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Ionic effects on intrinsic gill muscles in the freshwater bivalve, *Dreissena polymorpha*

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Abstract

The intrinsic gill muscles in *Dreissena polymorpha* are arranged to affect interfilament distance and ostial dimension. This orientation suggests a role in the general control of gill function including the regulation of water flow. Muscle contraction in the gill results in a decrease in overall surface area and we used this change in dimension as an indirect measure of muscle contraction. The Ringer's solution bathing the gill was experimentally manipulated to assess the effects of ionic composition on muscle activity elicited by acetylcholine application. Eliminating $CaCl_2$ in the Ringer's and adding 1 mM EGTA, or replacing the $CaCl_2$ with $CoCl_2$ or $MnCl_2$ abolished the contractile response. A proper balance between NaCl and KCl was also critical for the maintenance of muscle response. The optimal KCl concentration was between 1 and 2 mM and became most important at higher NaCl concentrations. Acclimation to hyperosmotic conditions was dependent in part on the ouabain-sensitive activity of a Na^+/K^+ATPase. Overall, these muscles respond to their ionic environment as one might expect from typical molluscan smooth muscles. An alteration of muscle function experienced during changing environmental conditions may limit the distribution of this species.

Keywords: Ions; Bivalve; Gill; Muscle; Zebra mussel; Acclimation; Environment; Freshwater

1. Introduction

The lamellibranch gills of bivalve molluscs are important respiratory and feeding structures. The intrinsic gill muscles in some eulamellibranchs are oriented to control the dimensions and posture of the demibranchs, but the importance of the intrinsic musculature to integrative responses such as feeding and respiration is currently unresolved. Most contemporary studies have focused on the ciliary function of the gill, with emphasis given to its role in water movement and feeding processes. Nevertheless, early investigators [1,14,42] underscored the importance of gill muscles in coordinating gross movements of the gill with these ciliary-based activities. We have recently studied the anatomy of the muscles and their responses to selected neurotransmitters in both unionid and dreissenid bivalves [18,27]. Briefly, the muscles in *Dreissena polymorpha* are arranged in two sets. One set is encased in connective tissue bands at the base of the gill filaments and is oriented so that contraction decreases interfilament distance. The second set is closely associated with the loose connective tissue sheets supporting the epithelial layers and is responsible for controlling the dimensions of the ostia and water canals of the gill (Fig. 1a). The intrinsic gill muscles of three major groups of freshwater bivalves (dreissenids, unionids, and corbiculids) share an ultrastructure that corresponds to a common type of molluscan smooth muscle (Fig. 1b,c) [28]. These muscles are potentially involved in the active regulation of gill function and it is important to understand their basic physiology.

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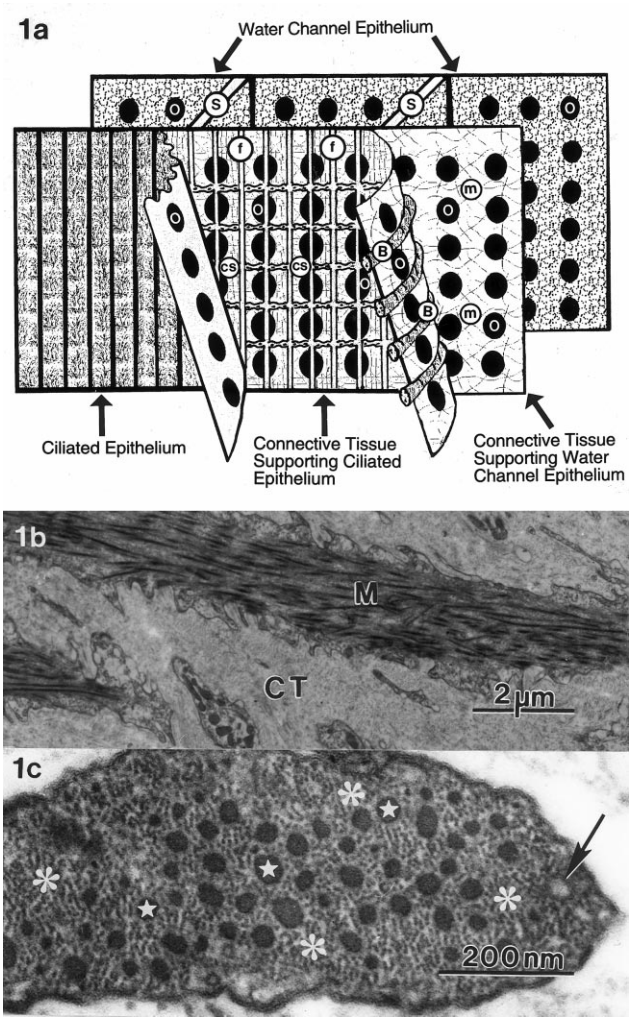

