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CRUSTACEAN MODELS FOR STUDYING CALCIUM TRANSPORT: THE JOURNEY FROM WHOLE ORGANISMS TO MOLECULAR MECHANISMS

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The crustacean moult cycle is a convenient model system in which to study calcium (Ca) homeostasis as vectorial movement across Ca transporting epithelia (gills, gastric epithelium, cuticular hypodermis, antennal gland) which occurs in either direction at different stages of the moulting cycle. Intermoult crustaceans are in relative Ca balance. During premoult, at the same time as the cuticle decalcifies, epithelia involved in Ca storage (e.g. gastric) calcify and/or increase their intracellular Ca stores. Premoult Ca balance is typically negative as Ca is excreted. During postmoult the soft new cuticle is remineralized largely with external Ca taken up across the gills and gastric epithelium (positive Ca balance); conversely during this time internally stored Ca is remobilized. This review (1) compares the relative roles of Ca transporting epithelia in Ca balance for crustaceans from different habitats; (2) proposes up-to-date cellular models for both apical to basolateral and basolateral to apical Ca transport in both noncalcifying and calcifying epithelia; (3) compares kinetics of the Ca pump and exchanger during intermoult; (4) presents new data on specific activity of calcium adenosinetriphosphatase (Ca^{2+}ATPase) during the moult cycle of crayfish and (5) characterizes a partial cDNA sequence for the crayfish sarcoplasmic reticular Ca^{2+}ATPase and documents its expression in gill, kidney and muscle of intermoult crayfish. The physiological and molecular characterization of Ca transporters in crustaceans will provide insight into the function, regulation and molecular evolution of mechanisms common to all eukaryotic cells.

INTRODUCTION

In the preface to his monograph on 'The crayfish' Huxley (1896) states "In writing this book about crayfish it has not been my intention to compose a zoological monograph on that group of animals. I have desired, in fact, to show how the careful study of one of the commonest and most insignificant of animals, leads us, step by step, from everyday knowledge to the widest generalizations and the most difficult problems of zoology; and, indeed of the biological sciences in general." The intention of this review is to demonstrate that the crustacean moult cycle presents an ideal model for studying the cellular and molecular mechanisms for calcium homeostasis which are critical for all eukaryotic cells. Recent review articles on crustacean Ca balance have focused on a whole organism approach (Greenaway, 1985, 1988; Neufeld & Cameron, 1993; Wheatly, 1996) or have discussed the structural bases of biomineralization (Lowenstam & Weiner, 1989; Simkiss & Wilbur, 1989). The purpose of the present article is briefly to relate whole animal Ca balance to the environment, and then to focus on the

physiological and molecular mechanisms that are employed at Ca transporting epithelia. Models will be proposed for epithelial Ca transport and new data will be presented on specific activity and molecular characterization of the Ca pump in the crayfish during the moult cycle.

WHOLE ORGANISM

The exoskeleton of crustaceans is heavily calcified with crystalline $CaCO_3$ (80% dry weight) and must be shed (ecdysis) to accommodate growth. The calcium dynamics of crustaceans are orchestrated into an elaborate moult cycle (Greenaway, 1985, 1988; Neufeld & Cameron, 1993; Wheatly, 1996). During premoult Ca is reabsorbed from the existing cuticle, and is either stored in amorphous form (in mineralized structures or intracellularly) or excreted (negative Ca balance). During postmoult rapid mineralization of the new soft cuticle is of adaptive advantage and uses Ca from all available internal and external sources (positive Ca balance). Crustaceans occupy a range of habitats extending from sea-water through brackish water to freshwater and ultimately via either origin to terrestrial habitats. Correspondingly they exhibit a range of behavioural and physiological adaptations for Ca conservation and mineralization (Figure 1). Circulating Ca remains remarkably constant throughout the moult cycle with the exception of freshwater land crabs which store Ca in haemolymph at ecdysis (review by Wheatly, 1996).

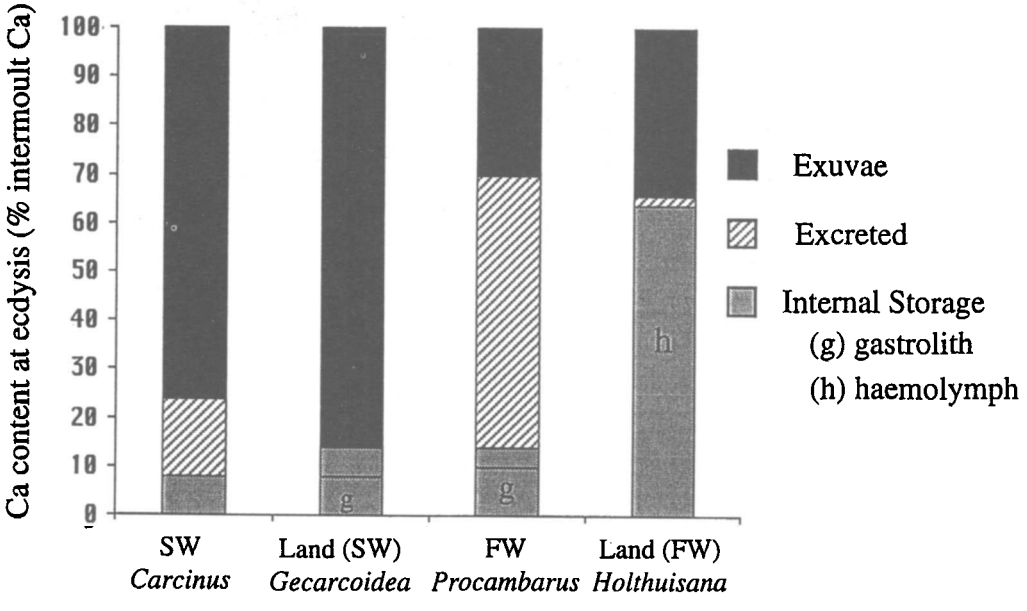

Figure 1. Calcium content at ecdysis (expressed as a percentage of intermoult Ca) of exuviae and soft body for a series of crustaceans representing different habitats: sea-water, *Carcinus maenas* (mass 22 g) data from Greenaway (1985); marine land crab, *Gecarcoidea natalis* (mass 100 g) data from Greenaway (1993); freshwater crayfish, *Procambarus clarkii* (mass 30 g) data from Wheatly & Ayers (1995); freshwater land crab, *Holthuisana transversa*, data from Sparkes & Greenaway (1984).

