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Evidence for Calcium Channels in Brine Shrimp: Diltiazem Protects Shrimp against Cadmium

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Much information is available on the accumulation and effects of toxic substances in aquatic organisms (Malins & Ostrander 1991). Fewer studies, however, have examined toxic mechanisms in marine biosystems. Present experiments provide information on the mechanism by which cadmium penetrates into tissues of the small marine organism known as the brine shrimp. Brine shrimp are of major importance in the aquaculture industry and they have been proposed as a uniform world-wide test system for toxicity of chemical substances (Vanhaeke et al. 1981) and for studies in developmental toxicology (Sleet & Brendel 1985).

In many mammalian systems, cadmium ion competes with calcium and slowly penetrates lipoid plasma membranes by way of calcium channels. Cadmium blockade can actually be used to distinguish between "N", "T" and "L" type calcium channels (Tsien et al. 1988). Cadmium flux through voltage-sensitive calcium channels is a major mechanism for cadmium entry into cells of an established pituitary cell line (Hinkle et al. 1987). Cadmium also penetrates calcium channels in cat sensory neurons (Taylor 1988). Furthermore, glucose stimulation can promote either cadmium or calcium uptake through voltage-sensitive calcium channels in pancreatic beta cells (Nilsson et al. 1987). It is clear that cadmium ion is able to enter mammalian cells through calcium channels. This is an essential step before cadmium can exert its toxic intracellular effects. The present study examines the possibility that cadmium may penetrate into tissues of a marine organism by way of calcium channels.

MATERIALS AND METHODS

Brine shrimp (Artemia salina, Leach) cysts (Living World, Metaframe Inc., Elmwood Park, NJ) were hatched

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in a shallow, rectangular dish filled with artificial seawater which was prepared from a commercial salt mixture (Instant Oceans, Aquarium Systems, Inc., East Lake, OH) and double distilled water. A plastic divider with several 2 mm holes was clamped into the dish to make two unequal compartments. The cysts (25 mg) were sprinkled into the larger compartment which was darkened, whereas the smaller compartment was illuminated. After 48 hrs, the phototropic nauplii were collected using a Pasteur pipette from the lighted side, having been separated by the divider from their shells. Procedures followed are those of Meyer et al. (1982), as modified by McLaughlin (1991).

The pH of the brine solution which is normally about 8 was adjusted to 7 to prevent precipitation of cadmium hydroxide. This also eliminated any possible pH-related variability in effectiveness of the weak bases employed in this study.

Ten shrimp were transferred to each sample vial, and artificial seawater was added to make 4 ml. Lidocaine HCl or diltiazem HCl were dissolved in distilled water and added 45 min before cadmium in small volumes (10 to 30 μl) to give the indicated concentrations. Cadmium chloride ($CdCl_2 \cdot 2\frac{1}{2} H_2O$) was also dissolved in distilled water and added in small volumes to make 100, 200 or 300 ppm. Controls received equal volumes of distilled water. Four separate batches of shrimp were used for experiments with lidocaine and for evaluating the protective effects of diltiazem against cadmium toxicity. After 24 hrs, the number of dead shrimp was noted in each vial and the LC_{50} (lethal concentration for 50% of the animals) values with 95% confidence intervals were estimated by computer using Finney's probit analysis (Finney 1971). Altogether over 2100 animals were used in this study.

RESULTS AND DISCUSSION

To determine concentrations of lidocaine and diltiazem which could be used without harming the shrimp, LC_{50} values were estimated for these substances. Using 100 shrimp, the LC_{50} for lidocaine HCl was found to be 567.9 ppm with a 95% confidence interval of 398.2 to 955.2. For diltiazem HCl (employing 120 shrimp) the LC_{50} value was 352.6 ppm with a 95% confidence interval of 260.5 to 488.8. Concentrations of these drugs up to about one-third of the LC_{50} values were selected for the present study.

When added 45 min prior to cadmium, 100 ppm diltiazem HCl increased the concentration of cadmium required to kill 50% of the shrimp in 24 hrs by 21% (Table 1). The

values are clearly different statistically since the 95% confidence intervals were exclusive of one another. Even 50 ppm of diltiazem HCl increased the LC_{50} by 19% (Table 1). There was considerable overlap, however, in the 95% confidence intervals at 50 ppm compared to the respective control. Taken together, these data indicate that cadmium is less toxic to brine shrimp when calcium channels are blocked.

It is evident from Table 1 that there is variation from one experiment to the next in the estimate of the LC_{50} for cadmium. All these data were collected in July and August so seasonal variation probably is not involved. It is possible that variations in room temperature occurred which affected cadmium toxicity (Spiragalli et al. 1983), but this remains to be established.

Table 1. Effect of diltiazem on cadmium toxicity in brine shrimp.