Fig. 1. (a) A schematic representation of a single demibranch of *D. polymorpha* gill (adapted from Medler and Silverman [27]; not to scale). Each demibranch consists of two opposing lamellae, connected by septa (S). Each lamella is composed of several layers: outermost is the ciliated epithelium of the filaments; a loose connective tissue sheet underlies this epithelium and is associated with filament supports (f) and cross struts (cs) that maintain interfilament distance in the absence of muscular contraction; a second loose connective tissue sheet is associated with the internal water-channel epithelium that lines the central channel of the gill. Ostia (o) are openings in the external and internal part of the gill allowing passage of water into the central water channel of the gill. One set of muscle fibers is in bands (B) at the base of the filament at right angles to the filaments and are encased within connective tissue. These muscles are oriented to pull the filaments closer to one another. The second set of fibers (m) is associated with the loose connective tissue sheets of both the internal and external epithelial layers. This set of muscles is arranged to change the dimensions of the internal and external ostia. (b) Smooth muscles (M) embedded in collagenous connective tissue (CT) from a region deep to the water channel epithelium of the gill. The majority of the cytoplasm is filled with contractile filaments and a well developed internal membrane system is absent. (c) Higher magnification of smooth muscle fiber in cross section. Thick filaments (stars) and surrounding regions of thin filaments (asterisks) fill the majority of the cytoplasm. Small volumes of sarcoplasmic reticulum (arrow) are visible near the cell membrane.

D. polymorpha, the zebra mussel, is a relatively recent inhabitant of fresh water with records dating only to the Miocene [34], as compared to the Triassic for the unionids [20]. The zebra mussel belongs to the bivalve subclass Heterodonta, which contains many brackish water species that are less well adapted to freshwater than, for example, the unionid clams of the subclass Paleoheterodonta [7]. Correspondingly, ionic tolerances described for *D. polymorpha* differ from the tolerances seen in the other freshwater bivalves. For example, zebra mussels do not survive in deionized water [8,32,38] while unionids survive for months under such conditions [8]. Wilcox and Dietz [46] suggested that the rapid ion turnover and an inability to reduce ion loss in fresh water are physiological indications of the zebra mussel's incomplete adaptation to freshwater. These rapid ion fluxes are reflective of an unusually 'leaky' epithelium with rates of paracellular ion movement exceeding those observed in other freshwater bivalve species [9–11,51]. Under hyperosmotic conditions, the response of the animals is to osmoconform, with blood ion composition closely following that of the bathing medium [10,11,47]. A critical balance between Na^+ and K^+ in this medium is essential for the survival of these animals [8,10,11,47]. Exposure to hypertonic NaCl is toxic when the K^+ concentration is too low [10,11,23], yet relatively low levels of K^+ are inherently toxic as well [10,11,17]. Finally, *D. polymorpha* has a unique requirement for Mg^{2+} in the bathing medium [8].

Zebra mussels in Europe are often inhabitants of brackish and estuarine waters, but apparently are limited in their distribution by critical salinity levels [44]. Data from natural populations [44] and from recent laboratory measurements [10,11,47] indicate that this species is especially sensitive to sudden fluctuations in salinity. When *D. polymorpha* are exposed to dilute artificial sea water (ASW), the concentration of blood ions increases to reflect the ionic content of the bath. Namely Na^+, Cl^-, K^+, and Mg^{2+} concentrations become elevated [47]. Alteration of muscle function might be expected when any of these ions are elevated in the blood and several studies have suggested that the sensitivity to K^+ in particular, is related to direct effects on excitable membranes [10,11,23,46]. Extracellular calcium availability is probably important as well, since the muscle fibers are small (1–2 μm in diameter) and lack a well developed internal membrane system (Fig. 1b,c) [27,28]. These morphological features suggest a reliance on external calcium for muscle activation.

In this study, we were interested in the basic ionic requirements for muscle contraction in the gill and in the effects caused by elevated ions as experienced during an acute exposure to dilute artificial sea water. We first examined the dependence of muscle activity on extracellular Ca^{2+}. In the second part of the study, we

examined the effects caused by elevated Na^+, Cl^-, K^+, and Mg^{2+} on muscle contraction in the gills of *D. polymorpha*.

2. Materials and methods

2.1. Animal maintenance

D. polymorpha were collected from Lake Erie at the mouth of the Raisin River in Monroe, MI and from the Mississippi River near Plaquemine, LA. The animals were maintained in artificial pondwater (see below) in aerated aquaria under laboratory conditions of approximately 22–24°C.