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As marine crabs (Neufeld & Cameron, 1993) reside in a vast reservoir of Ca (10 mmol l^{-1}) that is always available for postmoult calcification, little Ca storage between moults is required. Marine land crabs (Greenaway, 1988, 1993) store increasing amounts of Ca both internally in gastroliths ($CaCO_3$ concretions in the stomach) and haemolymph, and externally in the exuviae which may be ingested. Moulting in burrows facilitates this behaviour. Crustaceans such as the crayfish that successfully evolved in freshwater (Ca <1 mmol l^{-1}) store Ca in gastroliths (Wheatly & Gannon, 1995) although they do not store appreciably more than marine species. On the other hand, and against their best interest, they excrete significant amounts of Ca during premoult and do not routinely reingest the exuviae. This strategy suggests that ambient Ca in freshwater is every bit as accessible as Ca in sea-water. Efficient postmoult Ca influx mechanisms operate at the gill and antennal gland to ensure cuticular remineralization. Calcium conservation reaches its zenith in the freshwater land crab *Holthuisana transversa* (Sparkes & Greenaway, 1984) which stores 60% of body Ca between moults in the haemolymph.

Crustaceans provide an adaptive model for the study of Ca balance as, for a given Ca transporting epithelium, there are temporal differences with regard to the direction of Ca transfer at different stages of the moult cycle. In most other calcifying systems net accretionary growth patterns predominate. In crustaceans, whole animal Ca balance alternates between calcification and decalcification the two processes can often occur simultaneously at different epithelia. During premoult Ca is reabsorbed from the skeleton while deposition occurs at internal storage sites (e.g. gastrolith disc). During postmoult calcified stores are demineralized while the exoskeleton is undergoing mineralization. This suggests that regulation of Ca balance involves time and tissue specific regulation of Ca transporters. Control is even more complex in the woodlouse *Oniscus* sp. which sheds the posterior region of the cuticle first, and then uses Ca from the anterior region to calcify the posterior region before the anterior region is shed (Steel, 1982). To do this it must be able to simultaneously effect calcification and decalcification of the cuticle in different regions of the body.

EPITHELIAL

Four crustacean epithelia are specialized for bidirectional Ca exchange. (1) The gills are the site of both passive diffusional Ca efflux and active or passive uptake from the environment in aquatic species. They can also post-renally modify voided urine that trickles into the branchial chamber in terrestrial species. (2) The gastric epithelium is adapted for Ca uptake from food (including cast exuviae) into the haemolymph. Alternatively Ca can be transferred from the haemolymph into regions of the gut (hepatopancreas, gastroliths) for temporary storage. (3) The cuticular hypodermis is adapted either for reabsorption or deposition of exoskeletal $CaCO_3$. (4) At the antennal gland (kidney), filtered Ca (efflux) can be reabsorbed (influx) in the kidney tubule. Table 1 summarizes the relative roles of these four Ca transporting epithelia during intermoult and postmoult in a series of crustaceans from a range of habitats. Rates of transepithelial Ca flux were determined using whole animal isotopic methodology (branchial rates in aquatic species), antennal gland cannulation (renal) or isolated

Table 1. *Relative role of the four primary transporting epithelia in Ca flux[1] during intermoult and postmoult (24 h) in crustaceans from different habitats.*

Habitat / Species	Temperature (°C)	Mass (g)	Gills I Efflux (μmol kg⁻¹ h⁻¹)	Gills I Influx (μmol kg⁻¹ h⁻¹)	Gills P Efflux (μmol kg⁻¹ h⁻¹)	Gills P Influx (μmol kg⁻¹ h⁻¹)	Gastric epithelium[2] P (μmol kg⁻¹ h⁻¹)	Cuticular hypodermis I (nmol cm⁻² h⁻¹)	Cuticular hypodermis P (nmol cm⁻² h⁻¹)	Antennal gland I Filtered (μmol kg⁻¹ h⁻¹)	Antennal gland I Reabsorbed (μmol kg⁻¹ h⁻¹)	Reference
Sea-water												
Callinectes sapidus	25	64–211				+3000ⁿ	+800	+13·1(D2)	+102			Henry & Kormanik (1985); Neufeld & Cameron (1994)
Carcinus maenas	10	>20	-674	+513		+1800ⁿ		+34	+88	-48·2	+17·6	Zanders (1980); Greenaway (1976, 1983); Roer (1980)
Cancer magister	11	750								-10·5	+5·5	Wheatly (1985)
Homarus gammarus	12	700								-83·2	+40	Whiteley & Taylor (1992)
Freshwater												
Procambarus clarkii	23	8–16	-250	+83	–250	+1350	+6000					Wheatly & Ayers (1995)
Astacus fluviatilis	16								+400			Welinder (1975)
Pacifastacus leniusculus	12	26	-40ⁿ							-39	+35	Wheatly (1989); Wheatly & Toop (1989)
Austropotamobius pallipes	10	10–20	+3·4	+2000ⁿ						-46	+40·2	Greenaway (1974); Tyler-Jones & Taylor (1986)
Terrestrial (sea-water)												
Birgus latro	25	375								-41	+40·4	Greenaway et al. (1990)
Gecarcoidea natalis	25					+4600 store +7600 food[3]				-20·3		Kormanik & Harris (1981); Greenaway (1993)
Leptograpsus variegatus	25	9–60	+92ⁿ	+87ⁿ								Morris & Greenaway (1992)
Gecarcinus lateralis										-17·3ⁿ		Harris (1977); Wolcott & Wolcott (1991)
Terrestrial (freshwater)												
Cardisoma hirtipes		150								-6·8	+0·5	Greenaway (1989)
Holthuisana transversa	25	4–30		1935ⁿ					+50,000 μmol kg⁻¹ h⁻¹	-1·6ⁿ		Sparkes & Greenaway (1984)

[1], By convention positive values represent uptake into the animal (haemolymph or tissue) from external sources (water, Ca stored in gut, food); Negative values indicate loss from animal; n, net Ca flux. [2], Includes gastrolith disc or hepatopancreas and assumes that Ca is remobilized within 24 h postmoult. [3], Assumes the Ca from reingested exuviae is used to calcify skeleton over a period of one week. I, intermoult; P, postmoult.

hypodermis/integument (cuticular hypodermis). Gastric epithelium flux were calculated from the amount of Ca stored and the time required for remobilization.

