JOURNAL OF EXPERIMENTAL ZOOLOGY 283:620–640 (1999)

Calcium Homeostasis in Crustacea: The Evolving Role of Branchial, Renal, Digestive and Hypodermal Epithelia

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ABSTRACT Crustaceans serve as an ideal model for the study of calcium homeostasis due to their natural molting cycle. Demineralization and remineralization of the calcified cuticle is accompanied by bidirectional Ca transfer across the primary Ca transporting epithelia: gills, antennal gland (kidney), digestive system, and cuticular hypodermis. The review will demonstrate how a continuum of crustaceans can be used as a paradigm for the evolution of Ca transport mechanisms. Generally speaking, aquatic crustaceans rely primarily on branchial Ca uptake and accordingly are affected by water Ca content; terrestrial crustaceans rely on intake of dietary Ca across the digestive epithelium. Synchrony of mineralization at the cuticle vs. storage sites will be presented. Physiological and behavioral adaptations have evolved to optimize Ca balance during the molting cycle in different Ca environments. Intracellular Ca regulation reveals common mechanisms of apical and basolateral membrane transport as well as intracellular sequestration. Regulation of cell Ca concentration will be discussed in intermolt and during periods of the molting cycle when transepithelial Ca flux is significantly elevated. Molecular characterization of the sarco-/endoplasmic reticular Ca pump in aquatic species reveals the presence of two isoforms that originate from a single gene. This gene is differentially expressed during the molting cycle. Gene expression may be regulated by a suite of hormones including ecdysone, calcitonin, and vitamin D. Perspectives for future research are presented. *J. Exp. Zool. 283:620–640, 1999.*

Crustaceans have served comparative physiologists as useful paradigms for studying the evolutionary transition from water to land since they occupy habitats ranging from salt water (SW) to freshwater (FW) and via either origin onto land (Burggren and McMahon, '88). Examining calcium (Ca) homeostasis in a continuum of crustaceans may assist in understanding evolutionary changes in Ca regulation during the transition from aquatic to terrestrial environments. In recent years crustaceans have emerged as an ideal nonmammalian model in which to study Ca homeostasis by virtue of their natural molting cycle that involves erosion/deposition of the $CaCO_3$ cuticle (Wheatly, '97). In premolt Ca is reabsorbed from the old cuticle that is shed (ecdysis). The new cuticle is mineralized primarily with external (environmental) Ca^{2+} and with internal stores. Four crustacean epithelia are specialized for bidirectional Ca^{2+} exchange: (1) gills are the site of passive Ca^{2+} diffusional loss and active uptake in aquatic species and may postrenally modify Ca^{2+} content of voided urine in terrestrial species; (2) the digestive epithelium effects Ca^{2+} uptake from food (including reingested exuviae) and drink, and from Ca deposits stored in regions of the digestive system; (3) the antennal gland (analog of kidney) may be involved in postfiltrational reabsorption of urinary Ca^{2+}; and (4) the cuticular hypodermis can effect demineralization or mineralization at different stages of the molting cycle. Aquatic species rely primarily on their gills for Ca uptake from environmental water; in terrestrial species the digestive epithelium predominates as diet provides external Ca. This review will discuss very recent research that elucidates evolutionary strategies for Ca homeostasis in crustaceans at four levels (Fig. 1): (1) whole organism strategies related to environmental Ca; (2) extracellular regulation (hemolymph, shell, calcified deposits); (3) intracellular regulation; and (4) molecular regulation. Ca homeostasis is of fundamental significance given the supreme biological importance of Ca in living organisms.

Grant sponsor: NSF; Grant number: 9603723.
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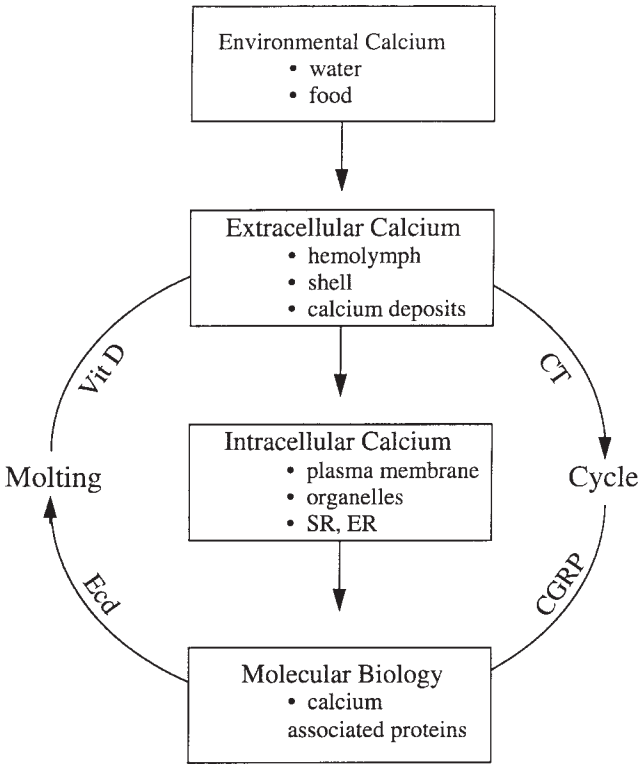

Fig. 1. Hierarchy for calcium homeostasis in crustaceans. SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; Ecd, ecdysone; CT, calcitonin; CGRP, calcitonin gene-related product.

ENVIRONMENTAL CALCIUM: EVOLUTIONARY STRATEGIES FOR WHOLE ORGANISM CALCIUM HOMEOSTASIS

Intermolt calcium balance is a product of the environment

Aquatic environments afford unlimited reservoirs of Ca be they marine (10 mM) or inland waters (<1 mM). Aquatic strategies for Ca homeostasis have focused on adult decapods. Marine crabs maintain intermolt extracellular (EC) and urine Ca isoionic with respect to SW (Greenaway, '85; Neufeld and Cameron, '92); Ca is rarely limiting in marine or brackish environments.

Intermolt FW crayfish hyperionically regulate EC Ca primarily through reabsorbing 95% Ca from urinary filtrate (Wheatly and Toop, '89). Inland waters can vary in their Ca content and this, in turn, can influence whole organism acid-base and ion balance (Table 1). The minimum level for Ca homeostasis in crayfish is around 50 μM (Wheatly, de Souza and Hart, unpublished). In nature, crayfish can maintain Ca balance in Ca-poor water only if the diet is Ca rich (Hessen et al., '91). External Ca can affect the electrical (Kirschner, '94) as well as chemical gradient across epithelia. However it has little effect on apparent permeability of FW crustaceans (Rasmussen and Bjerregaard, '95) since this is already minimal. Water Ca concentration can also influence physiological response to environmental change. For example, acid is more toxic to crayfish in high Ca water (Ellis and Morris, '95).

Calcium homeostasis in larval/juvenile crustaceans has not been well studied; however, due to their small size, early life-history stages have a larger SA: volume than adults and this will affect Ca fluxes in hyperregulating species. Early development of the crab *Metopaulias depressus* is completed in small FW reservoirs of bromeliad leaf axils that cannot supply the Ca demand for the developing brood. Female crabs behaviorally supplement Ca within the nursery by placing snail shells in the reservoir thus elevating the Ca concentration for nonfeeding stages; they also provide Ca-rich millipede diet to young crabs (Diesel and Schuh, '93).

TABLE 1. Effect of environmental Ca on hemolymph acid base and ion balance in freshwater crayfish

Species	Water [Ca] (μM)	Temp (°C)	Hemolymph (mM) pH	Ca	Na	Cl	Reference
High calcium							
Procambarus clarkii	1100	15	8.0	9	199	196	Morgan and McMahon, '82
Pacifastacus leniusculus	3200	15	7.9	9	200	201	Wheatly and McMahon, '82
Intermediate calcium							
Procambarus clarkii	600	23	7.6	12	218	192	Wheatly et al., '96
Cherax destructor	500	20	7.6	19	325	ND[1]	Ellis and Morris, '95
Orconectes propinquus	100	10	7.9	10	193	178	Wood and Rogano, '86
Astacus astacus	100	15	8.0	10	190	175	Jensen and Malte, '90
Low calcium							
Procambarus clarkii	50	23	7.6	8.5	205	170	Wheatly, unpubl.
Cherax destructor	50	20	7.6	19	161	ND	Ellis and Morris, '95

[1]ND, not determined.

Terrestrial strategies of Ca homeostasis have centered on land crabs (decapods) as well as amphipods and isopods. Brachyuran crabs have invaded land via SW (Ocypodidae, Gecarcinidae, Grapsidae) or FW origins (Potamidae). Intermolt land crabs regulate EC Ca (15 mM) by controlling input via food and drink (Greenaway, '85, '93). Most land crabs are omnivores. Leaf litter predominates their diet but has been shown to be growth limiting in *Gecarcinus lateralis* (Wolcott and Wolcott, '84). Mineral deficiency can nutritionally modulate carnivory and cannibalism (Wolcott, '88). Dietary Ca intake has recently been investigated in the herbivorous land crabs *Cardisoma hirtipes* (Greenaway and Raghaven, '98) and *Gecarcoidea natalis* (Greenaway and Linton, '95). Brown leaves contained twice the Ca content of green leaves but assimilation coefficient for *Cardisoma hirtipes* remained constant (20%) on either type of leaf. Calcium retention was proportional to dietary Ca intake. Assimilation was higher (37%) in *Gecarcoidea natalis*; however most of the ingested Ca was lost in the feces. Table 2 compares the role of different epithelia in intermolt Ca balance in an evolutionary continuum of decapods. In intermolt, crustaceans are typically in Ca balance; net fluxes via gills or food are relatively low.