2.2. Solutions

Artificial pondwater (APW) in mM consisted of 0.5 NaCl, 0.4 $CaCl_2$, 0.2 $MgSO_4$, 0.2 $NaHCO_3$, 0.05 KCl [8]. Artificial sea water (ASW) in mM consisted of 449.1 NaCl, 27.5 $MgSO_4$, 24.4 $MgCl_2$, 9.9 $CaCl_2$, 6.6 KCl, 2.4 $KHCO_3$, 0.8 KBr, 0.4 H_3BO_3; 1076 mOsm kg^{-1} total solute concentration; 35 ppt salinity [5,47]. Ringer's solution in mM was made of 5 NaCl, 5 $CaCl_2$, 5 $NaHCO_3$, 0.5 KCl, 5 $NaSO_4$, 0.5 $MgCl_2$; 48 mOsm kg^{-1} [8]. 'Elevated' Ringer's consisted of the same components as the standard Ringer's but with 45 NaCl, 2 KCl, and 5 $MgSO_4$; 130 mOsm kg^{-1}. The Ringer's solution was made to approximate the blood composition of animals in APW, while the 'elevated' Ringer's approximated the blood of animals exposed to 15% ASW.

Various experimental Ringer's solutions were made by adjusting single components of both Ringer's solutions.

Three different Ca^{2+}-free Ringer's solutions were made as follows. One solution was made by omitting $CaCl_2$ and including 1 mM EGTA. The difference in osmolality was corrected by the addition of NaCl. In two other cases, either $MnCl_2$ or $CoCl_2$ were substituted for $CaCl_2$. In each of the Ca^{2+}-free solutions, osmolality and pH were the same as in the Ringer's solution.

2.3. Gill preparation

Gills were excised into the appropriate Ringer's solution by freeing the anterior and posterior connections of the gills with forceps and then gently freeing the gills from along the body of the animal. Lateral and medial demibranchs from each side of an animal were separated by cutting the basal attachment with surgical scissors. Thus, each animal provided four demibranchs and these were randomly distributed among treatment groups for all experiments. Lateral and medial demibranchs were considered to be the same physiologically. Allocating tissues from a single animal to each treatment group eliminated between animal differences as a factor in statistical analysis.

2.4. Preparation for transmission electron microscopy (TEM)

Prior to gill excision, hemolymph samples were taken from animals by inserting a 26 gauge needle between the valves and into the pericardial space [8]. Hemolymph osmolality (approximately 40–60 mOsm) was measured on a Precision Systems freezing point osmometer. Tissues were fixed for 1 h in a 2% glutaraldehyde solution and rinsed twice with phosphate buffer adjusted to match the hemolymph osmolality. All gills were post-fixed in 1% OsO_4 for 1 h, rinsed twice in phosphate buffer, and dehydrated in a graded ethanol series. Gill strips (about 3 mm wide) were embedded in LR White (London Resin) medium grade resin by placing them in a 1:1 mixture of ethanol and resin for 24 h. They were transferred to 100% resin for 12 h, and embedded flat in fresh resin at 60°C for 24–48 h.

Gills were sectioned with a Reichert-Jung ultracut E ultramicrotome at 60–90 nm thickness with glass knives. Sections were stained with 3% uranyl acetate for 8 min followed by Reynolds' [39] lead citrate for 2–5 min and examined with a JEOL 100CX transmission electron microscope (TEM) operating at 80 kV.

2.5. Demibranch contraction assay

We have previously described an in vitro assay designed to examine the response of intrinsic demibranch muscles to neurotransmitters [27]. Demibranchs from animals were excised into a Ringer's solution and after 30–45 min of equilibration, the demibranchs were placed into a small volume of Ringer's solution on a glass slide. The gills were left in this position for about 1 min to ensure that the muscles of the demibranch had relaxed. The Ringer's solution was gently aspirated to leave the demibranch spread flat across the slide. A solution containing 1 mM acetylcholine (ACH) in Ringer's was quickly applied to the gill and immediately aspirated, leaving the demibranch flat on the slide. Acetylcholine is a dose-dependent stimulator of muscle contraction in the intrinsic gill muscles of *D. polymorpha* [27], eliciting strong responses at concentrations near 1 mM. Over the next 1–2 min, the demibranch decreases in area as the intrinsic muscles contract. During this procedure, gills were video taped on VHS tape at a magnification of about 10 × through a dissecting microscope. Digitized-video-images were analyzed with Image-1 computer software (Universal Imaging). Gill area was measured prior to transmitter

exposure and at timed intervals after transmitter application. This process represents tonic contraction and the reduction in gill area approaches an asymptote after about 1 min. The reduction in gill area (% of the initial area) after 1 min of contraction will be referred to as the 'contractile response'.

It is crucial that the demibranchs start in a relaxed state, since all subsequent measurements are expressed as a function of this initial state. Resting demibranch surface areas, prior to addition of transmitter, were compared between treatment groups to ensure that shrinkage did not confound the contractile response. Any departures from these assumptions are explicitly stated in the results.