Intermoult marine crabs exhibit high rates of branchial Ca exchange diffusion (Greenaway, 1976, 1983) and produce an essentially isosmotic urine with minimal Ca reabsorption (Wheatly, 1985). During postmoult they rely heavily on passive branchial influx from external sea-water for calcification of the cuticle (Neufeld & Cameron, 1992). Calcification in dilute sea-water involves active processes (Neufeld & Cameron, 1994). Remobilization of Ca stored in the hepatopancreas contributes <20% of the required Ca, and may be used for early hardening of mouth parts for resumption of feeding. Intermoult freshwater crayfish (Wheatly & Gannon, 1995) tolerate passive branchial efflux, with virtually negligible active influx. Meanwhile the antennal gland exhibits significant Ca reabsorption (influx) from primary filtrate which contributes to the production of dilute urine (Wheatly & Toop, 1989). During postmoult, Ca reabsorption from gastroliths occurs within 24 h at a rate that is more than double the branchial influx rate, indicating the importance of stored Ca during postmoult mineralization (Wheatly & Ayers, 1995). Postmoult mineralization in freshwater crayfish involves active branchial uptake mechanisms (Wheatly & Ignaszewski, 1990) that are massively activated in the first four days of postmoult and are subsequently deactivated (Wheatly & Gannon, 1993).

Land crabs typically control Ca input via water and food, although they can post-renally modify their isosmotic urine as it seeps into the branchial chamber (Wolcott & Wolcott, 1985; Morris et al., 1991). In marine land crabs during postmoult (Greenaway, 1988), the gastric epithelium shows significant Ca absorption rates both from internally stored Ca (gastroliths) and externally stored Ca (shed exuviae that are reingested). In this case the branchial epithelium is relatively unimportant; the antennal gland attempts to conserve Ca although few data exist to substantiate its function. Freshwater land crabs use Ca stored in the haemolymph to recalcify the skeleton during postmoult (Sparkes & Greenaway, 1984) at calculated transcuticular flux rates that are substantial. The branchial epithelium in land crabs is also adapted to take up Ca from available water.

To summarize, the relative importance of the four Ca transporting epithelia in crustaceans from different habitats appears to reflect environmental Ca availability and accessibility.

CELLULAR

Whole organism/tissue studies have indicated that transepithelial Ca transport involves the following mechanisms: (i) a Na^+/Ca^{2+} antiporter (Ahearn, 1978; Roer, 1980; Wheatly & Gannon, 1993) that is driven by the Na^+ gradient maintained by Na^+/K^+ATPase (Henry & Kormanik, 1985; Towle & Mangum, 1985) and (ii) active uptake (Greenaway, 1974) described by saturation kinetics (half maximal activation concentration, Km=0·13 mM; saturation=0·4 mM; maximum velocity, Vmax=2 mmol g^{-1} h^{-1}) that are characteristic of Ca^{2+}ATPase. In the present study levels of Ca^{2+}ATPase activity were determined for a variety of crayfish tissues during intermoult and postmoult (24 h). Methodology closely followed Morris et al. (1991) and Morris & Greenaway (1992). The microsomal fraction was prepared by differential centrifugation

as outlined in Flik et al. (1983, 1985). The activity of Ca^{2+}ATPase was determined in a two buffer system where the inhibitory buffer lacked $CaCl_2$. Calcium dependency was investigated by preparing a range of Ca-EGTA (ethylene glycol-bis N,N,N',N'-tetraacetic acid) buffers (Ghijsen et al., 1980) to provide concentrations in the range of 0–400 μmol l^{-1}. Co-purification of Ca^{2+}ATPase with Na^+/K^+ATPase suggested co-localization on the basolateral membrane.

In Table 2 Ca^{2+}ATPase activities are compared for different transporting epithelia of crustaceans from different habitats during intermoult and postmoult.

Table 2. *Specific activity of Ca^{2+}ATPase in tissue homogenates of intermoult and postmoult crustaceans from different habitats.*

Habitat Species	Temperature (°C)	Tissue	Ca^{2+} ATPase specific activity (nmol min^{-1} $mg\ pr^{-1}$) I	P (24 h)	Reference
Sea-water					
Callinectes sapidus	25	gill	10 ±2	15 ±3	Cameron (1989)
		cuticular hypodermis	3 ±0·5	13·5 ±2	Cameron (1989)
		hepatopancreas	400 (20)	nd	Fox & Ranga Rao (1978)
Freshwater					
Procambarus clarkii	23	gill	27 ±7	25 ±4	Present study
		cuticular hypodermis	28 ±9	76 ±2	Present study
		antennal gland	91 ±10	107 ±9	Present study
Terrestrial (Sea-water)					
Birgus latro	25	gill	11 (9)	nd	Morris et al. (1991)
Leptograpsus variegatus	25	gill*	8·0 (0·034)	10·3 (0·007)	Morris & Greenaway (1992)

Values in parentheses are Km in μM; nd, not detected; *, determined on membrane-enriched fraction; I, intermoult; P, postmoult.

It should be noted that the *Callinectes* sp. data (Cameron, 1989) may represent nonspecific alkaline phosphatase since the Km of the transporter in that study exceeded 1 mM. Within a species, intermoult Ca^{2+}ATPase activity at a tissue appears to be correlated with the degree of transepithelial Ca flux. Thus in crayfish (present study), levels in the antennal gland, known to actively reabsorb 95% of filtered Ca at intermoult, are three fold higher than at the gill (negligible Ca influx in intermoult) or hypodermis. In the intermoult blue crab, the hepatopancreas, a tissue known to store large amounts of Ca (Becker et al., 1974) has Ca^{2+}ATPase activity (Fox & Ranga Rao, 1978) that is an order of magnitude above levels in either the gill or cuticular hypodermis (Cameron, 1989). Furthermore, the hepatopancreatic Ca^{2+}ATPase requires a high concentration of Ca (20 mM) for maximal activity associated with the role of this organ in Ca storage (Chen et al., 1974). While Ca^{2+}ATPase levels in the gills of two terrestrial species of

marine crabs (*Birgus latro*, Morris et al., 1991; *Leptograpsus variegatus*, Morris & Greenaway, 1992) were similar in magnitude to that of the blue crab, the crayfish had branchial levels that were three fold higher (present study) associated with living in freshwater. Presumably in terrestrial species the branchial Ca^{2+}ATPase is involved in ion reclamation from the urine (Wolcott & Wolcott, 1985).