Optimality models propose that intestinal nutrient transporters are inducible and regulated by dietary intake depending on the cost vs. benefit of absorption (Diamond, '91). In mammals, restricted Ca intake stimulated intestinal Ca^{2+} uptake (Ferraris and Diamond, '89), whereas a high-Ca diet had the effect of downregulation (Bronner, '96). In insects, Ca intake increased with dietary content and was postabsorptively regulated at the Malpighian tubules (Taylor, '85). This implicates use of more economical passive intake mechanisms that are more conducive to rapid dietary switching. Land crabs will serve as a future model to assess the regulation of intestinal Ca transporters by dietary Ca intake.

Land crabs modify the Ca content of the excretory product by postrenally reabsorbing 80% of the Ca in their isoionic urine after it has seeped into the branchial chamber (Wolcott and Wolcott, '82, '85, '91; Greenaway and Morris, '89; Morris et al., '91). Annual breeding migrations of land crabs *Gecarcoidea natalis* can present a challenge for Ca balance (Greenaway, '94). Feeding is curtailed for the seven days of the migration and continuous movement is incompatible with urinary reprocessing. These two factors combine to lower hemolymph Ca. Crabs rectify this upon arrival at the coast by "dipping" activity which restores EC Ca balance.

Molting in different calcium environments

In adult marine crabs, relatively little Ca (<20%) is stored between molts in amorphous form in regions of the gut. In full-strength SW, Ca enters postmolt *Callinectes* passively via the gills at rates of 10 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (Cameron and Wood, '85; Cameron, '89; Neufeld and Cameron, '92). In dilute SW (2 ppm), postmolt crabs are able to calcify as rapidly and accumulate as much Ca as

TABLE 2. Relative role of Ca transporting epithelia in whole organism Ca flux in intermolt crustaceans from different environments

		Ca^{2+} flux ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)			
Species	Epithelium	Net flux	Influx	Outflux	Reference
Sea water					
Carcinus maenas	Gill	–161	+513	–674	Greenaway, '76; Zanders, '80
	Antennal gland	–31	+18	–48	
Cancer magister	Antennal gland	–5	+6	–11	Wheatly, '85
Homarus gammarus	Antennal gland	–43	+40	–83	Whiteley and Taylor, '92
Fresh water					
Procambarus clarkii	Gill	–130	+12	–142	Wheatly and Gannon, '95
Pacifastacus leniusculus	Gill	–40			Wheatly, '89
	Antennal gland	–4	+35	–39	Wheatly and Toop, '89
Austropotamobius pallipes	Gill	+3			Greenaway, '74
	Antennal gland	–6	+41	–46	Tyler-Jones and Taylor, '86
Terrestrial					
Birgus latro	Antennal gland	–1	+40	–41	Greenaway et al., '90
Leptograpsus variegatus	Gills	+90			Morris and Greenaway, '92
Cardisoma hirtipes	Digestive	+104	+542	–438	Greenaway and Raghaven, '98
	Antennal gland	–6	+1	–7	Greenaway, '89
Gecarcoidea natalis	Digestive	+58	+158	–100	Greenaway and Linton, '95

crabs in SW using active uptake processes (Neufeld and Cameron, 1992).

In adult FW crayfish, Ca storage between molts (in gastroliths) approximates that of marine species (14%). Postmolt mineralization involves active uptake of Ca primarily through the gills during the initial 4–5 days postmolt (Wheatly and Ignaszewski, '90; Wheatly and Gannon, '95); any deficit is restored by ingestion of exuviae (30%) and other food items. A parchment shell is produced within hours with complete recalcification occurring over a few days. Removal of external Na or HCO_3 reduced Ca uptake by 55%. In Ca-free medium, postmolt crayfish remained in Ca balance. When Ca was reintroduced into the medium after four days, calcification commenced, but at reduced rates. Thus active Ca-uptake mechanisms appear to be maximally active in the initial postmolt period. However a secondary increase in Na uptake upon reintroduction of external Ca, confirmed that Ca uptake is partially Na-dependent (Wheatly and Gannon, '95). During five days in acidic water (pH 5.2), Ca uptake was 60% reduced, and total body Ca was 40% lower compared with postmolt crayfish in neutral pH (Zanotto and Wheatly, '95). These effects could not be reproduced in high CO_2 acid water suggesting that the response was due to lowering of ambient CO_2 rather than a direct effect of pH. In alkaline water (pH 9.2) Ca uptake was 30% elevated.

Juvenile/larval Ca balance during the molting cycle has been studied in crayfish *Procambarus clarkii*. The calcium content of whole body, exuviae and gastroliths had scaling exponents of 0.93–1.27 (Wheatly and Ayers, '95). Large crayfish demineralize the cuticle more effectively in premolt and store less mineral compared to smaller crayfish. Small crayfish remineralize more rapidly in postmolt commensurate with increased molting frequency. Allometric relationships (slope 0.85–0.98) have recently been demonstrated on postmolt day 1 for both net Ca flux and unidirectional Ca influx (Zanotto et al., unpublished).

Marine land crabs store Ca internally in gastroliths (5–15%) although only at the same level as their aquatic counterparts; instead they rely on reingesting Ca in the shed exuviate. Wheatly ('97) estimated the rate of postmolt digestive Ca^{2+} uptake in the land crab *Gecarcoidea natalis* to be around 12,000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. Ca from storage sites and reingested exuviae is used for immediate calcification of critical regions. Once feeding resumes, dietary Ca will make up the deficiency. Behavioral adaptations enable many land crabs to maintain Ca balance. Many retreat to sealed burrows (Ocypode) where access to exuvial Ca is assured (Greenaway, '93). Often land crabs remain in their sealed burrows from three to four weeks during which time they do not feed (*Birgus*). They may also extract Ca from available burrow water using branchial uptake mechanisms (e.g., *Cardisoma*, Wood and Boutilier, '85; Pinder and Smits, '93). Certain intertidal species obtain Ca from rock pools, cliffs, or calcareous sand (*Grapsus, Gecarcinus*). FW land crabs (*Holthuisana*) use the hemolymph to increase Ca conservation to 65% (Sparkes and Greenaway, '84).

In terrestrial amphipods, the best-developed model is *Orchestia cavimana* which resides on the beach (family Talitridae, Meyran et al., '84, '86). Calcium reabsorbed from the old cuticle is temporarily stored in the posterior caeca (PC) which are diverticula of the midgut.

Terrestrial isopods (suborder Oniscoidea) have emerged in recent years as an interesting crustacean model for Ca homeostasis by virtue of their biphasic molting cycle which has been modeled on the species *Oniscus asellus* (Steel, '93), and *Porcellio scaber* (Ziegler, '96). Apolysis and epicuticle secretion occur synchronously throughout the animal until day 10 of premolt. At this time, Ca reabsorbed from the posterior cuticle is deposited in the anterior 4 sternites in the space between the old cuticle and the sternal epithelium (20% body Ca). Additional Ca is stored in the epimeral plates and throughout the general cuticle of the anterior region with total Ca storage totaling 48%. Ecdysis of the posterior half (Ep) occurs on day 16 followed by rapid remineralization using the transposed Ca (12 hr). Following a period called *intramolt* (24 hr), anterior ecdysis (Ea) follows on day 17, followed by calcification of the new anterior cuticle with Ca from the hemolymph. Synchrony is regained during days 17–23 postmolt. The isopods preferentially reingest their exuviae in order to recycle additional Ca. Complete mineralization requires Ca from ingestion of other food items.

EXTRACELLULAR CA REGULATION

In crustaceans, the majority of internal Ca is located extracellularly either in the acellular hemolymph or regions of the body that are calcified/decalcified associated with the molting cycle.

Intermolt hemolymph Ca regulation

Circulating Ca is held at a constant level in a range of decapods (around 12 mM). In intermolt

crabs diffusible Ca is in equilibrium; unidirectional Ca fluxes are equivalent both into and out of the animal (Table 2) suggesting exchange diffusion between internal and external pools (Greenaway, '76). In intermolt crayfish, active Ca influx mechanisms are silent in intermolt and unidirectional efflux results in a small negative Ca balance (Table 2, Wheatly and Gannon, '95). Investigators have used a battery of environmental parameters in an attempt to alter circulating Ca; however, in most cases it has been found that EC Ca is very tightly regulated.

Exposure of crayfish to low-Ca water (50 μM; Table 1) resulted in a reduced hemolymph Ca in *Procambarus clarkii* (Wheatly et al., unpublished) while not in *Cherax destructor* (Ellis and Morris, '95). Since the crayfish does not actively take up Ca in intermolt (Table 2), an increased gradient for passive diffusion could increase efflux. Low external Ca also produced an EC acidosis, and lowered both circulating Na and Cl due to increased permeability. In the wild, crayfish acclimated to low Ca (75 μM) continue to take up Ca throughout intermolt (Hessen et al., '91). In high Ca water, the hemolymph Ca did not rise (Table 1); however, inspection of crayfish at the conclusion of the experiment revealed well-developed gastroliths suggesting that excess Ca was stored.