LC_{50} Cadmium alone	with Diltiazem, 100 ppm
143 ppm	173.7 ppm
131.4 - 155.8	156.6 - 192.2
N = 510	N = 470
LC_{50} Cadmium alone	with Diltiazem, 50 ppm
267.7 ppm	314.8 ppm
235.4 - 321.9	265.7 - 427.5
N = 180	N = 180

Diltiazem HCl was added 45 min prior to cadmium. The LC_{50} with 95% confidence intervals is given in ppm for $CdCl_2 \cdot 2\frac{1}{2} H_2O$. Mortality data were collected 24 hrs after the addition of cadmium.

Table 2. Effect of lidocaine on cadmium toxicity in brine shrimp.

LC_{50} Cadmium alone	with Lidocaine 50 ppm	100 ppm	200 ppm
246.3 ppm	232.1 ppm	228.6 ppm	264.2 ppm
222.4-279.5	201.9-274.8	204.4-261.1	233.7-313.3
N=300	N=120	N=270	N=180

Lidocaine HCl was added 45 min prior to cadmium. The LC_{50} with 95% confidence intervals is given in ppm for $CdCl_2 \cdot 2\frac{1}{2} H_2O$. Mortality data were collected 24 hrs after the addition of cadmium.

The concentrations of diltiazem used in this study were 0.11 and 0.22 mM. Levels of calcium channel blockers of this magnitude may have a local anesthetic effect

and block sodium as well as calcium channels (Fleckinstein 1977). To determine whether sodium channel blockade could explain the effects of diltiazem, the local anesthetic lidocaine was also tested. It is clear from Table 2 that lidocaine HCl (50-200 ppm) did not protect brine shrimp from the toxic effects of cadmium. The protective effect of diltiazem against cadmium toxicity, therefore, is not due to a non-specific membrane stabilizing action including blockade of sodium channels.

Like calcium, cadmium is a divalent cation. Cadmium ion is able to displace Ca^{2+} or act like Ca^{2+} in many biological systems. Cadmium releases adrenal catecholamines even in the absence of extracellular calcium (Hart and Borowitz 1974). Cadmium releases calcium from isolated rat liver microsomes (Zhany et al. 1990). It also activates calmodulin in a calcium-like manner (Thulin et al. 1984). Calcium decreases the influx of cadmium in hepatocytes (Sorenson 1988). Lastly, cadmium increases the exocytotoxic release of insulin from pancreatic islets isolated from obese hyperglycemic mice in a manner not involving changes in cellular calcium (Nilsson et al. 1987). Since cadmium is so similar to calcium biologically and has an ionic radius similar to that of calcium, it is not surprising that Cd^{2+} is able to pass through calcium channels. Reduction of cadmium's toxic action by a calcium channel blocker in the present study suggests that cadmium passes through calcium channels to exert its toxic effects in brine shrimp.

The existence of calcium channels in marine biosystems has been demonstrated repeatedly (Au et al. 1991; Olson et al. 1991; Bolger et al. 1986). Actually, the electric organ of the eel is one of the richest sources of calcium channels known to man (Marsal et al. 1980). It appears that calcium channels are functionally important in many marine systems and are therefore very old phylogenetically. The data of the present study provide the first evidence that cadmium ion enters cells of brine shrimp by way of calcium channels. The results further suggest that calcium channels are functionally important in brine shrimp as they are in other marine species.

Toxicity of cadmium in fish is thought to be due to hypocalcemia. The disturbance of calcium ion regulation occurs at the level of the gills where Ca^{2+}-ATPase is responsible for calcium uptake. This enzyme is directly inhibited by cadmium (Verbost et al. 1988). The present results, however, suggest that cadmium toxicity in marine organisms may also involve toxic

intracellular actions since the protective action of diltiazem most likely involves calcium channels and not Ca^{2+}-ATPase.

Many drugs which have potent effects in mammals also have potent effects in brine shrimp. Physostigmine salcylate (cholinesterase inhibitor), propranolol HCl (beta adrenergic receptor blocker), amphetamine sulfate (stimulant), pargyline HCl (monoamine oxidase inhibitor), pentazocine HCl (opiate) promethazine HCl (antihistamine) and pentobarbital sodium (sedative) have LC_{50s} between 8 and 261 ppm. Also, atropine blocks the toxicity of physostigmine and antihistamines block the lethal effects of histamine in brine shrimp. Furthermore, certain agents which have antibacterial (penicillin G sodium, erythromycin estolate, antiamoebic (chloroquine phosphate) chemopherapeutic (streptozotocin) or antimalarial (pamaquine naphtholate) activity are not toxic to the shrimp (J. McLaughlin, Purdue Univ., unpublished data). Thus the nature of the systems in brine shrimp which respond to drugs (including diltiazem) appears to be similar to those in mammals.

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