Branchial Ca^{2+}ATPase levels did not increase significantly during postmoult in any of the marine species studied. This result was unexpected as Ca entry into postmoult *Callinectes* sp. is believed to be largely passive (Cameron, 1989); similarly reclamation of ions from the urine in the gill chamber is unlikely to contribute significantly to postmoult calcification in *Leptograpsus* sp. (Morris & Greenaway, 1992). However, the lack of increase in crayfish gill and antennal gland Ca^{2+}ATPase activity was surprising as postmoult Ca influx is necessarily active (Greenaway, 1974). However, Ca^{2+}ATPase activities in the cuticular hypodermis increased 3–4 fold in both the blue crab and crayfish. In a cytochemical study, Ca^{2+}ATPase activity increased in crayfish gastric epithelium both during premoult gastrolith calcification and during postmoult gastrolith decalcification (Ueno & Mizuhira, 1984).

It is possible to construct models for cellular Ca transport in crustaceans appreciating that transepithelial movement can occur in either direction (that is apical to basolateral including decalcification (Figure 2); or basolateral to apical, including calcification (Figure 3)). Separate models have been constructed for noncalcifying tissues (gills, antennal gland) and calcifying tissues (cuticular hypodermis, gastric epithelium, posterior caeca) since they differ in a number of respects including the composition of the fluid on the apical surface.

The model for apical to basolateral transfer in noncalcifying epithelia (gills, antennal gland; Figure 2A) was derived primarily from vesicle studies in intermoult crabs and lobsters (basolateral inside out vesicles of *Carcinus* sp. gill, Flik et al., 1994; apical and basolateral vesicles of lobster antennal gland and hepatopancreas, Ahearn & Franco, 1993; Ahearn & Zhuang, 1996; Zhuang & Ahearn, 1996). Calcium passively enters the cytosol from external water/luminal fluid through apical verapamil-inhibited Ca channels. Apical Ca transport may also involve: (i) an electrogenic, amiloride-sensitive antiporter $1Ca^{2+}/1H^{+}$ ($1Na^{+}$) and (ii) an electroneutral, amiloride-insensitive exchanger $1Ca^{2+}/nNa^{+}$ (nH^{+}). Basolateral Ca transport may involve: (i) a Ca^{2+}/Na^{+} antiporter and (ii) a calmodulin dependent Ca^{2+}ATPase.

The kinetics of the basolateral Ca pump and exchanger are compared for crab gill (50% sea-water) and lobster hepatopancreas (100% sea-water; Table 3). The Na^{+}/Ca^{2+} exchanger has a lower affinity but higher capacity and is the primary vectorial mechanism for basolateral Ca efflux into the haemolymph. In contrast, Ca^{2+}ATPase, typically, has high affinity and low capacity suggesting that it is a 'housekeeping' enzyme involved in fine-tuning intracellular Ca levels. The kinetics of the Ca pump and exchanger in crustaceans agree qualitatively and quantitatively (except for the hepatopancreas Na^{+}/Ca^{2+} exchanger) with published values for fish plasma membranes (Flik et al., 1990; Flik & Verbost, 1993; Flik et al., 1996). The Ca^{2+}ATPase had higher affinity and capacity in *Carcinus* sp. in 50% sea-water than in lobster in full strength sea-water. The Na^{+}/Ca^{2+} antiporter also had a higher affinity in dilute sea-water.

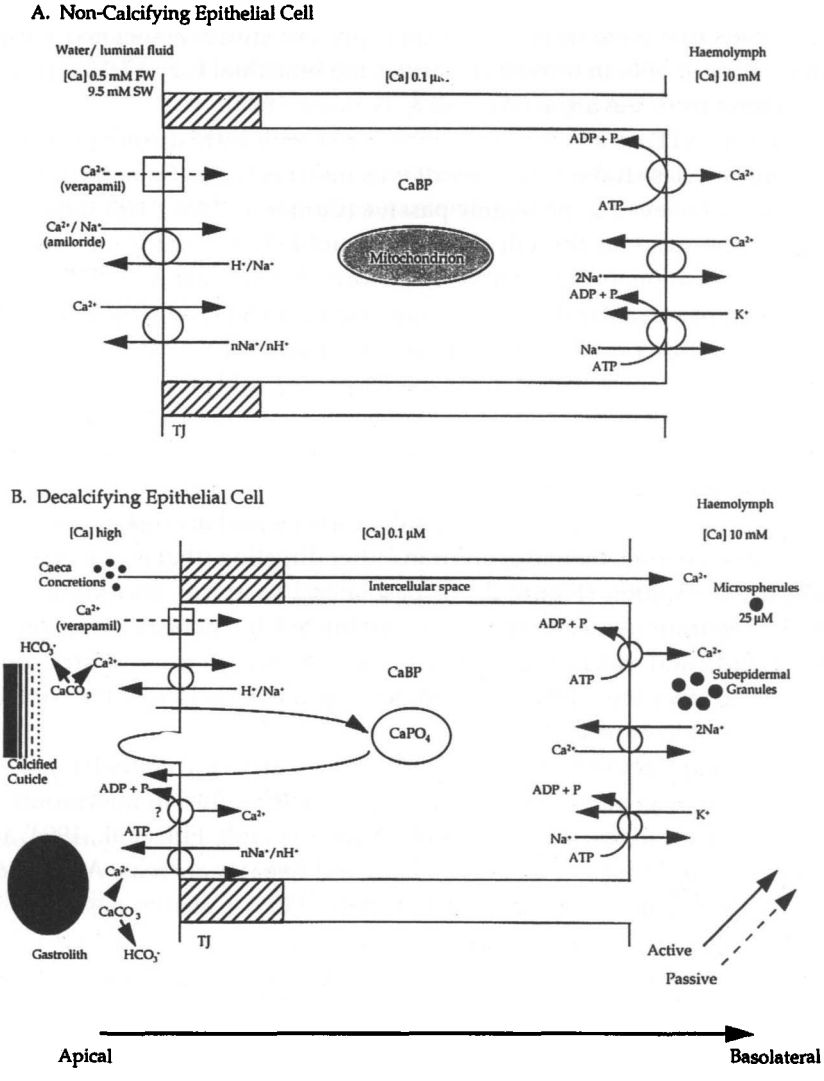

Figure 2. Models for apical to basolateral Ca transfer in (A) noncalcifying epithelial cell (gill, antennal gland); (B) decalcifying epithelial cell (cuticular hypodermis, gastrolith epithelium, hepatopancreas, posterior caeca). CaBP, calcium binding protein; TJ, tight junction.