In the past, whenever an elevation in EC Ca has occurred in response to EC acidosis there has been a tendency to associate this circumstantially with exoskeletal $CaCO_3$ mobilization for purposes of buffering H^+ (Morris et al., '86). The only definitive study addressing the involvement of carapace in buffering hypercapnic acidosis was performed in *Callinectes* (Cameron, '85) and showed that its role was negligible. It was postulated that this strategy was employed preferentially in air when standard branchial ion exchange mechanisms for acid-base regulation are inoperative (Wheatly and Henry, '92). A number of recent studies have continued this debate using external acidification or removal of water breathers into air as experimental stimuli.

Calcium balance during acidification in FW crustaceans is a complex issue that appears to be affected by water hardness, acid type, and evolutionary history. Several studies have reported a significant rise in Ca associated with severe EC acidosis upon external acidification of crayfish (*Orconectes*, Ca, 0.1 mM, Wood and Rogano, '86; *Procambarus clarkii*, Ca, 1 mM, Morgan and McMahon, '82). In other studies, where the EC acidosis was less pronounced, hemolymph Ca was unaltered (*Procambarus clarkii*, Ca, 0.6 mM, Wheatly et al., '96; *Cambarus*, Ca, 0.05 mM, Hollett et al., '86; *Astacus*, Ca, 0.1 mM, Jensen and Malte, '90). As in fish, the hemolymph acidosis appears to be more pronounced in harder water resulting from a loss of cations in excess of anions. The Ca ion offers a protective effect against severe ion loss in hard water arising from the weak ionic interactions with surface ligands on the gill which serves to stabilize the apical membrane (McDonald, '83). Aside from water hardness, evolutionary history is also important in the EC Ca response to acidification. Species (such as *Cambarus*) evolving under softer water conditions of mountain streams evolved ionoregulatory mechanisms that preadapted them to withstanding low-pH stress more successfully than species originating from central basins of great rivers (*Orconectes*). While sulfuric acidification raises Ca, nitric acid does not (McMahon and Stewart, '89).

Ellis and Morris ('95) attempted to resolve the debate by examining external acidification of the Southern Hemisphere crayfish *Cherax destructor* during long term (21 days) exposure to H_2SO_4 in either low- (50 μM) or high-Ca water (500 μM). Acidification in low-Ca water produced a metabolic acidosis compensated by a respiratory alkalosis and accompanied by a 3.2 mM increase in hemolymph Ca. However, this Ca increase was not accompanied by elevated HCO_3 or reduced carapace Ca content. Thus it was concluded that it originated, if anything, from other storage sites such as muscle or hepatopancreas. In low-Ca acid water, hemolymph Na was depressed suggesting that Ca has a role in mediating Na^+ and H^+ movements across the apical surface of branchial cells. The low-Ca level employed (50 μM) is at or below the minimum Ca for normal homeostasis. Under these conditions, crayfish appeared to maintain acid-base balance at the expense of ion balance by reducing H^+ permeability, stimulating H^+ excretion, shutting down Cl^-/HCO_3, and/or conserving HCO_3 to buffer entering H^+ (Wheatly and Henry, '92).

Along the same lines, aerial exposure of aquatic decapodans has in some cases resulted in elevated EC Ca (*Austropotamobius*, Morris et al., '86). This is typically observed in species that are poorly adapted/evolved for air breathing and that incur a significant hemolymph acidosis. Species routinely exposed to air in nature encounter mini-

mal acid-base disturbance and correspondingly maintain hemolymph Ca (*Procambarus*, Wheatly et al., '96; *Carcinus*, de Souza, and Taylor, '91).

So the degree of acidosis appears to be the major factor in influencing EC Ca homeostasis, namely that hemolymph Ca only rises under severe EC acidosis. Confirming this hypothesis was a recent study on exposure of *Carcinus* to lethal Cu levels (Boitel and Truchot, '89). An induced metabolic acidosis later reinforced by hypercapnia and accumulation of lactic acid resulted in restricted gas exchange. A steep increase in hemolymph Ca concentration occurred immediately before death. However all studies to date are in agreement that any EC Ca appearing in the hemolymph is translocated from soft body tissues and not the carapace (de Souza, '92).

Hemolymph calcium regulation during molting cycle

It is fairly well established that hemolymph Ca is well regulated during the molting cycle of aquatic species (Greenaway, '85; Zanotto and Wheatly, '93) even when whole-body Ca fluxes are profound. Total Ca typically increases by 40% in premolt associated with Ca reabsorption and decreases in postmolt associated with mineralization (Wheatly and Hart, '95). The premolt increase is primarily in the bound moiety; free Ca which is more relevant biologically, and can now be measured with ion selective electrodes, remains constant (Neufeld and Cameron, '92). Crayfish precipitated into ecdysis by multiple limb autotomy (MLA) exhibited an earlier premolt Ca peak and those precipitated into ecdysis by eyestalk removal (ER) did not exhibit a premolt Ca peak suggesting that Ca balance in artificially induced molts is not equivalent to those molts that occur naturally (Wheatly and Hart, '95).

Some FW land crabs (such as *Holthuisana*) store Ca in the form of $CaCO_3$ granules in the hemolymph during ecdysis (Sparkes and Greenaway, '84). This strategy enables storage to approach 65% with hemolymph concentration rising by 150-fold to 2 M. These granules are formed in subepidermal connective tissue cells (Greenaway and Farrelly, '91); their small size (0.26 μm) allows them to pass through the circulatory system. The hemolymph of *Ocypode* also appears milky immediately after ecdysis and Ca concentration reaches 70–100 mM (Greenaway, '93). In the amphipod *Orchestia cavimana,* total and ionized Ca change in parallel; they increase in premolt and decrease in postmolt (Meyran et al., '93). In the isopod *Oniscus,* the hemolymph becomes milky and viscous around ecdysis again suggestive of storage in the hemolymph (Steel, '93).

Calcified deposits

In intermolt crustaceans, most of the whole body Ca (80%) resides in the outer acellular layers of the cuticle in the form of crystalline $CaCO_3$. During premolt the cuticle is weakened in place by $CaCO_3$ reabsorption. Some Ca is stored in amorphous form in EC mineralized structures such as gastroliths in crayfish and crabs, and sternal deposits in isopods. Some is stored in amorphous form in IC granules and a significant amount is excreted. At ecdysis, the exuviae still contain significant quantities of Ca (30–70%); this Ca can be reingested in postmolt. Calcified deposits are remobilized in postmolt to provide Ca for immediate cuticular mineralization. The bulk of required Ca however comes from external sources (water/food). This area has been reviewed elsewhere (Greenaway, '85; Wheatly, '96, '97). Calcified deposits exhibit some common characteristics. First, their Ca flux is exactly the opposite of that at the cuticle (e.g., gastroliths are deposited while the cuticle is being reabsorbed and vice versa). This presents some interesting questions with respect to synchrony of control mechanisms. In other words, reabsorption and deposition of $CaCO_3$ are routinely exhibited simultaneously at different epithelia. Second, while the calcified deposits are deposited over the space of several weeks, they are reabsorbed within a period of hours.

The best-studied calcified deposits are the gastroliths which are paired $CaCO_3$ concretions formed by the gastrolith disc in the cardiac stomach of crayfish and some crabs. They are formed beginning at 40 days before ecdysis from hypertrophied mitochondria that slough off into the lumen of the stomach. At ecdysis only 20% body Ca remains in the soft crayfish and 17% of this is contained in the gastroliths (Wheatly and Ayers, '95). The gastroliths are solubilized within 24 hr and the Ca is used to preferentially harden mouthparts, gastric ossicles and dactyls of walking legs so that motility and feeding are resumed.

In the terrestrial isopods *Oniscus* and *Porcellio,* amorphous $CaCO_3$ deposits combined with an organic matrix are stored between the epithelium and old cuticle of the anterior sternites (Steel, '93; Ziegler, '94). After Ep, reabsorption of the Ca in these deposits is synchronized with calcification

of the new posterior exocuticle. In the FW land crab *Holthuisana* Ca reabsorbed from the cuticle in premolt is stored as large EC noncirculating granules in narrow intercellular channels between epidermal cells (large 0.8–4 μm, $CaPO_4$). These granules essentially get trapped so as to avoid obstruction of small hemolymph lacunae and arterial branches (Greenaway and Farrelly, '91). In amphipods, calcareous concretions are stored in the PC lumen (Meyran et al., '84).

Paracellular calcium pathway

One model for transepithelial Ca transport involves an extracellular or paracellular pathway that consists of passive movement between cells and across cell junctions along an electrochemical gradient. The paracellular pathway has been proposed for Ca storage in the midgut PC of the amphipod *Orchestia* (premolt, Meyran et al., '84) and the anterior sternal epithelium (ASE) of *Porcellio* (during resorption of deposits in intramolt, Ziegler, '96). In *Orchestia,* Ca enters the basal surface of the caecal epithelium cells from the hemolymph and is pumped into narrow IC channels to enter the lumen of the caeca predominantly via a paracellular route, although some may be pumped across the apical membrane directly into the lumen. After exuviation of amphipods, the Ca stored in the PC lumen concretions is transported from the caecal lumen to the hemolymph within 24–48 hr and used to mineralize the new cuticle (Graf and Meyran, '83, '85). The PC epithelium has a network of EC channels like that of the ASE of *Porcellio*. During postmolt resorption of the calcareous concretions, spherical granules appear in the EC network. These granules are secreted by epithelial cells and disintegrate into smaller particles (still EC) and ultimately into ionized Ca that enter hemolymph. There is ultrastructural similarity between the ASE of *Porcellio* and the midgut epithelium of PC of *Orchestia*.