2.6. Ca^{2+}-free experiments

Several experiments were conducted to examine the relationship between extracellular Ca^{2+} and muscle contraction. In one experiment, gills were excised into a Ca^{2+}-free solution containing 1 mM EGTA before exposure to the 1 mM ACH. After 1 min of ACH exposure without Ca^{2+}, 1 mM ACH in the Ringer's solution with Ca^{2+} was administered to the gills. Contractile responses for each solution were measured. In two other experiments, the inorganic Ca^{2+} antagonists $MnCl_2$ or $CoCl_2$ were substituted for $CaCl_2$ in the Ringer's solution, while control gills were placed in 'normal' Ringer's solution. In control experiments, gills exposed to ACH in the presence of Ca^{2+} antagonists were returned to the 'normal' Ringer's solution to examine whether the antagonistic effects were reversible. In each of the experiments, the contractile responses were compared with a t-test ($n = 10$ demibranchs per treatment group).

2.7. Artificial sea water experiment

A group of about 30 animals were transferred to 15% artificial sea water (ASW) diluted with artificial pondwater (APW) in two salinity steps over 4 days. Between 80 and 90% of these animals survived the transfer and remained alive throughout the experiment. A second group of animals remained in APW. The gills from animals in each of these acclimation regimes were dissected as described above and placed in either 'elevated' Ringer's or Ringer's solution. Thus, the experiment had a 2×2 factorial treatment arrangement with one factor being the water to which the animals were acclimated (15% ASW or APW), a second being the Ringer's solution to which the excised gill was exposed ('elevated' Ringer's or Ringer's). The contractile response was measured for each treatment group ($n = 20$ demibranchs per treatment group).

2.8. $MgSO_4$ experiment

The effect of $MgSO_4$ on demibranch muscle contraction was examined with both Ringer's solutions. The four excised demibranchs from 10 APW-acclimated animals were distributed to the Ringer's solution or to the 'elevated' Ringer's solution with either 0.5 mM $MgSO_4$ or 5 mM $MgSO_4$. Thus, the experiment had a 2×2 factorial treatment arrangement, with one factor being $MgSO_4$ concentration (0.5 or 5.0 mM) and the second being the Ringer's solution ('normal' or 'elevated'). The contractile response was measured for each of the demibranchs ($n = 10$ demibranchs per treatment group).

2.9. KCl experiments

The effects of KCl concentration on muscle contraction in the 'elevated' Ringer's solution were examined in a set of four experiments, each with a different NaCl concentration (2, 10, 15, 45 mM). The experimental design was to vary KCl concentration (0, 1, 2, 4 mM) while holding NaCl concentration constant for each experiment. All other ions were equal in concentration to those in the 'elevated' Ringer's solution. In each experiment, the four demibranchs from 10 APW-acclimated animals were distributed to one of the four KCl concentrations. The demibranchs were exposed to 1 mM ACH in the contraction assay and the contractile responses were measured ($n = 10$ demibranchs per treatment group).

2.10. NaCl experiments

The effect of NaCl concentration in the 'elevated' Ringer's solution was directly examined in two experiments with different KCl concentrations (0 or 2 mM). In each experiment, the four demibranchs from each of 10 APW-acclimated animals were distributed to 'elevated' Ringer's solutions containing one of four NaCl concentrations (2, 15, 30, or 45 mM). Therefore, these experiments were reciprocal to the KCl experiments: NaCl concentration was variable while KCl concentration was held constant. The contractile response from each experiment was plotted and simple linear regression was used to determine whether the response changed as a function of the NaCl concentration ($n = 10$ demibranchs per treatment group).

2.11. Ouabain experiments

The role of the Na^+/K^+ATPase on the ability of demibranch muscles to recover from an acute expo-

sure to 'elevated' Ringer's solution with no KCl was examined. The cardiac glycoside, ouabain, was used to block Na^+/K^+ ATPase activity and KCl was provided to some of the demibranchs following the acute exposure. The demibranchs from 10 APW-acclimated animals were excised and placed into 'elevated' Ringer's solution without K^+ for between 25 and 30 min. After this incubation, the demibranchs were distributed to an 'elevated' Ringer's solution containing either 0 or 2 mM KCl. Additionally, each Ringer's solution contained either 0 or 1 mM ouabain. Thus, the experiment had a 2×2 factorial treatment arrangement with one factor being the presence or absence of KCl, the second being the presence or absence of ouabain. After about 40 min in these solutions, the demibranchs were exposed to 1 mM ACH in the demibranch contraction assay and the contractile responses were recorded ($n = 10$ demibranchs per treatment group).