Based on higher Na^+ turnover in marine species, one can predict a more pronounced role for the exchanger in full strength sea-water as illustrated by the relative capacities. Presently, the kinetics of both mechanisms in basolateral membrane vesicles of freshwater crayfish at different stages of the moulting cycle are being determined.

Transepithelial Ca flux can be extensive during certain phases of the moulting cycle and yet cytosolic levels must be kept low to protect Ca sensitive metabolic processes. During transit between the apical and basolateral membranes, Ca is commonly bound to calcium binding protein (CaBP) or sequestered as $CaPO_4$ granules in organelles such as mitochondria. It was demonstrated that mitochondrial sequestration is associated

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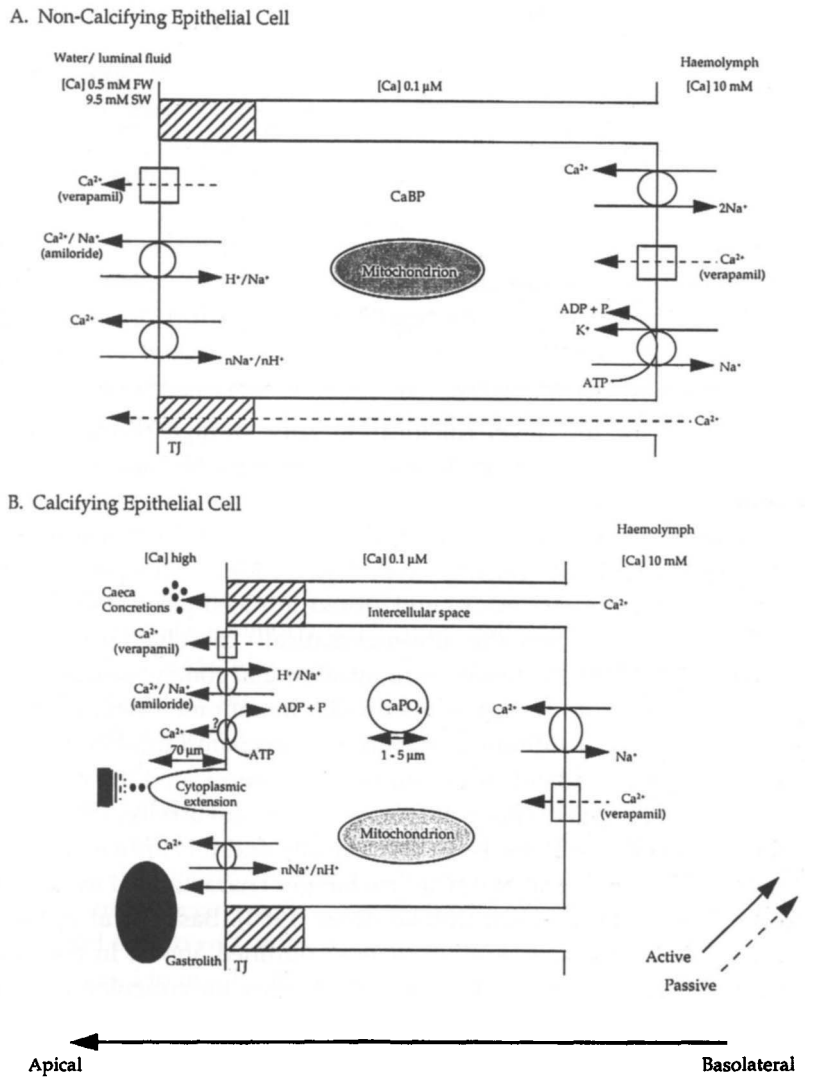

Figure 3. Models for basolateral to apical Ca transfer in (A) noncalcifying epithelial cell (gill, antennal gland); (B) calcifying epithelial cell (cuticular hypodermis, gastrolith epithelium, hepatopancreas, posterior caeca). CaBP, calcium binding protein; TJ, tight junction.

with elevated transepithelial Ca flux at calcifying epithelia (Ueno, 1980). Indeed, there is a positive correlation between mitochondrial Ca accumulation and increased Ca flux in the noncalcifying epithelium of the distal nephridial canal of freshwater crayfish antennal gland. In these cells mitochondrial accumulation was maximal at four days postmoult with stores remaining elevated for several weeks (J.V. Rogers & M.G. Wheatly, unpublished data). Freshwater crustaceans experience haemodilution at ecdysis due to loading of freshwater. Water balance is restored within 48 h postmoult by increased urinary filtration and subsequently increased renal Ca reabsorption. The fact that intramitochondrial Ca remained elevated, suggests that Ca stored in soft

Table 3. *Kinetics of Ca pump and exchanger in intermoult crustacean basolateral membrane vesicles.*

Species	Tissue	Medium	Mechanism	Vmax (nmol min^{-1} mg pr^{-1})	Km (μm)	Reference
Carcinus maenas	gill	50% SW	Ca^{2+}ATPase	1·73	0·15	Flik et al. (1994)
		Na^+/Ca^{2+}	9·88	1·78		Flik et al. (1994)
Homarus americanus	hepatopancreas	100% SW	Ca^{2+}ATPase	0·18	2·5	Ahearn & Zhuang (1996)
		Na^+/Ca^{2+}	74	310		Ahearn & Zhuang (1996)

Vmax, maximum velocity; Km, half maximal activation concentration.

body tissues is used for low level calcification after storage products have been exhausted (24–48 h) and branchial uptake mechanisms have become inactivated (four days postmoult).