There is less support for involvement of the paracellular route in reabsorption/mineralization of the cuticle. In the premolt FW land crab *Holthuisana*, Greenaway and Farrelly ('91) do not support the paracellular route for Ca movement from molting fluid to hemolymph. Calcium would have to diffuse through the thickness of the procuticle to reach the apical junctions and it is unlikely that such a concentration gradient exists since both solutions are saturated with respect to $CaCO_3$. Additionally the apical junctions are "tight." Onset of reabsorption may be regulated by solubilization of $CaCO_3$. During cuticular mineralization the matrix behaves like a Ca sink; however, free diffusion of Ca would allow for continuous uncontrolled mineralization which does not occur.

INTRACELLULAR CALCIUM REGULATION

Transcellular transport in intermolt

Transepithelial Ca flux typically involves a transcellular pathway summarized in Figure 2. Since cytosolic Ca is kept low (0.1–0.5 μM), this will involve passive Ca influx across the plasma membrane on one side of the cell and active extrusion across the membrane on the opposing side. Most crustacean Ca transporting epithelia exhibit bidirectional Ca transport at different stages in the molting cycle. Membrane transporters in intermolt crustaceans have been identified in lobster hepatopancreas and antennal gland, crab gills, and in all three crayfish tissues using vesicle approaches. Entry into the cell across the outer apical membrane may be passive or via a Ca^{2+}/Na^+ (H^+) antiporter. However active extrusion from the cell across the inner basolateral membrane is via Ca^{2+} ATPase or indirectly via Ca^{2+}/Na^+ exchanger. These active extrusion mechanisms can be used either to fine tune IC levels (housekeeping) or to accomplish Ca^{2+} translocation. This pathway requires the presence of intercellular junctions that limit paracellular movement. A major constraint on this mechanism is that Ca must be transported through the cell without increasing IC Ca concentration above the submicromolar range (see below). Transmembrane Ca transfer will be governed by both the electrical and chemical gradients. In crayfish transepithelial potential (TEP) is very sensitive to external Ca (Kirschner, '94). At 0.034 mM Ca TEP was –18 mV; as external Ca increased, TEP became more positive. The TEP is attributed to the diffusive efflux of NaCl across the gills, with a Cl/Na perm ratio of 1. The Ca dependence of the TEP originates from the fact that active inward Ca transport is electrogenic.

Apical mechanisms

Apical transport at the lobster, *Homarus*, antennal gland (Ahearn and Franco, '93), and hepatopancreas (Zhuang and Ahearn, '96) involves three independent processes, two of which involve carrier-mediated facilitated diffusion through antiporters that can exchange Ca^{2+} for either Na^+ or H^+: (1) amiloride sensitive electrogenic $2Ca^{2+}/$

H^+ (H^+ preferred to Na^+) antiport that is unique to crustaceans—this transporter exhibits 2 external cation binding sites with markedly dissimilar apparent binding affinities and a single IC binding site ($K_{0.5}$, 0.05 mM; V_{max}, 7 nmol · mg^{-1} · 2.5 sec^{-1}); (2) amiloride insensitive electroneutral $1Ca^{2+}/2Na^+$ antiport (Na^+ preferred to H^+, $K_{0.5}$, 0.23 mM; V_{max}, 2.27 nmol · mg^{-1} · 2.5 sec^{-1}); (3) simple diffusion through a verapamil-sensitive Ca channel that is dependent upon membrane potential.

Basolateral mechanisms

Basolateral membrane vesicles (BLMV) have been employed to characterize basolateral Ca transport in a range of intermolt crustaceans (Table 3). BLMV from gills of intermolt crab, *Carcinus*, acclimated to 50% SW (Flik et al., '94) exhibited ATP-dependent uptake (Ca2+ ATPase with $K_{0.5}$, 149 nM; V_{max}, 1.73 nmol · mg^{-1} · min^{-1}) and Na gradient-dependent uptake (Na^+/Ca^{2+} with $K_{0.5}$, 1780 nmol; V_{max}, 9.88 nmol · mg^{-1} · min^{-1}). Affinity of both mechanisms matched physiological IC Ca. At cytosolic Ca concentrations up to 500 nM, the Ca^{2+} ATPase is the primary mechanism of efflux; however, above 500 nM the exchanger predominates. The same two independent processes of energized Ca transport were demonstrated in BLMV of lobster hepatopancreas (Zhuang and Ahearn, '98) namely a vanadate-sensitive high-affinity but low-capacity calmodulin-dependent plasma membrane Ca^{2+} ATPase (PMCA; $K_{0.5}$, 65 nM; V_{max}, 1.07 pmol · μg^{-1} · 8 sec^{-1}) and a low-affinity high-capacity $Ca^{2+}/3Na^+$ (or Li^+) antiporter ($K_{0.5}$, 14.6 μM). When Li^+ replaced Na^+ in exchange for Ca^{2+}, the apparent affinity for Ca influx was not significantly affected ($K_{0.5}$ for Na 14 μM, Kt for Li 20 μM) but the V_{max} was reduced by factor of 3 (V_{max} for Na^+, 2.72 pmol · μg^{-1} · 8 sec^{-1}; V_{max} for Li^+, 1.03 pmol · μg^{-1} · 8 sec^{-1}). The Ca^{2+} ATPase kinetics approximated to those reported by Flik et al. ('94) in crab gill. At IC Ca activities that might occur in a typical cell (100–500 nM) greater than 90% of Ca efflux takes place via the Ca ATPase. At IC Ca of 1,000–10,000 nM, the $Ca^{2+}/3Na^+$ assumes a greater role in Ca efflux from the cell. It has been postulated that such concentrations may be attained during elevated transcellular Ca flux associated with storage and remobilization of Ca deposits in hepatopancreas.

Flik and Haond (unpublished) have compared kinetics of both Ca^{2+} ATPase and Na^+/Ca^{2+} exchanger in full-strength SW and after 2-week acclimation to 70% SW in branchial tissues (gills, epipodites, and branchiostegites) of the lobster (*Homarus gammarus*), a weak hyperregulator (Table 3). Affinity of both transporters was unchanged upon dilution. However, the transport capacity of both transporters in epipodites and branchiostegites increased significantly while corresponding levels in the gills decreased. A related study (Haond et al., '98) using confocal laser scanning microscopy revealed that the epipodites (lamellar organs inserted between the gills) and inner side of the branchiostegites are covered by a well-differentiated osmoregulatory epithelium that becomes increasingly differentiated during external dilution. Basolateral infoldings develop, apical microvilli increase in height, and vesicles proliferate. Meanwhile the trichobranchiate gills remain poorly differentiated and their ultrastructure is unchanged upon dilution. The epipodites and branchiostegites serve as ancillary ion transport epithelia to compensate for ion loss through the permeable cuticle and the limited ionoregulatory capacity of the gills.

My lab has recently characterized ATP-dependent Ca^{2+} uptake into osmotically reactive inside-out resealed BLMV (Wheatly et al., '99) from hepatopancreas, gill, and antennal gland of intermolt FW crayfish (Table 3). ATP-dependent $^{45}Ca^{2+}$ uptake (Wheatly et al., '99) was abolished by pretreatment with either vanadate or the ionophore A_{23187}. Calcium affinity was equivalent in hepatopancreas and gill ($K_{0.5}$, 0.28 μM) but was higher in antennal gland (0.11 μM). Maximal uptake was 20.26 pmol · mg pr^{-1} · min^{-1} in hepatopancreas and was 5–6× higher in gill and antennal gland. An ATP titration curve indicated a $K_{0.5}$ of 0.01 mM in hepatopancreas and gill and 0.039 mM in antennal gland. EGTA treatment of hepatopancreas and antennal gland vesicles decreased Ca^{2+} uptake by 50%; uptake was restorable by calmodulin. However, in gill Ca uptake was unaffected by EGTA treatment and calmodulin decreased uptake. Q_{10} ranged from 1.43 to 2.06. Addition of Na (5 mM) caused a 60% increase in Ca uptake that was inhibitable by preincubation with ouabain indicating that the Na pump generates a Na^+ gradient favorable for Ca accumulation via the Na^+/Ca^{2+} exchanger. Capacity for $^{45}Ca^{2+}$ uptake was an order of magnitude greater in gill than in either hepatopancreas or antennal gland. In vitro branchial $^{45}Ca^{2+}$ uptake capacity was 6× greater than in vivo unidirectional influx; however, in antennal gland Ca pumps operate at capacity. Sodium gradient-dependent uptake into vesicles is being examined using standard techniques and flow cytometry. Comparison of kinetics across the evolutionary

TABLE 3. Kinetics of Ca pump and exchanger in intermolt crustacean basolateral membrane vesicles

			Ca^{2+} ATPase		Ca^{2+}/Na^{+} exchanger[1]		
Species	Tissue	Temp °C	$K_{0.5}$ (μM)	V_{max} (nmol · min^{-1} · mg^{-1})	$K_{0.5}$ (μM)	V_{max} (nmol · min^{-1} · mg^{-1})	Reference
Sea water							
Homarus americanus	Hepatopancreas	20	0.07	133[2]	14.60	339	Zhuang and Ahearn, '98
Homarus gammarus	Gills	37	0.15	20	2.74	248	Flik and Haond, unpubl.
	Epipodites	37	0.24	3	4.26	256	Flik and Haond, unpubl.
	Branchiostegites	37	0.21	8	4.34	332	Flik and Haond, unpubl.
Dilute SW (60%)							
Carcinus maenas	Gills	37	0.15	8	1.78	45	Flik et al., '94
Homarus gammarus	Gills	37	0.15	6	3.24	130	Flik and Haond, unpubl.
	Epipodites	37	0.21	8	4.02	310	Flik and Haond, unpubl.
	Branchiostegites	37	0.23	13	3.95	391	Flik and Haond, unpubl.
Fresh water							
Procambarus clarkii	Gills	37	0.28	0.63	ND	ND	Wheatly et al., '99
	Hepatopancreas	37	0.27	0.14	ND	ND	Wheatly et al., '99
	Antennal gland	37	0.11	0.58	ND	ND	Wheatly et al., '99

[1]ND, not determined.
[2]V_{max} is corrected for membrane configuration.

continuum (Table 3) reveals that affinities of both Ca pump and exchanger (except for lobster hepatopancreas) were comparable in all species. However V_{max} decreased with evolution into fresh water, probably associated with reduced permeability and passive fluxes.