In a separate control experiment, demibranchs were placed in either Ringer's containing 1 mM ouabain or in Ringer's alone as a control. This experiment was performed to test the effects of ouabain alone on gill muscles in the absence of a hyperosmotic stress. The contractile responses of both groups were measured over a time period from 10 min to about 2 h following initial incubation in ouabain. A simple linear regression was used to determine whether the responses changed over time. In addition, the final gill areas following ACH treatment from the control and ouabain-treated groups were compared with a t-test ($n =$ 20 demibranchs per treatment group).

2.12. Statistics

In most experiments, the contractile response was compared between treatment groups with an ANOVA. A Tukey post-ANOVA test was then used to make pair-wise comparisons between individual treatment means (experiment-wise error rate = 0.05 in all experiments). In the NaCl experiment, a simple linear regression was used to test whether the contractile response changed as a function of NaCl concentration. Frequency histograms of the data and of the residuals demonstrated normality, therefore no further transformation was required. Variances were homogeneous between treatment groups. Statistics were performed with SAS version 6.10 [41].

3. Results

3.1. Muscle ultrastructure

The morphological features of these muscles are described in greater detail elsewhere [27], but a brief description includes the following. They are smooth muscles that share an ultrastructure with those found in other molluscan species [28]. The majority of the cytoplasm is occupied by the contractile filaments, with an undeveloped internal membrane system (Fig. 1b,c). An abundance of collagenous connective tissue surrounds the fibers and makes electrical recordings difficult (Fig. 1b).

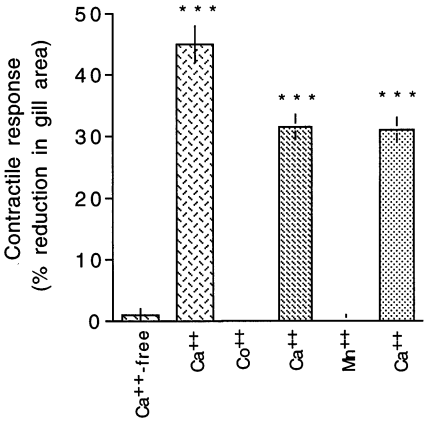

Fig. 2. Three independent experiments, each shaded with a different pattern, demonstrating the effect of extracellular Ca^{2+} on muscle contraction. In one experiment, demibranchs were bathed in Ca^{2+}-free Ringer's solution containing 1 mM EGTA (Ca^{2+}-free). In the second and third experiments, the $CaCl_2$ in the Ringer's solution was replaced by the Ca^{2+} antagonists $CoCl_2$ (Co^{2+}) and $MnCl_2$ (Mn^{2+}), respectively. In each of the three experiments, the demibranchs with Ca^{2+} available in the Ringer's solution (Ca^{2+}) contracted significantly more than those without Ca^{2+} (Ca^{2+}-free, Co^{2+}, Mn^{2+}) (***$P < 0.0001$; mean $\pm$ S.E., $n = 10$).

3.2. Ca^{2+}-free experiments

In each of the individual experiments, the gills in the Ca^{2+}-free solutions failed to contract when exposed to 1 mM ACH, while the control groups contracted normally ($P < 0.0001$ for each experiment) (Fig. 2). The control experiments showed that demibranchs exposed to $MnCl_2$ or $CoCl_2$ rapidly regained their contractile activity when returned to the Ringer's solution with Ca^{2+} (data not shown).

3.3. Artificial sea water experiment

The gills of animals acclimated to 15% ASW showed the same level of muscle contraction when stimulated by 1 mM ACH in both 'elevated' Ringer's and Ringer's solutions (Fig. 3). These responses were the same as those of the APW-acclimated gills stimulated with 1 mM ACH in Ringer's. The gills from APW-acclimated animals exposed to 'elevated' Ringer's solution showed a significant depression in the ACH-stimulated contractile response when compared with the other treatment groups (Fig. 3).

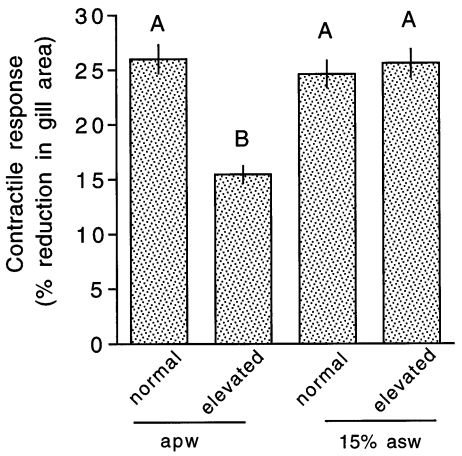

Fig. 3. Contractile responses of gills acclimated to 15% ASW (15%) or to APW (apw) and exposed acutely to Ringer's (normal) or to 'elevated' Ringer's (elevated). The APW-acclimated animals acutely exposed to 'elevated' Ringer's solution show depressed contractile activity as compared with the other treatment groups. The 15% ASW-acclimated animals had the same level of demibranch contraction as the APW-acclimated animals in Ringer's. Contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test (mean ± S.E., $n = 20$).