Apical to basolateral Ca transfer in decalcifying tissues (postmoult gastric epithelium and posterior caeca; premoult cuticular hypodermis; Figure 2B) takes a variety of forms. Gastroliths (amorphous $CaCO_3$) and stored intracellular $CaPO_4$ granules are released into the gut lumen, where they are enzymatically dissolved using extremes of pH (Ueno & Mizuhira, 1984). Posterior caeca intraluminal concretions are solubilized via a Na^+/Ca^{2+} exchanger on the apical microvillar membrane (Meyran et al., 1986). Ionic Ca enters the apical membrane passively via verapamil-inhibited Ca channels down an electrochemical gradient. Some studies have implicated the involvement of Ca^{2+}ATPase in apical Ca uptake (Roer, 1980; Greenaway & Farrelly, 1991) but there is no direct evidence that Ca^{2+}ATPase is located apically and it is unlikely that it would pump Ca into the cell. The apical Na^+/Ca^{2+} exchanger characterized in noncalcifying epithelia could participate in apical uptake (Roer, 1980). Basolateral uptake would involve the Ca^{2+}ATPase and Na^+/Ca^{2+} exchanger outlined above. In the case of the freshwater land crab, *Holthuisana* sp., Ca reabsorbed following cuticular decalcification is deposited as granules that are either stored subepidermally or circulate in the haemocoel (Greenaway & Farrelly, 1991). This Ca is transported across the cell in a nonionized form (granules, vesicles, bound) or it travels to the haemolymph via paracellular channels. Intraluminal Ca concretions stored in the posterior midgut caeca of the terrestrial amphipod *Orchestia* sp. travel from the apical to basolateral surface in an ionized form (Graf & Meyran, 1985; Meyran et al., 1986). Calcium is reabsorbed from the crystalline calcite in cuticle under conditions of reduced pH. Ionic Ca typically enters epithelial cells through the apical microvilli (cytoplasmic extensions) that begin to regrow as pre-exuvial layers are deposited in late premoult (Compère & Goffinet, 1987).

Basolateral to apical Ca transfer in noncalcifying epithelia primarily involves passive diffusion through paracellular routes (Figure 3A). The basolateral membrane could admit Ca from the haemolymph (10 mM) via a verapamil-inhibited channel or via reversal of the Na^+/Ca^{2+} exchanger. While the Ca pump is also technically reversible, it is physiologically unlikely that the basolateral Ca^{2+}ATPase would ever pump in the

direction of the cytoplasm since intracellular levels are maintained at a low level. Apical transport could occur via Ca channels (although this is unlikely given the existing gradient) or again via reversal of the Na^+/Ca^{2+} exchanger. During translocation Ca could be sequestered in mitochondria or bound to CaBP.

Basolateral to apical Ca transfer in calcifying tissues (premoult gastric epithelium and posterior caeca; postmoult cuticular hypodermis) is illustrated in Figure 3B. Large quantities of Ca entering the haemolymph (either from reabsorption of skeletal Ca or branchial uptake) could enter the epithelial cell through verapamil-inhibited Ca channels down an electrochemical gradient or through Na^+/Ca^{2+} exchange. Gastric epithelial cells of the crab hepatopancreas store Ca as electron-dense $CaPO_4$ granules (associated with Mg, adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to prevent solubilization) that become enveloped by a membrane (Becker et al., 1974). Alternatively cytosolic Ca can be actively accumulated and stored/translocated as $CaPO_4$ in mitochondria (Chen et al., 1974). Ueno (1980) and Mizuhira & Ueno (1983) proposed that gastroliths originate as hypertrophied Ca-rich mitochondria that have sloughed off from the gastrolith disc. However, Simkiss & Wilbur (1989) consider that intramitochondrial Ca storage is an indirect effect of large transepithelial flux at this tissue. Intracellular Ca sequestration buffers cytoplasmic free Ca thereby protecting Ca sensitive metabolic processes (Simkiss, 1974). In the terrestrial amphipod, *Orchestia* sp., Ca^{2+} moves from the haemolymph through extracellular networks. Water is drawn from the cell to the intercellular space due to active Na transport. Calcium accumulation results from activity of the Ca pump and Na^+/Ca^{2+} exchange. Calcium becomes insolubilized into calcareous concretions in the posterior caeca lumen (Graf, 1971; Meyran et al., 1984).

The postmoult cuticular hypodermis transfers Ca into the outer layers of the cuticle via cytoplasmic extensions (up to $4x10^6$ mm^{-2}) that are contained in pore canals (Roer & Dillaman, 1984). These provide a large cell surface for apical transport. Calcification of chitin/protein fibres begins in the most external regions and proceeds proximally with crystals aligned end to end along the axis of the pore canal (Travis, 1963). Apical Ca transfer is believed to involve Na^+/Ca^{2+} exchange and possibly an apical Ca^{2+}ATPase. Roer (1980) proposed that the direction of transhypodermal Ca flux in crustacean cuticle reflected the relative surface area of the apical vs basal membrane. Thus in postmoult Ca will be pumped outward into the cuticle due to an extensive network of cytoplasmic extensions on the apical surface. As these extensions are severed in early premoult, basal transport of Ca into the haemolymph will predominate. Basic chemistry stipulates that a calcifying microenvironment is alkaline and this has been confirmed in both blue crab (Cameron & Wood, 1985) and crayfish (Wheatly et al., 1991).

MOLECULAR

In the laboratory work has begun to clone the genes for Ca^{2+}ATPase and the Na^+/Ca^{2+} exchanger in freshwater crayfish tissues and to quantify expression of these in Ca transporting epithelia at different times during the moult cycle. As data from the epithelia indicated, the Ca pump, which is inactive during intermoult, is activated significantly for a brief four day period during postmoult. This suggests that Ca pumps

are differentially expressed/activated in the initial postmoult period. Recently we have cloned a partial complementary deoxyribonucleic acid (cDNA) fragment from the crayfish muscle Ca pump and have used it as a probe to quantify expression in intermoult gills, kidney, hepatopancreas and muscle.