In intermolt aquatic crustaceans, active Ca influx at the gills is minimal (Table 2). Ca influx across isolated perfused gills of intermolt shore crab (Pedersen and Bjerregaard, '95) occurred mainly by passive mechanisms. The electrochemical gradient is directed into marine crabs at 30 and 20 ppt and out of the animal below 6 ppt (Neufeld and Cameron, '92, '93). Similarly, in FW crayfish there is virtually negligible active Ca influx at the gills in intermolt; however, production of a dilute urine necessitates continuous reabsorption of Ca from urinary filtrate at the nephridial canal cells of the antennal gland.

In land crabs, Ca^{2+} ATPase activity has been reported in whole gill homogenates associated with Ca reabsorption from voided urine (*Birgus latro*, Morris et al., '91; *Leptograpsus variegatus*, Morris and Greenaway, '92). In the terrestrial anomuran *Birgus latro,* the specific activity (10 nmol $\cdot$ min^{-1} $\cdot$ mg pr^{-1}) and affinity ($K_{0.5}$ < 9 μM; Morris et al., '91) were typical of those reported in aquatic species (Table 3). In the supratidal brachyuran *Leptograpsus variegatus* the affinity of Ca^{2+} ATPase was 6–34 nM and specific activity was 8 nmol $\cdot$ min^{-1} $\cdot$ mg pr^{-1} (Morris and Greenaway, '92). Digestive epithelia would be continuously involved in uptake of Ca from food in intermolt, the primary time for feeding.

Bidirectional transcellular transport during molting cycle

The models for apical to basolateral Ca movement or vice versa (basolateral to apical) have been explored in Wheatly ('97) for both mineralizing (hypodermis, gastrolith disc) and nonmineralizing epithelia (gill, antennal gland, and hepatopancreas). Basolateral to apical transport (postmolt cuticular mineralization or premolt gastrolith formation) requires that Ca flows from hemolymph into cells through channels in the basolateral membrane (Fig. 2). Ca is sequestered in mitochondria/organelles and the rest is transported out of the apical membrane by the amiloride-insensitive carrier system or Ca ATPase for delivery to the mineralizing matrix of cuticle/gastroliths. Transcellular Ca^{2+} transport was proposed for *Carcinus* (Roer, '80) and *Callinectes* (Cameron, '89) during cuticle calcification. Apical to basolateral (postmolt branchial uptake, gastrolith remobilization, premolt cuticular reabsorption, digestive absorption) requires that Ca enters the apical membrane via channels and exchangers outlined above. Inside the cell, Ca is released from IC stores. Transport across the basolateral membrane is via Ca^{2+} ATPase and $Ca^{2+}/3Na^{+}$.

If Ca^{2+} ATPase is functional during bidirectional transcellular Ca transport then one would expect its activity to switch location between basolateral and apical at different stages of the molting cycle. For example, in hypodermis one would expect it to be located basally during premolt and apically during postmolt. In *Callinectes* hypodermal quercetin-sensitive Ca^{2+} ATPase (Greenaway et al., '95) was localized along basolateral membranes below the apical junction in premolt (stage D2). By postmolt stage, A2 Ca^{2+} ATPase was localized along apical membranes and microvilli. At B2, similar activity was seen and the apical cytoplasm contained small vacuoles with activity. Minimal activity was observed in intermolt. Similarly, during basal to apical transport Ca^{2+} ATPase activity was evident in apical microvilli of the storage epithelium in *Orchestia* (Meyran and Graf, '86). Thus the Ca pump can be located on either membrane depending upon the directionality of transport. The Na^{+}/Ca^{2+} exchangers derive their energy from a Na^{+} gradient established by the Na^{+} pump. The Na^{+} pump was localized in basolateral membrane of the ASE and PSE of the isopod *Porcellio scaber* (Ziegler, '97a). It remained localized exclusively in basolateral membranes irrespective of directionality of the Ca transfer and was present during formation and resorption of $CaCO_3$ deposits. The ASE exhibited greater Na pump activity than the PSE. Activity was maximal during premolt and intramolt when basolateral membrane area increased.

Typically, Ca^{2+} ATPase levels are elevated at mineralizing sites. Ca^{2+} ATPase activity was elevated three- to fourfold in cuticular hypodermis during postmolt of both blue crab (Cameron, '89) and crayfish (Wheatly, '97). One caveat is that the data from *Callinectes* may represent nonspecific alkaline phosphatase (Flik et al., '94). In a cytochemical study, Ca^{2+} ATPase increased in crayfish gastrolith disc both in premolt calcification and postmolt reabsorption (Ueno and Mizuhira, '84). Specific activity of Ca^{2+} ATPase did not alter during the molting cycle at the gill in either study or in the supratidal crab *Leptograpsus* (Morris and Greenaway, '92).

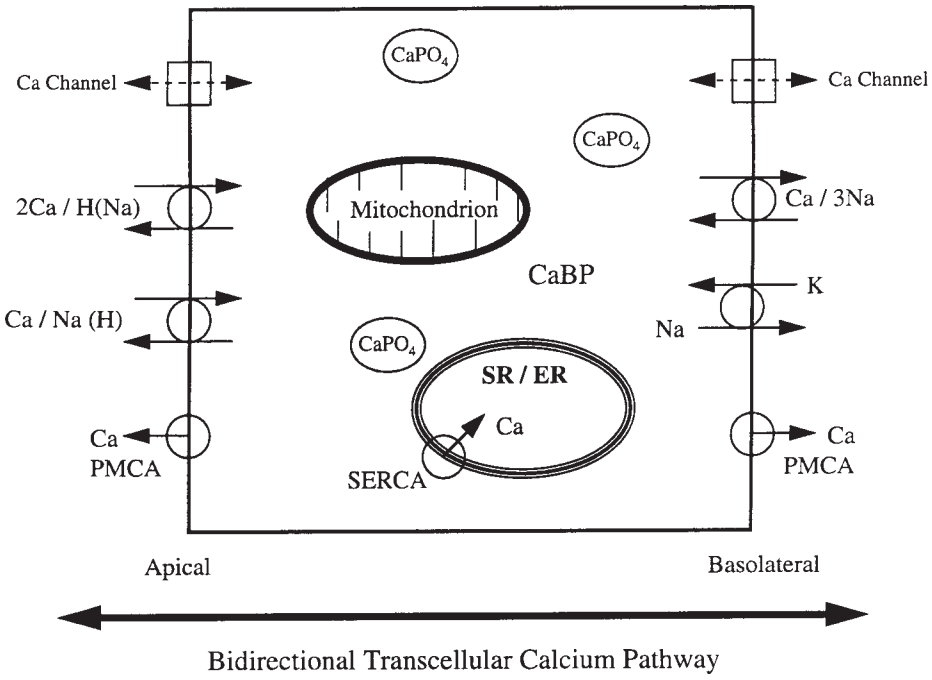

Fig. 2. Model of transcellular calcium pathway in crustaceans. CaBP, calcium binding protein; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, sarco-/endoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum.

The postmolt cuticular hypodermis transfers Ca into the outer layers via cytoplasmic extensions ($5 \times 10^6 \cdot mm^{-2}$) that are contained in pore canals (Compère and Goffinet, '87). An ultrastructural study of the cuticle of *Carcinus* using K-pyroantimonate and X-ray microanalysis (Compère et al., '93) demonstrated that plasma membranes of the cytoplasmic extensions are densely lined by particles of antimony precipitate indicating location of transporting sites for Ca^{2+} ATPase and Na^+/Ca^{2+} exchanger. Gills are covered in a noncalcified cuticle that must be shed at ecdysis. The ultrastructure of the gill filament epithelium of the crayfish *Procambarus clarkii* was examined throughout the molting cycle (Andrews and Dillaman, '93). Compared to the mineralizing cuticle, preexuvial cuticle deposition is greatly delayed in the gill filaments occurring only during the last 10% of premolt so that increased diffusion distance is minimized. Also postexuvial cuticle is not deposited at the gills, again leaving ion uptake uninterrupted.