3.4. $MgSO_4$ experiment

The concentration of $MgSO_4$ (0.5 vs. 5 mM) had no effect on the degree of ACH-induced contraction in either Ringer's solution (Fig. 4). When pooled, gills in the 'elevated' Ringer's solution did contract less than those in the Ringer's solution ($P < 0.012$). This depression is consistent with the results of the ASW experiment (Fig. 3).

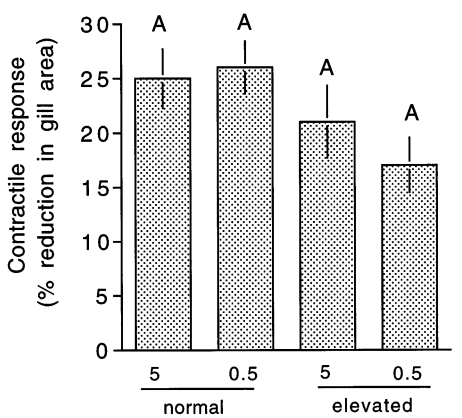

Fig. 4. Contractile responses of gills in Ringer's (normal or 'elevated') with different concentrations of $MgSO_4$ (5 or 0.5 mM). $MgSO_4$ concentration did not have a significant effect on muscle contraction. When pooled, the demibranchs in the 'elevated' Ringer's solution contracted less than those in the normal Ringer's solution. The contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test (mean ± S.E., $n = 10$).

3.5. KCl experiments

The effects of KCl concentration on ACH-induced contraction in APW-acclimated mussel gills were examined in four independent experiments, each with four different NaCl concentrations in the 'elevated' Ringer's solution. Maximum contraction corresponded to either 1 or 2 mM KCl in each of the experiments (Fig. 5). Comparison across the four experiments suggests that when KCl is absent from the Ringer's solution, contraction decreases as a function of increasing NaCl concentration.

3.6. NaCl experiments

The effects of NaCl concentration on ACH-induced contraction in APW-acclimated mussel gills were examined in two experiments with either 0 mM or 2 mM KCl in the 'elevated' Ringer's. Contraction of the demibranch muscles significantly decreased as a function of increasing NaCl concentration when the KCl concentration was 0 mM ($P < 0.0001$, $r^2 = 0.36$). However, when 2 mM KCl was present the contractile response did not change with increasing NaCl concentration ($P < 0.655$, $r^2 = 0.006$) (Fig. 6).

3.7. Ouabain experiment

The effect of ouabain on ACH-induced muscle contraction was examined in both the presence and absence of KCl following an acute 25- to 30-min incubation in 'elevated' Ringer's without KCl. The treatment group with 2 mM KCl and no ouabain showed significantly greater contraction than the other treatment groups (Fig. 7). The mean response of the treatment group with KCl alone was over three times larger than the mean response when KCl was present with ouabain and over 1.5 times greater than the response of the group with neither KCl nor ouabain added.

In the control experiment, the demibranchs exposed to ouabain in Ringer's became partially contracted prior to ACH treatment, making our standard comparisons untenable. Nevertheless, the contractile response of the ouabain-treated gills did not decrease over time ($P < 0.585$, $r^2 = 0.017$) and the final demibranch area following ACH-induced contraction was not different from that of the control group ($P < 0.799$) (data not shown). Thus, ouabain treatment alone did not block the ACH-induced response of control gills in Ringer's solution over the experimental time period.