Calcium adenosinetriphosphatase, belongs to a category of ATPases ('P' phosphorylated ion motive enzymes) which includes the P-type H^+ATPase and other enzymes found in eukaryotes, fungi and bacteria that last shared a common prokaryotic ancestor ~$1{\cdot}5x10^9$ years ago (Pederson & Carafoli, 1987; Serrano, 1988). Structurally, ATPases are 1000 residue multipass transmembrane proteins. Their primary structures have been established from the nucleotide sequence of their cDNAs: Na^+/K^+ (Shull et al., 1985, 1986); H^+/K^+ (Shull & Lingrel, 1986); and Ca^{2+} (MacLennan et al., 1985; Brandl et al., 1986). These ATPases have four conserved functional domains, three of which are common to all ATPases: (i) the phosphorylation and transduction domain, an aspartate followed by KTGT; (ii) the ATP binding and kinase domain, a lysine residue that is inhibited by fluorescein 5'-isothiocyanate (FITC site); and (iii) the hinge region which brings the nucleotide binding and phosphorylation domain into close spatial contact, a region of highest homology (15 identical nonsequential residues) that can be labelled by the ATP antagonist 5'-(p-fluorosulfonyl) benzoyladenosine (termed the FSBA site). The fourth functional domain is involved in cation specific binding and transport across the membrane and is located in hydrophobic transmembrane stretches that anchor the protein in the bilayer, and intra- and extracytoplasmic regions close to the membrane (stalk regions). Brandl et al. (1986) proposed the existence of a high affinity cytoplasmic Ca binding site located in five alpha-helical structures immediate to the five most N-terminal transmembrane regions (stalk region 1–5). More recently, Clarke et al. (1989) showed that mutation of six charged amino acid residues located in this region resulted in complete loss of Ca transport activity.

In mammals, two Ca^{2+}ATPases have been characterized, one from sarcoplasmic reticulum of muscle (SRCA) and the other from plasma membrane (PMCA). The SRCA is used to recover Ca released into the cytoplasm following cell depolarization (Inesi, 1985); its stoichiometry involves an electrogenic exchange of $2Ca^{2+}/2H^+$ for each ATP hydrolysed. MacLennan et al. (1985) have cloned two rabbit genes which are 84% homologous, one coding for the enzyme in fast twitch skeletal muscle, the other in slow twitch, cardiac muscle and nonmuscle tissue. The PMCA regulates intracellular Ca concentration; it is calmodulin sensitive and has a stoichiometry of $1Ca^{2+}/1–2\ H^+$ per ATP. Shull & Greeb (1988) have sequenced two genes from rat brain that encode two different isoforms of the PMCA with 82% homology.

Prior to the present study, there was one existing crustacean sequence in the literature (Palmero & Sastre, 1989). These authors used a whole animal preparation (primarily muscle) of the brine shrimp, *Artemia* sp., to isolate a cDNA clone that was highly homologous to mammalian SRCA. The amino acid sequence predicted for the coding region of *Artemia* sp. SRCA was 71% similar to that of slow and fast twitch rabbit muscle SRCA even though the two species are related but distantly in evolutionary terms (600 million years). The sequence is less than 25% similar to other ion transporting 'P' ATPases. The homology is especially high in the functional domains outlined above. In particular the putative Ca binding site identified by Clarke et al. (1989) was conserved

in the *Artemia* sp. protein. Probes of this likely *Artemia* sp. SRCA are hybridized to two mRNAs of 5·2kb and 4·5 kb suggesting the existence of alternative splicing or of multiple genes as for mammalian Ca^{2+}ATPase (Brandl et al., 1986; Shull & Greeb, 1988). Further studies indicated that the 5·2 kb mRNA is a precursor of the 4·5 kb mRNA.

Preliminary studies have used nondegenerate primers designed to highly conserved transmembrane regions of the published *Artemia* sp. SRCA (Palmero & Sastre, 1989) to generate a partial cDNA probe from crayfish muscle using RT(reverse transcription)-PCR (polymerase chain reaction). The oligonucleotide primers were designed to amplify a 460 bp fragment spanning transmembrane regions 5, 6, and 7. The sense primer had the sequence 5'-GAA ATT TCC GCT ATG ACT GG-3' (corresponding to nucleotide positions 2176-2197). The antisense primer had the sequence 5'-AC AGT GGC AGC ACC AAC ATA-3' (corresponding to nucleotide positions 2617–2636). First strand cDNA was prepared from mRNA by reverse transcription using the SuperScript preamplification system (Gibco BRL no. 18089-011). Quality of cDNA was determined by PCR of a 677 bp β-actin fragment. The target cDNA was amplified using the designed primers via PCR. Polymerase chain reaction products were analysed by electrophoresis in 0·8–1% agarose gels run in tris acetate EDTA (TAE) buffer, stained with ethidium bromide and visualized in UV. The PCR product was a single band of 460 bp. Fresh PCR product with the 3'A-overhangs was ligated with the pCRII vector which has single 3' T residues (Invitrogen no. K2000-01 and K2000-40), then transformed into INVαF' cells. Recombinant colonies were identified by their blue-white colour selection as well as restriction enzyme digestion. As the pCRII vector has M13 forward (-20 and -40) and M13 reverse priming sites, the recombinant DNA could be sequenced directly without subcloning. The PCR product was sequenced using Sequenase 2·0 systems (United States Biochemical (USB) 70770). Sequencing products labelled with ^{35}S were analysed by electrophoresis on 6% polyacrylamide denaturing gels followed by exposure of the dried gel to X-ray film.