The anterior sternal epithelium (ASE) and posterior sternal epithelium (PSE) of the terrestrial isopod *Porcellio* (Ziegler, '96, '97b) show structural differentiations associated with secretion and resorption of $CaCO_3$. During formation of $CaCO_3$ deposits (late premolt and intramolt) the basolateral plasma membrane of the ASE increases in area due to an elaborate interstitial network (IN) of interstitial dilations and channels. Also, mitochondrial volume increases. Such ultrastructural changes are typically associated with elevated transepithelial ion transport. After Ep, the direction of transepithelial Ca transport across the ASE has to be reversed and the rate of transport has to increase dramatically in order to degrade the $CaCO_3$ deposits within the short intramolt period (<24 hr). During resorption of $CaCO_3$ deposits numerous Ca containing osmiophilic granules are formed on the basolateral membrane. These detach, move basally and disintegrate into small osmiophilic particles when they cross the basal lamina. Their function might be to limit the Ca activity within the IN during resorption of the $CaCO_3$ deposits in order to maintain a specific Ca gradient across the plasma membranes of the epithelial cells. During resorption of the CaCO deposits, the apical plasma membrane of the ASE is increased by many subcuticular folds. The PSE does not contain IN, osmiophilic granules, or apical subcuticular folds. Cellular extensions of the epithelial cells into the cuticle occur in both the ASE and PSE but are more prominent in the former and may play a role in transport of ions involved in cuticle calcification. Although the ASE

of *Porcellio* is ectodermal, as is gastrolith disc, the ultrastructure is actually more similar to the amphipod midgut PC which is of endodermal origin.

Intracellular sequestration

Intracellular (IC) Ca^{2+} mediates signal transduction mechanisms that modulate many physiological functions in the cell. Therefore eukaryotic cells use active and passive mechanisms to maintain free Ca^{2+} within a very narrow range (100 nM). Regulation of cytosolic Ca is especially challenged during periods of elevated transcellular Ca flux associated with the molting cycle. Sequestration mechanisms include attachment to Ca-binding proteins (calsequestrin in SR, calmodulin, calreticulin), formation of $CaPO_4/CaSO_4$ granules, or sequestration among many IC organelles including ER/SR, mitochondria, nucleus, Golgi, endosomes, or lysosomes using ATP-coupled pumps. Existence of a Ca gradient through the cell cytosol may drive transport through the cell.

The sarco-/endoplasmic reticulum is a well-developed membranous structure which controls cytosolic free Ca through the release of Ca from channels and Ca resequestration via an ATP-dependent Ca-uptake pump—Sarco-/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA)—that is regulated by phospholamban (Lytton and MacLennan, '92). SERCA has been purified from skeletal muscle of the FW land crab *Potamon* (Tentes et al., '92). The enzyme, which has a molecular mass of 120 kDa, has a lower affinity for Ca than crustacean PMCA (Tables 3 and 4). It has two $K_{0.5}$ for ATP (40, 330 mM at ATP concentrations of 10–75 and >75 μM, respectively). The pH optimum is 7.5 and the enzyme is highly temperature dependent (17–40°C). In a separate study, SERCA activity was characterized in vesicles from SR of both fast (deep extensor abdominal muscle medialis) and slow (flexor muscle of carpopodite in meropodite) crayfish striated muscle (Ushio and Watabe, '93). Uptake rate was higher in fast muscle than in slow muscle possibly due to existence of different isoforms or differential modulation via proteins such as calmodulin and phospholamban. Finally, different phospholipid and fatty acyl-chain composition may alter SERCA activity in different muscle types. A comparison with PMCA kinetics (Tables 3 and 4) reveals that SERCA activity is almost three orders of magnitude greater than PMCA. This difference reflects relative abundance of pumps and has been confirmed by molecular studies (see below).

Using histochemical techniques we recently demonstrated that intramitochondrial electron-dense Ca precipitates increased significantly in crayfish nephridial-canal cells in postmolt corresponding with elevated transepithelial Ca flux (Rogers and Wheatly, '97). Ca reabsorbed from the FW land crab *Holthuisana* cuticle in premolt may be stored as IC granules (0.4–2 μm, $CaCO_3$) in epidermal cells (Greenaway and Farrelly, '91). Membrane-bound vesicles are formed in the apical cytoplasm by the Golgi. Lamellae appear and $CaCO_3$ is deposited in the organic matrix. Granule masses move basally and may be stored in the connective tissue. The hepatopancreas can play a role in Ca sequestration since amorphous $CaPO_4$ granules (1–5 μm) are found within the absorptive epithelial R cells in inter- and premolt of *Callinectes* and *Cancer* (Becker et al., '74). These are then released into the hemolymph for incorporation into the new exoskeleton during postmolt. Trace metals other than Ca, such as Zn and Cd, can be incorporated into these granules, making the crab hepatopancreas an environmental monitor for toxicological studies (Bjerregaard and Depledge, '94; Taylor and Simkiss, '94; Simmons et al., '96; Zhuang and Ahearn, '96). The Ca content of muscle increases in premolt in the white prawn (Vijayan and Diwan, 96). In postmolt this Ca is remobilized for mineralization.

MOLECULAR CA REGULATION

Molecular work on crustacean Ca pumps has followed the same chronology as in vertebrates where SERCA pumps were initially cloned in the mid-'80s from muscle, a tissue in which they are highly expressed. Within a few years this led to the cloning and sequencing of PMCA. At present the only crustacean Ca transporting proteins cloned are SERCA

TABLE 4. Characterization of sarco-endoplasmic reticulum Ca^{2+} ATPase in crustacean muscle

Species	Tissue	Temp (°C)	V_{max} (μmol · min^{-1} · mg^{-1})	$K_{0.5}$ (μM)	Reference
Procambarus clarkii	Slow muscle	20	0.270	ND[1]	Ushio and Watabe, '93
	Fast muscle	20	0.390	ND	
Potamon potamios	Slow muscle	37	0.450	2	Tentes et al., '92

[1]ND, not determined.

pumps in *Artemia* and *Procambarus*. My lab has recently obtained a partial cDNA fragment from crayfish PMCA.

Mammalian SERCA (MacLennan et al., '85) pumps Ca against its electrochemical gradient using the energy from hydrolysis of ATP, mediated through a covalent attachment of the γ phosphate to the polypeptide chain and subsequent conformational transitions. SERCA belongs to the superfamily of P-type ATPases which includes Na^+/K^+ ATPase, H^+/K^+ ATPase, yeast and plant H^+ ATPase, and bacterial K^+ ATPase. A molecular evolution study of the entire family was published recently (Axelson and Palmgren, '98). The protein has 1,000 amino acids forming 10 transmembrane domains each in a helical conformation, 4 in the NH_2-terminal and 6 in the COOH terminal. The first five transmembrane helices extend into the cytoplasm to form a pentahelical stalk domain. A cytoplasmic loop between stalk sectors 2 and 3 folds into a domain composed of entirely β strands, whereas the large polypeptide loop between stalk sectors 4 and 5 folds into two separate but communicating domains that bind ATP (nucleotide binding site) and accept the γ phosphate (phosphorylation site). The domains involved in the energy transduction process are highly conserved among P-type ATPases.

Mammalian SERCA is encoded by a family of three homologous and alternatively spliced genes encoding five isoforms (Wu et al., '95) which have a distinct pattern of tissue-specific expression. SERCA1 gene codes for two isoforms: SERCA1a in adult fast-twitch muscle and SERCA1b in neonatal fast-twitch muscle. SERCA2a (shorter isoform) is found in slow-twitch skeletal and cardiac muscle (MacLennan et al., '85; Brandl et al., '87) and SERCA2b (longer isoform) is ubiquitously expressed in all cell types (Gunteski-Hamblin et al., '88; Lytton and MacLennan, '88). SERCA3 has only one isoform that is expressed in nonmuscle tissue. The genes for SERCA1 and SERCA2 are 84% homologous in rabbit (MacLennan et al., '85). The SERCA3 amino-acid sequence is 76% identical with SERCA1 and SERCA2 (Burk et al., '89). The SERCA2 gene is closest to the invertebrate gene (see below). Four distinct mRNAs have been revealed in a variety of tissues derived from the same gene transcript by alternative transcript processing (Wuytack et al., '92). The four mRNAs are translated into only two distinct proteins that differ only in the C terminal: a short tail of four amino acids in SERCA2a and a more extended tail of fifty residues for SERCA2b. This extension may contain an extra membrane spanning stretch that would put the protein's carboxy terminus in the lumen of the ER. This additional tail may modulate the intrinsic activity of the pump and interact with phospholamban, a well-known regulator of SERCA2 pumps. Mammalian SERCA genes are differentially expressed during development (Brandl et al., '87) and also in response to treatments that alter Ca^{2+} pumping activity such as: changing levels of thyroid hormone (Rohrer and Dillmann, '88), chronic muscle stimulation (Briggs et al., '90; Hu et al., '95), or cardiac hypertrophy (Nagai et al., '89). The crustacean molting cycle would appear to be an ideal model for correlating differential expression of SERCA/PMCA with transepithelial Ca^{2+} flux.