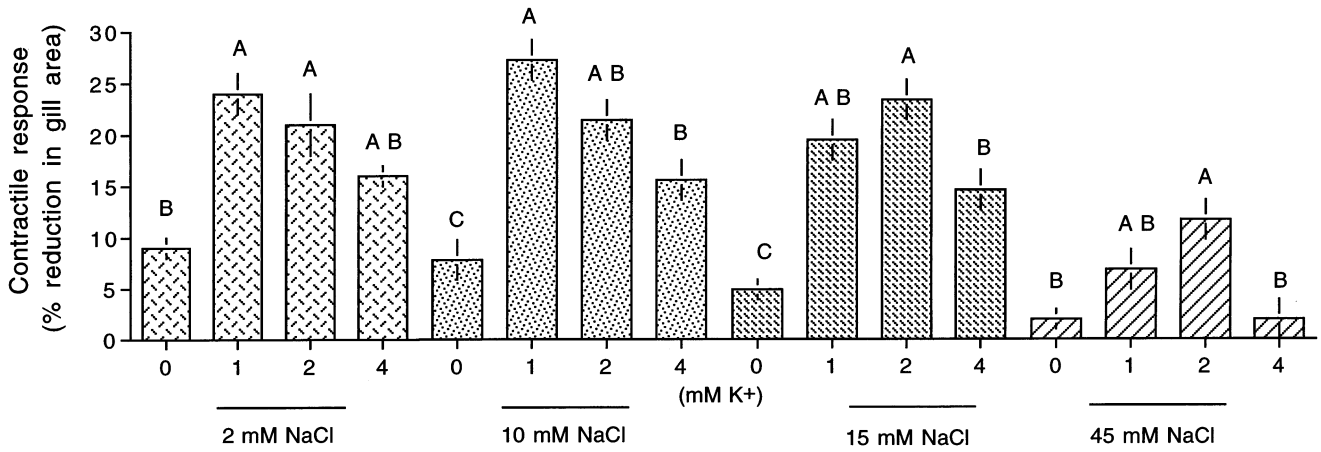

Fig. 5. Contractile responses of gills for the four independent KCl experiments, each at a different NaCl concentration indicated by a different shading pattern. The greatest degree of muscle contraction corresponds to either 1 or 2 mM KCl in each experiment. The contractile responses with the same letter within an experiment are not significantly different from one another when compared by a Tukey post-ANOVA test (mean ± S.E., $n = 10$).

4. Discussion

The gill muscles of *D. polymorpha* are dependent on external calcium for ACH-induced muscle activation: removing Ca^{2+} from the Ringer's solution or blocking the effects of Ca^{2+} with inorganic Ca^{2+} antagonists (Co^{2+} or Mn^{2+}) prevents contraction. The small size of the fibers and their lack of a well-developed internal Ca^{2+} storage system (Fig. 1b, c) [27,28] are consistent with these findings. Many muscles are dependent on external Ca^{2+} for activation, including the odontophore protractor of *Busycon canaliculatum* [24], the proboscis muscles of *Buccinum undatum* [30], the anterior byssus retractor muscle of *Mytilus edulis* [29], and other smooth muscles. Muscle contraction was sensitive to K^+ concentration with only a narrow range (1–2 mM) maintaining maximal responses. This sensitivity was heightened when the osmolality of the bath was elevated with NaCl.

Osmoconforming bivalves show continued cellular function over a wide range of ion concentrations. *Mytilus edulis* can adapt to between 25 and 125% sea water with ionic and osmotic conformity [48]. The nervous tissue from these animals continues to produce full-sized action potentials after acclimation to 25% sea water [49]. Isolated ventricle strips from three bivalve species continue to function over a wide range of salinities: good contractile activity was found over a

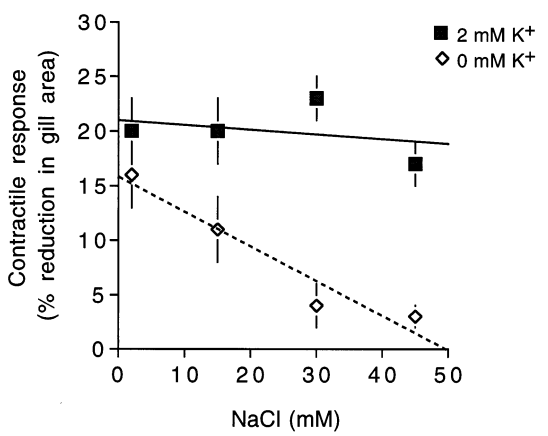

Fig. 6. Contractile responses as a function of increasing NaCl concentration in the 'elevated' Ringer's solution with either 0 or 2 mM KCl. When no KCl was present in the solution, contraction significantly decreased as a function of increasing NaCl concentration. When 2 mM KCl was provided, no such reduction was observed (mean ± S.E., $n = 10$).

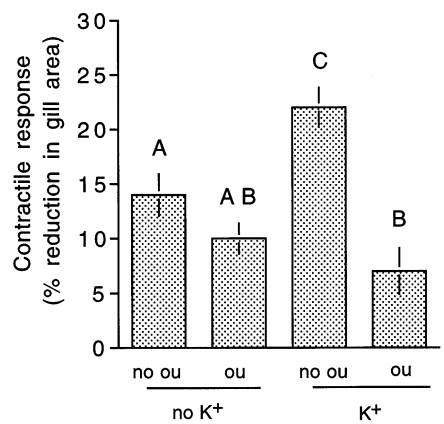

Fig. 7. Contractile responses of demibranchs treated with ouabain (0 or 1 mM) and/or KCl (0 or 2 mM). Gills were acutely exposed to 'elevated' Ringer's without KCl. After 25–30 min, demibranchs were distributed to treatment groups and allowed to acclimate for about 40 min. Treatment groups are 'elevated' Ringer's with (K^+) or without (no K^+) KCl and with (ou) or without (no ou) ouabain. When K^+ was present without ouabain, the greatest level of muscle contraction was measured. However, adding ouabain caused a significant reduction of this response. Contractile responses with the same letter are not significantly different from one another when compared by a Tukey post-ANOVA test (mean ± S.E., $n = 10$).

range of 40–160% sea water in *Mytilus edulis*, 70–120% sea water in *Ostrea edulis*, and 2–24% sea water in *Anodonta cygnea* [36]. Each of these animals also showed continued ciliary function over a broad range of salinities [37].