The partial nucleotide sequence for crayfish muscle SRCA cDNA and the deduced amino acid sequence are given in Figure 4. A search of the Genbank database using the

```
5' GAT GGT GTG AAT GAT GCA CCT GCC CTG AAG AAA GCT GAA ATT GGC ATT GCT ATG GGA TCT  60
   D   G   V   N   D   A   P   A   L   K   K   A   E   I   G   I   A   M   G   S

  GGT ACT GCT GTG GCC AAG TCT GCC TCT GAA ATG GTG CTG GCT GAT GAC AAC TTC TCC TCT  120
   G   T   A   V   A   K   S   A   S   E   M   V   L   A   D   D   N   F   S   S

  ATT GTG GCT GCT GTT GAA GAA GGT CGT GCT ATT TAC AAC AAC ATG AAG CAG TTC ATC CGT  180
   I   V   A   A   V   E   E   G   R   A   I   Y   N   N   M   K   Q   F   I   R

  TAC CTC ATT TCT TCC AAT GTT GGT GAG GTT GTT TCC ATC TTT TTG ACT GCT GCT CTA GGT  240
   Y   L   I   S   S   N   V   G   E   V   V   S   I   F   L   T   A   A   L   G

  CTT CCA GAA GCT CTT ATC CCA GTC CAG CTC CTG TGG GTC AAC CTT GTA ACT GAT GGC TTG  300
   L   P   E   A   L   I   P   V   Q   L   L   W   V   N   L   V   T   D   G   L

  CCT GCT ACT GCC TTG GGC TTC AAC CCT CCA GAT CTT GAT ATT ACG GAC AAA CCT CCC CGC  360
   P   A   T   A   L   G   F   N   P   P   D   L   D   I   T   D   K   P   P   R

  AGA GCT GAC GAG TCC CTC ATC TCT GGC TGG CTA TTC TTC CGT TAC ATG GCC ATT GGT GGC3'420
   R   A   D   E   S   L   I   S   G   W   L   F   F   R   Y   M   A   I   G   G
```

Figure 4. Partial nucleotide sequence and deduced amino acid sequence of the cloned muscle sarcoplasmic reticulum Ca^{2+}ATPase (SRCA) of crayfish *Procambarus clarkii*.

BLASTN algorithm (Altschul et al., 1990) revealed high scoring matches (75–85% identity) with published SRCA sequences ranging from *Artemia* sp. and *Drosophila* sp. to rabbit and chicken. Homology with published PMCA sequences and other known 'P' ATPases such as Na^+/K^+ATPase was minimal. The deduced amino acid sequence shared 94% identity with the published *Artemia* sp. sequence and 93% identity with rabbit sequence (Figure 5).

```
Crayfish  D G V N D A P A L K K A E I  G I  A M G S G T A V A K S A S E M V L A D D N F S S I  V A A V E E G R A I  Y N N M K Q F I  R
Artemia   . . . . . A . . . . . T . . . . . .
Rabbit    . . . . . T . . . . . T . . . . . .

Crayfish  Y L I  S S N V G E V V S I  F L T A A L G L P E A L I  P V Q L L W V N L V T D G L P A T L G F N P P D L D I  T D K P P R R
Artemia   . . . . . I . . . . . M N . . . . .
Rabbit    . . . . . C . . . . . M . R . . . S

Crayfish  A D E S L I  S G W L F F R Y M A I  G G
Artemia   . . . G . . . . . . . . . . T
Rabbit    P K . P . . . . . .
```

Figure 5. Comparison of the deduced partial amino acid sequence of *Procambarus clarkii* SRCA with (A) *Artemia* SRCA (Palmero & Sastre, 1989) and (B) rabbit fast twitch muscle SRCA (Brandl et al., 1986). Amino acids that are identical are indicated by a dot. Alignment of the amino acid sequences was performed using BLASTP software.

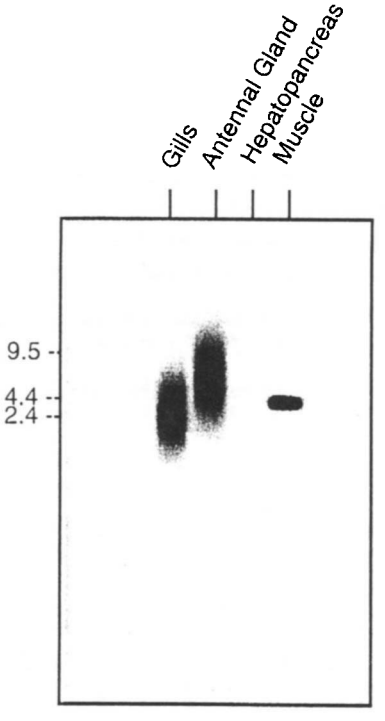

Figure 6. Expression of sarcoplasmic reticulum of muscle (SRCA) in intermoult *Procambarus clarkii* tissues. Messenger RNA was separated on a denaturing agarose gel, transferred to a nylon membrane and hybridized to ^{32}P labeled DNA probe sequenced in Figure 4.

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This partial cDNA crayfish SRCA fragment was subsequently used to probe a northern blot of crayfish mRNA taken from a variety of intermoult tissues to quantify the level of mRNA expression and to discern whether there are multiple isoforms (Figure 6). Probe of this likely crayfish muscle SRCA hybridized with a 4·5 kb mRNA in both gill and muscle. The *Artemia* sp. study (Palmero & Sastre, 1989) similarly reported hybridization with a 4·5 kb species. Expression was greater in muscle. In kidney the probe hybridized with a 10·6 kb mRNA suggesting that there are multiple isoforms as found in mammals.

FUTURE DIRECTIONS

Having obtained a cDNA clone of a central region of the crayfish muscle SRCA gene, PCR of the 5' end and 3' end regions will be undertaken using rapid amplification of cDNA ends (RACE) to obtain the full length clone. Our ultimate goal is to use similar approaches to characterize the PMCA. We will then compare the sequences for SRCA and PMCA and quantify relative expression in different Ca-transporting tissues at different stages of the moult cycle. The PMCA may be more important in transepithelial Ca transfer. A sequence comparison of SRCA and PMCA in mammals (Shull & Greeb, 1988) indicates a high degree of identity in amino acid sequence in the cytoplasmic region following the second transmembrane domain, around the phosphate site, FITC site and FSBA/CIRATP (γ-[4-N-2-chloroethyl-N-methylamino)] benzylamide ATP) site, and in and around hydrophobic domains 1, 2, 4, 5, 6, and 10. However, the fact that PMCA and SRCA share only 29% identity in the translocation region suggests that they may employ different mechanisms for Ca translocation.

Ecdysis is orchestrated by a late premoult peak in the steroid β-ecdysone which is associated with premoult Ca storage and postmoult Ca uptake (Hopkins, 1992). Our ultimate goal is to determine whether the expression of the gene for Ca^{2+}ATPase in crayfish Ca transporting epithelia is regulated by ecdysteroids, and if so, to delineate the molecular mechanism by which this occurs.

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