When my lab set out to clone SERCA pumps in crayfish, the only existing crustacean sequences were from muscle of brine shrimp, *Artemia* (Palmero and Sastre, '89; Escalante and Sastre, '93, '94). Two mRNAs (4.5 and 5.2 kb) are produced from a single gene via alternative splicing; both are present in cryptobiotic embryos. Expression of the 5.2 kb decreases after naupliar hatching while the 4.5-kb isoform increases during embryonic development. In larval tissue the 4.5-kb isoform is expressed in muscle fibers of appendages (predominantly fast-twitch). The 5.2-kb isoform is expressed in a variety of larval tissues (Escalante and Sastre, '96). The amino-acid sequence shares 71% homology with mammalian SERCA2a and -b especially in the region of the functional domains and putative Ca-binding site. The two isoforms differ only at the C-terminal end of the protein (Escalante and Sastre, '93). The last 6 amino acids of the 4.5 kb isoform are replaced by 30 hydrophobic amino acids in the 5.2 kb isoform that have the potential of forming an extra transmembrane domain. Both isoforms arise from the same gene by alternative splicing of the last two exons.

The *Artemia* SERCA gene is transcribed from two alternative promoters (Escalante and Sastre, '95). These two mRNAs also differ at the initial part of their 5′ untranslated region. The 5.2-kb mRNA-specific 5′ untranslated region is present as an independent exon whose transcription is regulated by a promoter different from the one previously described that regulates the expression of the 4.5-kb mRNA. The nucleotide sequence of the 5.2 kb-mRNA promoter and the transcription initiation site have been determined. Results suggest that the expression of the two protein isoforms is regulated in *Artemia* at the transcription-initiation step in con-

trast to the vertebrate SERCA genes 1 and 2 which have unique promoters for transcription of the two isoforms encoded by each gene. The donor-splicing site of the penultimate exon can either be recognized and fused to the last exon, giving rise to the mRNA coding for the shorter protein, or remain unrecognized, in which case a polyadenylation site is recognized before the last exon of the gene and the mRNA coding for the longer protein is originated (Escalante and Sastre, '93).

Genomic clones coding for *Artemia* SERCA have been isolated (Escalante and Sastre, '94). Nucleotide sequence of the mRNA coding regions show that the gene is divided into 18 exons separated by 17 introns. Compared with the rabbit SERCA1 gene, 12 of the introns are in the same position, 8 introns in the rabbit gene are absent from *Artemia*, 4 introns present in *Artemia* are not found in rabbit and the position of 1 intron is shifted one base between both genes. Primer extension and nuclease S1 protection experiments have shown the existence of two main regions of transcription initiation separated by 30 nucleotides. Transcription is initiated at both regions at two or three consecutive bases. A hexanucleotide that includes the initiation sites is repeated in both transcription-initiation regions. The nucleotide sequence of the promoter region shows the existence of several putative regulatory sites including some that are muscle specific such as one CArG box, three MEF-2, and eight putative binding sites for muscle transcription factors of the MyoD family.

There appear to be similarities between the vertebrate SERCA2 and the *Artemia* SERCA gene. Both genes can be transcribed and processed to give two different mRNAs by identical mechanisms: the use of two alternative polyadenylation sites that involve an additional splicing event if the more distal polyadenylation site is used. In both cases the proteins encoded by the different mRNAs are identical except for the carboxy terminal amino acids: the shorter form codes for 6 or 4 amino acids and the longer isoform codes for 30–40 amino acids. In vertebrates the shorter isoform is expressed in slow-twitch and cardiac muscle whereas the longer isoform is expressed in smooth muscle and nonmuscle cells. Despite these similarities the tissue specific expression of the two isoforms is regulated through different mechanisms. In vertebrates the two isoforms are transcribed from the same promoter and the generation of one or the other isoform is dependent on processing of the last exons of each gene which have been shown to be tissue-specific (Gunteski-Hamblin et al., '88). In *Artemia* there are two different tissue-specific mechanisms that regulate the expression of each isoform. One is the existence of two different promoters that independently regulate the expression of each isoform. The second mechanism would be the differential processing of the last two exons of the gene which is also tissue-specific. This second mechanism has been conserved between *Artemia* and vertebrates. It is interesting to note that the existence of alternative promoters in *Artemia* is possible because of the existence of one intron in the 5′ untranslated region that is not present in the vertebrate genes. This intron might have been generated in the evolutionary branch of the crustaceans after the divergence of protostoma from deuterostoma which would have allowed for the development of the internal alternative first exon with a new promoter. A second possibility could be the existence of the first intron and the alternative promoter in the ancestral gene and their loss during vertebrate evolution. The study of SERCA genes in crayfish will elucidate whether the existence of alternative promoters is specific to *Artemia* or a general feature of crustaceans.

My laboratory has recently cloned and sequenced SERCA from crayfish muscle (Wheatly, 1997; Zhang et al., unpublished) and heart (Chen et al., unpublished), and we also have partial cDNA clones for SERCA in gill and antennal gland. In muscle the complete 3,864-bp sequence includes a 145-nt noncoding sequence at the 5' end, an open reading frame of 3,006 nt (coding for 1,002 amino acids, molecular mass of 110 kd), and a 705-nt untranslated 3′ region. The complete heart sequence consists of 4,495 bp with a 3,060-bp open reading frame, coding for 1,020 amino acids. The nucleotide sequence shares 73% identity with *Artemia* and 71% with rabbit muscle. This gene differs from the crayfish muscle SERCA solely in its C-terminal amino acids where the final 9 amino acids are replaced by 27 hydrophobic amino acids that potentially form an additional transmembrane domain. The deduced amino acid sequence matched with more than 30 SERCAs from invertebrates and vertebrates, exhibiting 83% identity with *Drosophila*, 70% identity with *Artemia,* and 76% identity with rabbit fast-twitch muscle. Northern analysis of tissue SERCA distribution revealed five isoforms (4.5, 5.8, 7.6, 8.8, and 10.1 kb), some of which are tissue-specific. Southern analysis indicated that a single gene

codes for the five isoforms. Muscle SERCA expression was highest in intermolt and decreased in pre/postmolt. Whole organism/epithelial approaches have indicated that PMCA is inactive during the intermolt (above). At this time SERCA would be the primary mechanism available for IC Ca regulation. We expect PMCA expression to be elevated in postmolt when basolateral Ca efflux is elevated; correspondingly, SERCA's role in Ca regulation may diminish. SERCA expression was inversely correlated with PMCA expression in mammalian cells (Kuo, personal communication). Work continues in our lab to isolate genomic clones of crayfish SERCA in order to understand gene regulation.

The relationship between these two crayfish isoforms is similar to that observed for the two SERCA isoforms in *Artemia* (Escalante and Sastre, '93) and mammalian SERCA2 isoforms (Lytton and MacLennan, '88). The C-terminal extensions share their hydrophobic character but have no significant amino-acid homology. As mentioned earlier the extension has the potential of forming another transmembrane region; in mammals the C terminus of SERCA2a is in the cytosol whereas the C terminus of SERCA2b is in the ER lumen. The conservation of amino-acid sequence and the same alternative splicing mechanism (Shull and Greeb, '88) between species so distant in evolution (300 MY) suggests that the two isoforms play an important physiological role. Comparison of the genomic sequences in *Artemia* and mammals (Escalante and Sastre, '94) indicate that over 50% of the intron positions are conserved during the evolution of this gene. One interpretation of this phenomenon is that all the introns were present in the ancestral gene and are being lost during evolution. The ancestral gene should have had at least 26 introns, 8 were lost during evolution of *Artemia,* and 4 were lost in the evolution of SERCA1 gene. An alternative explanation is that introns are added to the genes during evolution. Sequence analysis reveals the same degree of homology of the crustacean SERCA gene with all three vertebrate genes. Researchers have hypothesized the existence of a unique (pleisomorphic) ancestral gene in a common protostome and deuterostome ancestor from which the unique arthropod gene and three vertebrate genes were then derived (apomorphs). The same alternative splicing was preserved in the invertebrate gene and the vertebrate SERCA 2 gene while it was lost in the evolution of the other two vertebrate genes. The similarity between *Artemia*, *Procambarus,* and SERCA2 suggests that this common ancestral gene might have possessed the same genetic structure and have been able to generate muscle-specific and non-muscle-specific isoforms though similar processing events.

Our long term objective is to characterize crayfish PMCA. PMCA and SERCA share structural features, the number and location of the putative transmembrane domains, and the distribution of the predicted secondary motifs of β sheets and the α helices are similar; both families are inhibited by orthovanadate and La^{3+}. In mammals PMCA is encoded by four different genes (Strehler, '91). Despite these similarities, there are a number of differences between PMCA and SERCA apart from their subcellular location and associated role in cell Ca regulation. PMCAs have a molecular mass of 134 kD while the SERCAs have a molecular mass of 110 kD (Carafoli, '94). PMCAs are regulated by calmodulin, while SERCAs are regulated by phospholamban.

Two genes for PMCA in rat brain have been sequenced (Shull and Greeb, '88); they encode two different isoforms with 82% homology. A partial sequence has recently been cloned from PMCA in crayfish heart and muscle (Fig. 3, Zhang et al., unpublished). The crayfish nucleotide and amino-acid sequences share 65% and 68% identity respectively with rat PMCA. When this partial PMCA sequence was used to probe a Northern blot of mRNAs from crayfish heart, hepatopancreas, gill, egg, muscle, and antennal gland, it hybridized with a 7-kb band in all tissues, with a prominent band in egg and antennal gland. There is an additional 5-kb band in egg indicating a possible isoform or precursor. Work continues to completely sequence this pump. Our long-term goal is to investigate whether the expression of Ca pumps in crayfish is hormonally responsive.

