{ie}$ reported in this study raise the following interesting questions: 1) Do these two mechanisms take place in the same gill cell type or are there specific cell subtypes for each of them? 2) Is ion regulation linked to A/B regulation in the gills of crustaceans? 3) What is the identity of the pH/[HCO$_3^-$] sensor(s) that regulates the activation of one mechanism over the other? Finally, given that the key ion-transporting proteins (e.g., Na$^+$-K$^+$-ATPase, V-H$^+$-ATPase, CA) involved in our proposed A/B regulatory mechanisms in crab gills are also present in ion-transporting epithelia from vertebrates, it is possible that they all share a similar direct activation by blood A/B variables. It is exciting to hypothesize that intrinsic cellular signaling pathways could have been exploited for further regulation via hormones during the course of evolution.

**ACKNOWLEDGMENTS**

We acknowledge the assistance by the staffs at Asentamiento Universitario San Martin de los Andes and Centro Nacional de Educación para el Desarrollo who were extremely helpful with troubleshooting. We are also grateful to Dra. Iara Rochetta, Mirna Ferrada, Gonzalo Fernandez, Ana Schiffrin, Pablo, Facundo, and Ivan Nonini and Mr. Sabatini (Sr.). We would also like to thank the input of three anonymous reviewers who recommended additional experiments that improved this manuscript.

**GRANTS**

This research was funded by the Izaak Walton Killam Memorial Scholarship, The Company of Biologists Travel Fund and The University of Alberta, Faculty of Graduate Studies and Research, Research Abroad Travel Grant (to M. T. Ehrenfeld), the Queen Elizabeth II doctoral scholarship and the University of Alberta, Faculty of Graduate Studies and Research. Travel Grant (to S. K. Parks), Consejo Nacional de Investigaciones Científicas y Técnicas PIP 6244 and material support from CIEDE to C. M. Luquet and an Natural Sciences and Engineering Research Council Discovery Grant to G. G. Goss.

**REFERENCES**