Despite such extreme changes in the ionic composition of the bathing medium, many invertebrates including annelids [4,31], snails [3,13] and bivalves [25,43,45,49,50] utilize the distribution of Na^+ and K^+ as the ionic basis of excitability. The results of the present study demonstrate the necessity for a proper balance between Na^+, K^+, and an absolute requirement for Ca^{2+} to maintain normal gill muscle function in *D. polymorpha*. The muscle fibers in the gill of *D. polymorpha* that are responsible for the contractility reported here are small (1–2 μm in diameter) and encased within a connective tissue matrix (Fig. 1b,c) [27,28], making electrical recordings elusive to date. However, interpreting our data in light of other studies of molluscan muscle activation [24,29,30], we can infer that the ACH-induced muscle contraction begins with the opening of 'slow' calcium channels following an initial membrane event such as a Na^+- or Ca^{2+}-dependent action potential. The entry of extracellular Ca^{2+} ultimately leads to the direct activation of the myosin heavy chain by Ca^{2+} ions and contraction ensues [40]. Since the resting membrane potential in molluscan muscles is primarily due to the ratio of intracellular and extracellular potassium ions [3,13], altering the extracellular K^+ concentration will directly affect the membrane potential. These muscles are clearly sensitive to K^+ concentration, with maximal contractile responses occurring only within a narrow range of K^+. This sensitivity becomes even more pronounced in the presence of hyperosmotic NaCl concentrations.

In addition to direct electrical effects on the muscle cell membrane, osmoregulatory processes may also be important for maintaining muscle function under the hyperosmotic conditions examined in this study. The movement of K^+ and other inorganic ions between intracellular and extracellular pools can be an important mechanism of volume regulation in invertebrates [12,19,35], vertebrates [21,22], and even in bacteria [15,16]. During hypoosmotic volume regulation (the case most studied in marine invertebrates), K^+ efflux from intracellular compartments appears to be a response to reduce cellular swelling during the initial phase of exposure (within minutes), while changes in the concentration of organic effector molecules such as free amino acids are thought to be important for volume regulation over a longer readjustment period (within hours) [19]. Freshwater bivalves differ from marine species in that they use K^+ as the primary intracellular regulator of cell volume, whereas marine bivalves use both free amino acids and K^+ [10,12]. Our results show that K^+ is critically important to maintain contractility in the face of elevated NaCl concentrations. Similarly, the epithelial cells of freshwater bivalve gills shrink rapidly when exposed to a hyperosmotic challenge of 45 mM NaCl, but a significant volume recovery is observed after the addition of 1 mM KCl to the medium [12].

While some inorganic ion movement may be passive [19], active transport is also important for maintaining ion balance. For example, Na^+/K^+ATPase activity is involved in the recovery of neural tissues from osmotic or ionic imbalances in polychaetes [2] and in bivalves [45,50]. The activity of a ouabain-sensitive sarcolemmal Na^+/K^+ATPase has also been linked to fatigue resistance in vertebrate skeletal muscle, where cellular extrusion of K^+ and accumulation of Na^+ is a contributor to fatigue [6,33]. Our results demonstrate that the recovery of muscle contractility following acute osmotic stress in *D. polymorpha* is ouabain-sensitive. The active exchange of Na^+ and K^+ in the gill muscles of *D. polymorpha* during hyperosmotic/ionic stress is likely to be important for two processes: [1] the cellular uptake of K^+ and extrusion of Na^+ directly linked to the maintenance of membrane potential and [2] the active accumulation of K^+ needed for expanding the cellular volume of the muscle fibers, as occurs in the gill epithelia under similar conditions [12].

Zebra mussels are sensitive to the ionic composition experienced during hyperosmotic exposures [10,11,47]. Other reports have indicated that these bivalves could survive in regions of elevated salinity, provided that salinity changes were not pronounced or rapid [11,26,47]. Major fluctuations in the ionic environment combined with an unusually 'leaky' epithelium culminate in dramatic changes in the ion composition of the extracellular fluid [11,47]. The smooth muscles of the gill show some capacity to acclimate under these conditions, but their responsiveness is clearly impaired when a proper balance of ions is disrupted. Muscle function impaired to the degree observed in this study would be expected to have direct effects on survival. Such direct physiological consequences are, no doubt, important in establishing the salinity boundaries observed in estuarine environments for these animals.

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