Hormonal regulation: evolutionary strategies

In arthropods, ecdysis is coordinated by the steroid hormone ecdysone (Hopkins, '92; Lachaise et al., '93). In decapods there is a transitory ecdysone peak in the hemolymph about three weeks before ecdysis that initiates entry into preecdysis (Ca reabsorption, gastrolith formation). This is followed days before ecdysis by a second peak of greater magnitude. In the isopod *Oniscus asellus,* an ecdysone peak in premolt is followed by a second smaller peak 1 hr after Ep (Johnen et al., '95). In the amphipod *Orchestia,* an ecdysone peak stimulates preexuvial Ca storage in the PC (Graf and Delbecque, '87). The ecdysone peak reported

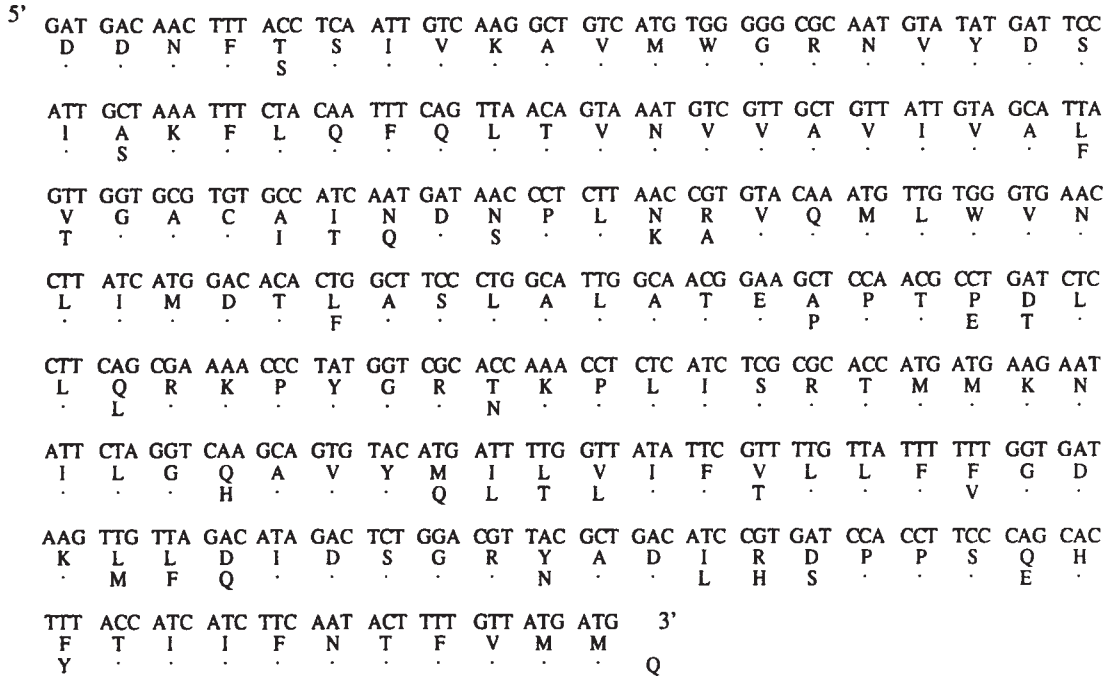

Fig. 3. Partial nucleotide sequence and deduced amino acid sequence of the muscle plasma membrane Ca^{2+} ATPase (PMCA) of crayfish *Procambarus clarkii*. Comparison of the deduced amino acid sequence with rat PMCA is illustrated in the third row. Amino acids that are identical are indicated by a dot.

in crayfish in induced molts (ER or MLA) was larger and more protracted than observed in natural molts (Wheatly and Hart, '95). Several labs are attempting to establish molecular links between ecdysone and expression of genes that code for proteins involved in Ca homeostasis.

When the ecdysone analogue ponasterone A (25 deoxy-20-hydroxyecdysone agonist of 20 hydroxyecdysone) was injected into crayfish it precipitated premolt (Ueno et al., '92a). Ecdysone receptors were present in nuclei and cytoplasm of the crayfish hypodermis, Leydig, and pillar cells at premolt (small gastrolith period) coincident with the first ecdysone peak and when Ca reabsorption is being induced. This suggests that cells involved in Ca transport are the target of ecdysone. Nuclear ecdysteroid binding is indicative of a genomic action of ecdysone which may be related to the induction of Ca transport proteins. In the gastrolith disc (Ueno et al., '92b), maximal binding to ponasterone A was observed in the cytoplasm of gastrolith epithelial cells in premolt (small but not large gastrolith phase) suggesting its involvement in induction of Ca secretion. The appearance of cytoplasmic receptors may reflect induction of receptor protein by ecdysone. In immediate postmolt (gastrolith reabsorption), binding was in both nuclei and cytoplasm implicating translocation of cytoplasmic receptors into the nucleus as is known for glucocorticoid receptors.

In vitro EC Ca can block MIH suppression of ecdysteroidogenesis in crustaceans (Watson et al., '89) and increased free-IC Ca can elevate ecdysteroid production by activating calmodulin (Mattson and Spaziani, '86). In lobster muscle in vivo ecdysteroids increased actin synthesis by promoting translational processing in addition to transcriptional regulation (Whiteley and El Haj, '97). While we have demonstrated differential expression of SERCA during the molting cycle, we have not associated this with ecdysone titers.

In vertebrates, the secosteroid vitamin D works in concert with calcitonin (CT) and parathyroid hormone to regulate Ca balance. Vitamin D affects calcium fluxes at transporting epithelia in bone, gut, kidney, and chick chorioallantoic membrane. It can stimulate Ca transport by genomic action (synthesis of basolateral Ca pumps and Ca binding proteins) and nongenomic action (rapid effect: transcaltachia, liponomic effect at brush border).

Immunoreactivity to vitamin D-like molecules has been identified in whole extracts of the amphipod *Orchestia* (Meyran et al., '91a). Receptors were found in nuclei of epithelial cells in the PC (Meyran et al., '91b), and immunoreactivity levels were highest during postmolt/intermolt. This period coincides

with cuticular stabilization, reduced circulating Ca (Sellem et al., '89), and reduced hemolymph ecdysone. This profile resembles the action of vitamin D on the vertebrate skeleton. Application of exogenous vitamin D resulted in a decrease in hemolymph Ca in pre- and intermolt and stimulated preexuvial Ca storage at the PC epithelium (Meyran et al., '93).

In vertebrates, calcitonin (CT) is a 32 amino-acid peptide (MW 3,500 daltons) that lowers blood Ca by inhibiting release from bone. It also has a variety of "neurotransmitter-like" functions. CT and calcitonin gene-related product (CGRP) are derived from the same ancient gene by alternative splicing of a common transcript. The CT-like molecules are derived from a phylogenetically old peptide. While it has evolved in parallel with the need for a calcified exo- or endoskeleton, it has no recognizable role in calcification in some organisms and so its ancestral function is unknown.

Recently CT-like molecules (27.2 kDa, larger than in vertebrates) have been reported in marine crustaceans (shrimp, lobster) exhibiting a maximal concentration in postmolt when hemolymph Ca is declining (Arlot-Bonnemains et al., '86; Fouchereau-Peron et al., '87). However, injections into intermolt/premolt animals failed to produce any effect. In the shrimp *Palaemon serratus*, CT and CGRP activity were both found in heart and eyestalk; CGRP was also identified in gills (Lamharzi et al., '92). In the blue crab *Callinectes,* CT-like immunoreactivity was found in hepatopancreas and other tissues with a small increase in premolt (2×, Cameron and Thomas, '92).

In the amphipod *Orchestia*, CT-like molecules peak in premolt (10-fold increase) correlated with increased hemolymph Ca (Graf et al., '89; Meyran et al., '93). Immunocytological localization (Graf et al., '92) revealed two reactive organs: the PC epithelium, where it may be involved in Ca flux, and the CNS, where it may play a role as a neurotransmitter. Based on temporal patterns of release, it has been suggested that ecdysone regulates expression of the CT gene via an ecdysone-response element in the crayfish CT gene promoter (L. Smith, personal communication).

In summary, vitamin D and CT appear to play an increased role in Ca regulation in terrestrial crustaceans where Ca storage is emphasized. The fact that the hormonal regulation of Ca homeostasis in terrestrial crustaceans appears to resemble that of vertebrates suggests that it may be associated with preadaptation to aerial life.

FUTURE PERSPECTIVES

At the whole animal/EC level there are still questions regarding mechanisms by which environmental Ca (either water or food) can regulate intermolt Ca homeostasis in crustaceans. Synchrony of Ca-flux mechanisms at mineralizing and nonmineralizing epithelia is also of extreme interest, as are the temporal relationships between mineralization and reabsorption. Research at the cellular level will comprehensively characterize Ca transporters in apical, basolateral, and internal membranes in a range of transporting epithelia and as a function of molting stage. Biochemical studies should be supported with ultrastructural work. Molecular studies will continue to sequence Ca-associated proteins from different crustacean tissues, and to determine their expression during elevated transepithelial Ca flux occurring during the molting cycle. Up- and downregulation of transporters by water Ca (aquatic species) or dietary Ca (terrestrial species) will also be studied. Additionally, it will be important to determine whether protein expression is responsive to hormones. Isolation of genomic clones will clarify the complete sequences of appropriate genes and their intron and exon structure together with splicing mechanisms. Analysis of the 5′ upstream sequences will elucidate the existence of the putative regulatory sites and associated factors involved in gene regulation.

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